GENETIC STUDIES IN CLEFT LIP AND PALATE PATIENTS FROM KERALA

Thesis submitted in partial fulfilment of the requirements for the Degree of DOCTOR OF PHILOSOPHY IN ZOOLOGY

Faculty of science

University of Calicut

By

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Under the supervision of

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2023

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I, SOUMYA RAJ, hereby declare that the work embodied in the thesis "**GENETIC STUDIES IN CLEFT LIP AND PALATE PATIENTS FROM KERALA**" submitted to the University of Calicut in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology is a bonafide record of my research work carried out by me under the supervision of Dr. Leyon Varghese, Assistant professor, Department of Zoology, Christ College, Irinjalakuda, Thrissur and no part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any university.

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ACKNOWLEDGEMENTS

Undertaking this Ph.D. has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people.

First and foremost, I want to thank my supervisor **Dr. Leyon Varghese**, Assistant Professor Department of Zoology, Christ College, Irinjalakuda; I appreciate all his contributions of time, and ideas to make my Ph.D experience productive and stimulating. The joy and enthusiasm have for his research were contagious and motivational for me, even during tough times in the Ph.D pursuit. Moreover, his exemplary guidance, monitoring, and constant encouragement during the course of research will linger in my memory throughout the journey I am about to embark. Thank you for giving me the opportunity to grow in this field of research.

I would like to express my profound gratitude to Dr Alex George, Scientist D, Jubilee Centre for Medical Research, JMMCRI, Thrissur, whose unwavering guidance and mentorship have been the cornerstone of my Ph.D. journey. Dr. Alex's exceptional expertise in the field of Genomics has not only shaped the direction of my research but has also inspired me to strive for excellence in my scientific pursuits. His willingness to lend a listening ear, offer insightful advice, and provide constructive feedback has greatly enriched my academic experience. I am grateful to Dr Alex George, for his immense guidance, suggestions, and support.

I would like to express my deep sense of gratitude to Dr. P. R. Varghese, Research Co-ordinator, Jubilee Centre for Medical Research for the constant support and motivation throughout my research. Dr. Varghese's commitment to advancing knowledge and his dedication to fostering a culture of curiosity within our research group have been a constant source of inspiration. His insightful feedback, constructive critiques, and encouragement have significantly enriched the quality of my work.

I am deeply indebted to Dr. Suresh Kumar R, Scientist B2, for his invaluable assistance and contributions to this Ph.D. thesis. I am also grateful for the personal sacrifices he has made to ensure that I had the time, space, and resources to focus on my research. Their unwavering commitment to my academic pursuits has been a driving force behind my achievements. Dr. Suresh Kumar has been an unwavering source of support and guidance

throughout my research journey, and his expertise has played a pivotal role in shaping the outcomes of this work.

I would like to express my sincere gratitude to Prof. Dr. D M. Vasudevan, MBBS, MD, FRCPath, Research Director, JCMR, JMMC&RI for his unwavering support and encouragement throughout my Ph.D. journey. His steadfast belief in my abilities, along with their selfless assistance, has been instrumental in my successful completion of this thesis.

Dr. Ranjith S, Assistant professor, Department of Anatomy, JMMC&RI, has been an unwavering pillar of support, providing invaluable guidance and motivation during both the high points and challenging moments of my doctoral studies.

I am deeply indebted to Dr. Hirji S Adenwalla (Late), Plastic surgeon, JMMC&RI, (Smile Train India) a true legend, meticulous and ever encouraging personality has been an unwavering pillar of support.

I would like to express my sincere gratitude to Dr. P. V. Narayanan, Associate Professor, The Charles Pinto Centre for Cleft lip and Palate and Craniofacial anomalies, JMMCRI, Thrissur providing sufficient facilities for the successful completion of work.

I owe my sincere thanks to Dr. John Thomas K, Associate Professor (Retd.), Department of Zoology, Christ college, Irinjalakuda and Scientist D, JMMC&RI, who has been a constant source of inspiration and driving force behind my achievements and rekindled the spirit of research in me.

I also extend my sincere gratitude to former Director Rev. Fr. Francis Pallikunnath, JMMC&RI, Thrissur for his timely support and blessings.

I also extend my sincere gratitude to Dr. Kumudham Unni, Nodal Officer, JMMC&RI, Thrissur for her timely support.

I would like to express my sincere gratitude to Dr. Kezia Kuruvilla, Scientist D, JMMC&RI, for her unwavering support and encouragement throughout my Ph.D journey.

I also extend my sincere gratitude to the Director Rev. Fr. Renny Mundankurian, JMMC&RI, Thrissur for his timely support and blessings.

I also extend my sincere gratitude to the Management, HR department, JMMC&RI, Thrissur for the timely support.

I am also thankful to the entire faculties and research scholars of Jubilee Centre for Medical Research for providing an intellectually stimulating environment that facilitated meaningful collaborations and valuable discussions. The support from my fellow researchers and colleagues has been instrumental in overcoming challenges and pushing the boundaries of our collective knowledge.

I would like to express my deep sense of gratitude to Dr. Prof. Nasser M, Pro-Vice Chancellor, University of Calicut for the constant support and motivation throughout my research.

I also extend my sincere gratitude to former principal Fr. Dr. Jose Thekkan C M I (Late) for his timely support and blessings.

My deep sense of gratitude also goes out to Fr. Dr. Jolly Andrews CMI, Principal-in-charge, Christ College, Irinjalakuda for providing sufficient facilities for the successful completion of work.

I express my immense gratitude to the Dr. Sudhikumar A. V, Assistant Professor, Head, Department of Zoology, Christ College, Irinjalakuda for the facilities provided.

My deep appreciation goes out to Dr. C. O. Joshi, Former Head, Department of Zoology, Christ College, Irinjalakuda for providing research facilities.

I extend my sincere thanks to all other teaching and non-teaching staffs of Christ College, Irinjalakuda for their kind support and encouragement.

I would like to express my sincere gratitude to Dr. Sreelata Nair, Consultant Geneticist, Lifeline Super speciality Hospital, Adoor.

The financial assistance from Smile train India is gratefully acknowledged.

The members of Jubilee Centre for Medical Research have contributed immensely to my personal and research work. JCMR has been a source of friendships as well as good advice and collaboration. I am also grateful to Dr. Ravindran Ankathil, Scientist G; Dr. Raghu K G, Scientist F; for their valuable advices. The work could not have gone ahead without the support of my colleagues, Dr. Mathew John, Scientist C; Dr. Dileep Vijayan, Scientist D; Mrs. Mridula Vellore, Mrs. Evelyn Mariya, Mrs. Liji M J, Mrs. Shiji Jesso and Dr. Anjali Annie George. I am obliged them for their valuable comments, suggestions and timely support throughout my study.

I am much indebted to Dr. Saliha C. K, Reader, Annoor Dental College and hospital, Ernakulam whose constant and timely support has helped me a lot.

I express my immense gratitude to Dr. Binu Ramachandran, Department of Zoology, University of Calicut, for his timely support and valuable suggestions and cooperation during the way of my investigation.

I owe special thanks to Dr. Pushpalatha, Professor, University of Calicut and members of the teaching and non-teaching staff of the University of Calicut for their co-operation during the period of my study.

I am deeply indebted to Dr. Anoop Kumar A. N, CMNPF, Department of Zoology, University of Calicut for the constant support and encouragement throughout my research and who has been an inspiration for me to do research.

I also utilize this opportunity to express my thanks to all the Research Scholars of Department of Zoology, Christ College, Irinjalakuda for their support and encouragement during the course of the study. A very special thanks to Dr. Praveen K, Ms. Madhuri Menon, Ms. Jimcy, Ms. Lekshmi Jayakrishnan, Dr. Dhrishya, Dr. Sudhin, Ms. Dhilna Sunny, Dr. Kashmeera N A, Dr. Sumesh for their encouragement and help.

My special thanks to Ms. Ragitha T S, Ms. Reema Rose Alappat, Fr. Jijo Francis, Fr. Roger Francis, Ms. Mary Martin, Ms. Meenakshi, Research scholars, JCMR. I am greatly indebted to them for the completion of this work in the specified period.

My special thanks to Dr. Preeji K.P., Assistant Professor, Department of Psychology, Sahridhaya College of Advanced Studies, Kodakara, for her timely support and suggestions.

I also utilize this opportunity to express my thanks to the families participated in the study for their co-operation and giving consent for the study.

I would like to extend my sincere thanks to Mr. Unnikrishnan U. G, Biostatistician, JMMC&RI for the timely support.

I express my deepest gratitude to Dr. Krishnachandran R, Head, Molecular Genetics, DDRC for his immense support and has been an incredible inspiration and a constant source of motivation for me. His valuable instructions and insights have been nothing short of inspiring.

I would like to extend my sincere thanks to my best friends Dr. Lalu Krishna N, Mrs. Aswija Shetty, Mrs. Pamala John, Dr. Ullas P T, Mr. Nidhish G, Mr. Vineeth Vasu, Mr. Ajith V Johnson, Mr. Vinu E V, Dr. Vidhya Gopalan, Dr. Muthulakshmi C M, Mrs. Presthiena Lofi E L, Mr. Sanesh Jose, Dr. Nikita Thomas; for their support.

I am deeply indebted to Mr. Rabin Rajendran, my spouse for the unwavering support he has provided throughout this incredible journey. I also want to acknowledge the sacrifices he has made to ensure that I could pursue my academic dreams. I have often found myself looking up to him as a role model, not just in terms of academic excellence but also as a person of character and integrity. His patience, understanding, and willingness to stand by my side during this time have meant more to me than words can express. His belief in me, even when I doubted myself, has been a constant source of strength. His presence in my life has been an anchor, and his encouragement has been the driving force behind my success.

Completion of the thesis, of course, ultimately due to the encouragement and support of my family. For my parents who raised me with a love of science and supported me in all my pursuits. I am grateful to my mother Mrs. Anitha, father Mr. Rajan, my uncle Mr. Ajit. M. S and family, Mr. Arun M.S and family and my sister Mrs. Remya Haneesh and my daughter Ms. Ishani for their immense love, patience, care and understanding in making the years of my research unforgettable. I would like to express my heartfelt gratitude to my in-laws for their unwavering support and understanding throughout my PhD journey.

Besides the above said persons, I express my profound gratitude to all those unknown persons who helped me in some way or the other during my research.

Above all, I thank the Almighty God from the bottom of my heart, who is the constant source of strength, wisdom, love, guidance, and inspiration and for giving me immeasurable blessing, for without him this could not be possible.

Thank you

Mrs. Soumya Raj

ABBREVIATIONS

NSCLP- Non-Syndromic cleft lip with or without cleft palate

CLP- Cleft lip and palate

NS- Non- Syndromic

OFCs- Orofacial clefts

MxP- Maxillary processes

MNP- Medial nasal processes

LNP- Lateral nasal processes

OD- Optical density

Rs- Reference SNP cluster ID

RhoA- Ras Homolog Family Member A

SNPs- Single nucleotide polymorphism

IRF6- Interferon regulatory factor6

DNA- Deoxyribonucleic acid

mRNA- Mitochondrial Reoxyribo nucleicacid

TGFb- Transforming growth factor b

MEE- Medial edge epithelium

TGFß- Transforming growth factor ß

SMAD- Suppressor of mothers against decapentaplegic

MAPK- Mitogen- activated protein kinase

SNA12- Snail family transcriptional repressor2

ZO-1- Zonula occludens1

RIPK4- Receptor-Interacting protein kinase 4

ROCK- Rho associated protein kinase

Bmp2- Bone morphogenic protein2

Bmp4- Bone morphogenic protein4

CT – Computed Tomography

Shh- Sonic hedgehog

LAHSN- Lip Alveolus Hard palate Soft palate and Nose

ISS- Initial Severity Score

PRS- Pierre Robin Sequence

CNC- Cranial neutral crest cells

NC- Neural crest

ECM- Extracellular matrix

MES- Middle line epithelial seam

Fgf- Fibroblast growth factor

VDWS- Van der woude syndrome

PPS- Popliteal pterygium syndrome

JAG2- Jagged canonical notch ligand 2

TGFA- Transforming growth factor alpha

EGF- Epidermal growth factor

EGFR- Epidermal growth factor receptor

Taq1- *Thermus aquaticus*

FOXE1- Forkhead box protein E1

FOX- Forkhead

MSX1- Msh homeobox1

SUMO1- Small ubiquitin -related modifier 1

GRHL3- Grainy head like transcription factor 3

VWS- Van der woude syndrome

HWE- Hardy-Weinberg equilibrium

LD- Linkage disequilibrium

CRISPLD2- Cysteine rich secretory protein LCCL domain 2

UCLP- Unilateral cleft lip and palate

BCLP-Bilateral cleft lip and palate

VPD- Velopharyngeal dysfunction

WNT- Wingless -related integration site

EDTA- Ethylene diamine tetra acetate

GIS- Geographic information system

PBLC- Peripheral blood lymphocyte Culture

ISCN- International system for human cytogenetic nomenclature

MAF- Minor allele frequency

PCR- Polymerase chain reaction

EtBr- Ethidium bromide

BLASTN-Basic local alignment search tool

IDT- Integrated DNA technologies

SPSS- Statistical Package for Social Sciences

TDT- Transmission Disequilibrium Tests

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Abstract

Research regarding various inferences allied with the orofacial clefts is crucial to address the lack of awareness among the general population and to understand how they can hinder personal growth and impact quality of life. For the past few years, progress in genetics and molecular biology has started unveiling the foundations of craniofacial development. Gaining insight into the causes of cleft lip and palate is essential for diagnosing, treating, and evaluating the risk of recurrence within families. In this regard, here the author has focused on the identification of association between Non syndromic cleft lip and palate (NSCLP) and Chromosome abnormalities as first strand. The second strand of the investigation have focussed on the identification of association between NSCLP and *IRF6* gene. The final strands of the study have directed the research in context with the identification of association between NSCLP and *CRISPLD2* gene. The cytogenetic analysis revealed that about 5 cases (3.84%) with abnormal karyotype in which a higher frequency of pericentric inversion - specifically in the chromosome 9 [inv(9)(p11p13)] - was observed. The second strand of the study have found a prominent link between the analysed elements such as NSCLP and *IRF6* gene. The last segment of the study also examined the potential involvement of *CRISPLD2* in initiating the abnormalities associated with craniofacial disorders, particularly cleft lip (CL) and cleft palate (CP). In the final remarks, the results obtained from these investigations could be acknowledged as a noteworthy endeavour in developing effective strategies for the prevention and treatment of individuals affected by these anomalies.

Keywords: Orofacial clefts, NSCLP, *IRF6, CRISPLD2*, karyotype, Chromosomes.

സംഗ്രഹം

വിള്ളൽ ചുണ്ടിനും അണ്ണാക്കിനും കാരണമാകുന്ന വിവിധ ഘടകങ്ങളേയും നിഗമനങ്ങളേയും കുറിച്ചുള്ള പഠനമാണിത്. പൊതുജനങ്ങൾക്കിടയിൽ അവബോധം വളർത്തുന്നതിനും ഇതുമൂലം വ്യക്തിഗത വളർച്ചക്കും ജീവിതനിലവാരത്തിനുമുണ്ടാകുന്ന വ്യതിയാനങ്ങൾ മനസ്സിലാക്കുന്നതിനും ഇത്തരം ഗവേഷണങ്ങൾ ഉപകാരപ്രദമാണ്. കഴിഞ്ഞ കുറച്ച് വർഷങ്ങളായി, ജനിതകശാസ്ത്രത്തിലെയും തന്മാത്രാ ജീവശാസ്ത്രത്തിലെയും പുരോഗതി ക്രാനിയോഫേഷ്യൽ വികസനത്തിന്റെ അടിത്തറ അനാവരണം ചെയ്യാൻ തുടങ്ങി. വിള്ളൽ ചുണ്ടിന്റെയും അണ്ണാക്കിന്റെയും കാരണങ്ങളെക്കുറിച്ച് ഉൾക്കാഴ്ച നേടുന്നത് കുടുംബങ്ങൾക്കുള്ളിൽ ആവർത്തനത്തിനുള്ള സാധ്യത നിർണ്ണയിക്കുന്നതിനും ചികിത്സിക്കുന്നതിനും വിലയിരുത്തുന്നതിനും അത്യന്താപേക്ഷിതമാണ്. ഇക്കാര്യത്തിൽ, നോൺ സിൻഡ്രോമിക് ക്ലെഫ്റ്റ് ലിപ് ആൻഡ് പാലറ്റ്(എൻഎസ്സിഎൽപി), ക്രോമസോം അബ്നോർമാലിറ്റികൾ എന്നിവ തമ്മിലുള്ള ബന്ധത്തെ ആദ്യ ഇഴയായി തിരിച്ചറിയുന്നതിലാണ് ഗവേഷക ഇവിടെ ശ്രദ്ധ കേന്ദ്രീകരിച്ചത്. എൻഎസ്സിഎൽപിയും ഐആർഎഫ്6 ജീനും തമ്മിലുള്ള ബന്ധത്തെ തിരിച്ചറിയുന്നതിലാണ് അന്വേഷണത്തിന്റെ രണ്ടാം ഭാഗം ശ്രദ്ധ കേന്ദ്രീകരിച്ചിരിക്കുന്നത്. എൻഎസ്സിഎൽപിയും സിആർഐഎസ്പിഎൽഡി2 ജീനും (CRISPLD2) തമ്മിലുള്ള ബന്ധം തിരിച്ചറിയുന്ന പശ്ചാത്തലത്തിലാണ് പഠനത്തിന്റെ അവസാന ഭാഗങ്ങൾ ഗവേഷണത്തിന് നേതൃത്വം നൽകിയത്. വിശകലനം ചെയ്ത മേഖലയിൽ പെരിസെൻട്രിക് ഇൻവെർഷന്റെ ഉയർന്ന ആവൃത്തി, പ്രത്യേകിച്ച് ക്രോമസോം 9 (inv(9)(p11p13)) നിരീക്ഷിക്കപ്പെടുന്ന അസാധാരണമായ കാരിയോടൈപ്പുള്ള ഏകദേശം 5 കേസുകൾ (3.84%) സൈറ്റോജെനെറ്റിക് വിശകലനം വെളിപ്പെടുത്തി. വിശകലനം ചെയ്ത മൂലകങ്ങളായ NSCLP, IRF6 ജീൻ എന്നിവ തമ്മിലുള്ള ഒരു പ്രധാന ബന്ധം പഠനത്തിന്റെ രണ്ടാമത്തെ ധാരയിൽ കണ്ടെത്തി. ക്രാനിയോഫേഷ്യൽ ഡിസോർഡേഴ്സ്, പ്രത്യേകിച്ച് മുറിച്ചുണ്ട് (CL), അണ്ണാക്കിന്റെ പിളർപ്പ് (CP) എന്നിവയുമായി ബന്ധപ്പെട്ട അസാധാരണത്വങ്ങൾ ആരംഭിക്കുന്നതിൽ CRISPLD2 ന്റെ സാധ്യതയുള്ള പങ്കാളിത്തവും പഠനത്തിന്റെ അവസാന ഭാഗം പരിശോധിച്ചു. അന്തിമ പരാമർശങ്ങളിൽ, ഈ അന്വേഷണങ്ങളിൽ നിന്ന് ലഭിച്ച ഫലങ്ങൾ, ഈ അവസ്ഥ തടയുന്നതിനും ചികിത്സിക്കുന്നതിനുമുള്ള ഫലപ്രദമായ പ്രതിവിധി വികസിപ്പിക്കുന്നതിനുള്ള ശ്രദ്ധേയമായ ശ്രമമായി അംഗീകരിക്കാവുന്നതാണ്.

CHAPTER I: INTRODUCTION

1. INTRODUCTION

1.1 Overview

1.1.1 Craniofacial Development

Craniofacial development is an intricate and multifaceted process that plays a pivotal role in shaping the human face. This complex procedure involves the orchestration of numerous transcription factors and intricate molecular signaling pathways. Any disruption within this intricate network of molecular signaling and associated proteins can lead to the development of facial clefts, a group of congenital conditions characterized by abnormalities in the formation of the face. The profound impact of craniofacial development on an individual's appearance and overall well-being underscores the significance of understanding the underlying mechanisms responsible for facial clefts. Research in the field of craniofacial development has shown that these conditions can be hereditary, with a notable tendency for facial clefts to recur within families. Studies by Jugessur and Murray (2005) shed light on the heritability of facial clefts, emphasizing the importance of genetic factors in their etiology. This observation has spurred extensive research efforts aimed at identifying the specific genes and molecular pathways responsible for Non-Syndromic Cleft Lip and Palate (NSCLP).

The quest to unravel the genetic underpinnings of NSCLP has been a prominent theme in scientific inquiry for years. Researchers have employed a diverse array of investigative approaches, ranging from genomic rearrangement studies to genome-wide association studies (Rahimov et al., 2012). These comprehensive investigations seek to pinpoint the genetic variations and alterations that contribute to the development of NSCLP, shedding light on the complex interplay between genetics and craniofacial development. Craniofacial disorders, with cleft lip and palate (CLP) as their central

structural manifestation, are among the most prevalent congenital birth defects globally. It is estimated that these conditions affect approximately one in every 700 live births (Dixon et al., 2011). The staggering frequency of CLP underscores the urgency and significance of research aimed at deciphering the root causes of these conditions. They represent a substantial healthcare burden, both in terms of medical care and the psychological well-being of affected individuals and their families. The etiological factors contributing to these craniofacial complications have been diverse and multifaceted. These factors can be broadly categorized into chromosome aberrations, single-gene mutations, sporadic occurrences without a known cause, or exposure to teratogenic agents (Lidral et al., 2008). This intricate web of causative factors necessitates extensive investigation to comprehend the complex genetic contributions to craniofacial development fully.

The classification of orofacial clefts has revealed distinct categories based on the presence of structural anomalies. One such classification distinguishes between non-syndromic (NS) and syndromic clefts (Gorlin et al., 2001). Notably, cleft lip with or without cleft palate (CLP) is considered a distinct phenotype within this classification. This distinction highlights the remarkable diversity and complexity of craniofacial conditions, underscoring the need for precise genetic and molecular investigations. Recent advancements in high-throughput genotyping techniques and the development of effective statistical methodologies have significantly expanded our ability to identify loci associated with susceptibility to multifaceted diseases, including craniofacial abnormalities (Altmüller et al., 2001). These cutting-edge approaches offer unprecedented opportunities to unravel the intricate genetic landscape underlying craniofacial development, potentially paving the way for innovative therapeutic strategies and interventions.

1.1.2 Cleft Lip and Palate: A Common Craniofacial Anomaly

Orofacial clefts (OFCs) represent one of the most prevalent congenital craniofacial anomalies, encompassing a range of conditions that result in openings or clefts in the facial structures. These anomalies can affect various parts of the face, including the lip and palate. The etiology of orofacial clefts is complex and multifactorial, involving a combination of genetic and environmental factors. In some instances, these clefts extend into the oral cavity and adjacent facial structures, giving rise to a spectrum of oral, facial, or craniofacial deformities (Saleem et al., 2019). The global prevalence of orofacial clefts is significant, with approximately 1 in 700 live births being affected by cleft lip and palate (CLP). Interestingly, gender plays a role in the presentation of these conditions. Males are more likely than females to have both cleft lip and cleft palate, while females have a higher risk of developing isolated cleft palates (Turner et al., 1998). The presence of a cleft lip and palate can have profound psychosocial implications, impacting an individual's self-esteem and social skills, which, in turn, can affect their overall well-being. This is particularly pertinent in a society increasingly focused on appearance, where individuals with visible differences may face unique challenges, especially girls. The management of orofacial clefts typically involves surgical repair, which can entail multiple surgeries and therapeutic interventions. These treatments can be costly, and despite medical interventions, individuals may still grapple with lifelong psychological issues and mental health concerns (Pisula et al., 2014). Thus, understanding the developmental origins and genetic underpinnings of orofacial clefts is crucial not only for improving medical interventions but also for addressing the broader psychosocial and societal implications associated with these conditions.

Orofacial clefts are a result of flawed or disrupted craniofacial developmental processes during embryogenesis. The development of the face initiates in the fourth week of

gestation and involves a series of intricate events, including cell growth, migration, differentiation, and apoptosis. During this period, neural crest cells migrate and give rise to five facial primordia: the frontonasal prominence, the paired mandibular processes, and the paired maxillary processes (MxP). Subsequently, the nasal placodes invaginate, forming the medial nasal processes (MNP) and the lateral nasal processes (LNP) (Serisier et al., 2013). Around the sixth and seventh weeks of gestation, the MxP fuses with the LNP and then merges with the MNP, ultimately forming the upper lip and the primary palate (Leslie and Marazita, 2013). Any disruption or failure in these intricate developmental processes can lead to the formation of orofacial clefts, affecting the upper lip, alveolus, and the primary palate (Smarius et al., 2017). Orofacial clefts are further categorized based on the extent of involvement, ranging from clefts affecting only the lip to those affecting both the lip and palate, or isolated cleft palates. These distinctions help clinicians assess the severity of the condition (Beriaghi et al., 2009). Despite the phenotypic diversity observed in orofacial clefts, they are generally considered a simple, qualitative trait. However, the genetic and developmental complexities underlying these conditions suggest a more intricate interplay of factors than initially meets the eye. In clinical practice, orofacial clefts are classified as syndromic or non-syndromic based on the presence or absence of associated physical or cognitive abnormalities. Syndromic clefting is often associated with additional features and can have diverse origins, including chromosomal abnormalities, Mendelian inheritance patterns, or sporadic mutations. Notably, over 275 syndromes have been identified to date that feature clefting as a primary characteristic. These syndromes typically result from mutations in single genetic loci, chromosomal abnormalities, or exposure to teratogenic agents (Pengelly et al., 2015). Understanding the intricate genetic and developmental factors contributing to orofacial clefts is paramount for advancing medical care, improving surgical techniques,

and enhancing the overall well-being of affected individuals. Furthermore, this knowledge can inform public health efforts to raise awareness about the psychosocial challenges faced by individuals with orofacial clefts and promote inclusivity and acceptance in society.

1.1.3 Syndromic and Non-syndromic Clefts: Unravelling Complexity

Cleft lip and palate conditions can be broadly classified into two main categories: syndromic and non-syndromic, each with its unique characteristics and genetic underpinnings.

1.1.3.1 Syndromic Clefts:

Van der Woude syndrome stands out as one of the most prevalent forms of syndromic clefting (Wong et al., 2001). Syndromic clefts are characterized by the presence of additional physical or cognitive abnormalities along with the orofacial cleft. These conditions are relatively rare but have become increasingly identifiable due to advancements in sequencing technology. In such cases, the genetic identification of the causative genes has been successful, shedding light on the underlying genetic mechanisms responsible for these syndromes.

1.1.3.2 Non-syndromic Clefts:

Conversely, non-syndromic clefts refer to orofacial clefts that occur independently, without being associated with any other syndrome or major genetic abnormalities (Barrow et al., 2002). They are the most prevalent form of orofacial clefts and represent one of the most common birth defects observed worldwide. Non-syndromic clefts typically manifest as isolated cleft lip and palate conditions without any additional features. Studies have revealed notable gender-based differences in the prevalence of non-syndromic clefts, with cleft lip and palate (CLP) occurring twice as frequently in males

compared to females, while cleft palate (CP) is more frequently observed in females (Souza and Raskin, 2013).

The distinction between syndromic and non-syndromic clefts is crucial for both clinical diagnosis and research efforts aimed at understanding the genetic and environmental factors contributing to these conditions. Investigating the genetic basis of non-syndromic clefts, in particular, has provided valuable insights into the intricate mechanisms underlying craniofacial development and the etiology of these common birth defects.

1.1.4 Etiology: Unravelling the Multifaceted Causes of Orofacial Clefts

Numerous studies have explored the genetic underpinnings of orofacial clefts. Notably, the comprehensive CLP scan conducted with British sib pairs identified specific genomic regions, including one on chromosome 16q, displaying significant associations (Prescott et al., 2001; Prescott et al., 2000). Subsequent investigations, spanning various populations, including the Indian region, consistently pinpointed the region of 16q21-24 as statistically significant in the context of CLP linkage (Field et al., 2004; Marazita et al., 2009; Marazita et al., 2004). Within the chromosome 16q24.1 region, researchers identified the 'cysteine-rich secretory protein containing LCCL domain 2' gene (*CRISPLD2*) as a promising candidate gene for non-syndromic cleft lip and palate (CL(P)) in Hispanic and Caucasian populations (Chiquet et al., 2007). Intriguingly, *CRISPLD2* exhibited expression in critical craniofacial development sites, including the palate, nasopharynx, and mandible, further underscoring its relevance in NSCLP etiology. To investigate the genetic basis of NSCLP, researchers utilized the Taqman OD assay to genotype four single nucleotide polymorphisms (SNPs) within the *CRISPLD2* gene domain: rs4783099 (3' UTR), rs1546124 (5' UTR), rs2326398 (Intron 8), and rs8061351 (Exon 4). Environmental and Socio-economic Factors: Beyond genetics, environmental

and socio-economic factors play pivotal roles in orofacial cleft development. Factors such as maternal smoking, alcohol consumption during pregnancy, and exposure to teratogens have been implicated as potential environmental contributors (Honein et al., 2007). Socio-economic disparities have also been associated with varying cleft prevalence rates, highlighting the complex interplay of these factors in cleft etiology (Wyszynski et al., 2010). Orofacial cleft occurrence exhibits geographic and ethnic disparities. Studies have noted variations in prevalence rates among different populations, emphasizing the importance of considering regional and ethnic factors when investigating cleft etiology (Mossey et al., 2009).

1.2 Influencing Factors:

1.2.1 Genetic Factors

A myriad of studies has illuminated the intricate genetic landscape underlying the pathogenesis of cleft lip and palate (CLP). Among the constellation of genetic factors implicated, Interferon Regulatory Factor 6 (*IRF6*) stands out as a prominent gene associated with CLP, contributing to approximately 2% of all global cases (Vyas et al., 2020). The *IRF6* gene positioned on chromosome 1q32.2, *IRF6* belongs to the Interferon Regulatory Factor family, a group of helix-turn-helix transcription factors characterized by a highly conserved N-terminal DNA binding domain and a less conserved protein-binding domain. The primary function of IRF family genes is regulating virus-induced interferon production. Upon viral infection, these genes are activated, and phosphorylation at serine residues transforms them into their active forms, facilitating transcription processes. Consequently, mutations in *IRF* genes predominantly affect the immune system. *IRF6*, exclusive to this family, plays a pivotal role in craniofacial development, influencing cell cycle, proliferation, periderm formation, and keratinocyte

differentiation (Hixon et al., 2017). *IRF6* variants were initially identified in Van der Woude syndrome and have been recurrently linked to non-syndromic cleft lip and palate (NSCLP) and Popliteal Pterygium Syndrome (Hixon et al., 2017). Similar to other genes in the IRF family, *IRF6* comprises two critical domains: the SMIR-IAD protein-binding domain and the helix-turn-helix DNA-binding domain (Kondo et al., 2002). However, it diverges in its regulatory targets. *IRF6* has emerged as the master regulator of epithelial proliferation and differentiation (Girousi et al., 2021). It plays a pivotal role in the formation and development of periderm, which covers the superficial layer of oral epithelium and embryonic skin. Studies in animals lacking *IRF6* have revealed hyperproliferative epithelia without proper stratification. Superficial cells exhibit poor keratinization, leading to apoptosis, pathological interepithelial adhesion on the apical surface, and increased apical protrusion (Richardson et al., 2006). *IRF6*'s role extends to elevating secondary palatal shelves and facilitating the differentiation and degradation of the medial edge of the epithelium (Xu et al., 2006).

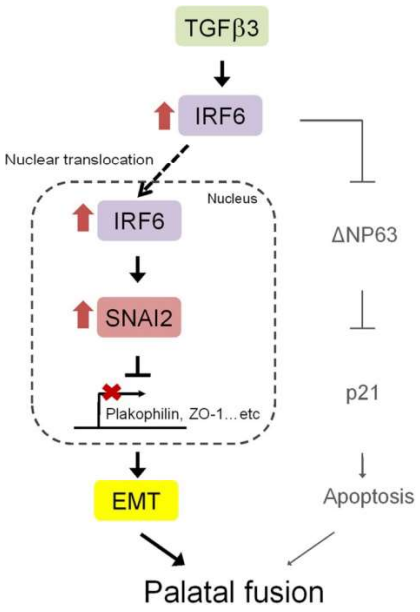


Figure 1.1 Palatal Fusion

In an animal study, epithelial-specific ablation of the TGFβ signaling receptor, TGFβR2, resulted in epithelial defects and oral facial clefts. *IRF6* downregulates TGFβ signaling during medial edge epithelium (MEE) regulation. *TGFβ* enhances *IRF6* expression through both the SMAD-dependent pathway and the p38 MAPK pathway, orchestrating MEE apoptosis via the *IRF6*/ΔNp63/p21 signaling cascade (Figure 1.1). *IRF6* also enhances the expression of a key epithelial-mesenchymal transition regulator, SNAI2, while suppressing the activation of proteins like ZO-1, plakophilin, and E-cadherin. Inhibition of SNAI2 negatively impacts palatal fusion, underscoring *IRF6*'s crucial role (Hammond et al., 2003).

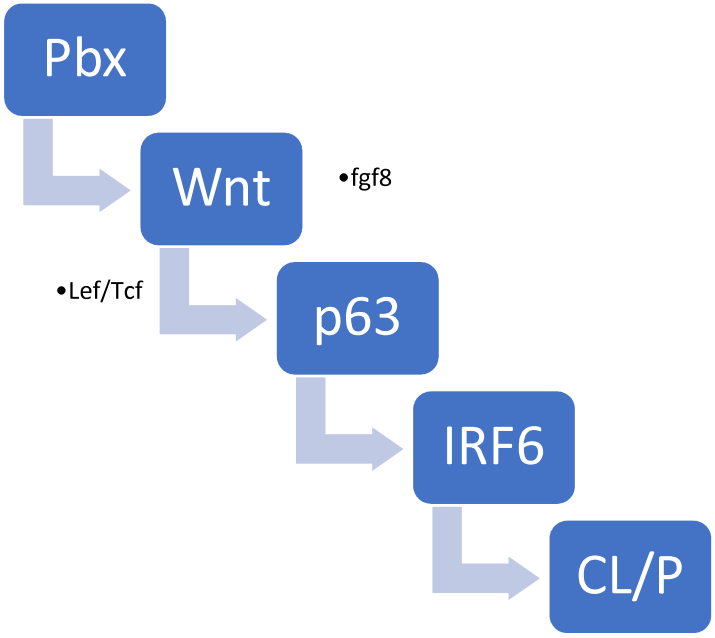


Figure 1.2 Role of specific genes

IRF6 doesn't work in isolation; (Figure 1.2) it's part of a complex network. Pbx genes, which influence epithelial proliferation and differentiation, interact with *IRF6*. Loss of Pbx genes affects the Wnt-p63-*IRF6* regulatory network and can lead to cleft lip and palate (Kurosaka et al., 2014). Another essential gene is P63, upregulated by Pbx genes through Wnt9b/Wnt3. P63, in turn, enhances *IRF6* expression in specific craniofacial domains, and its mutations can result in ectodermal dysplasia and CLP.

Recent research has unveiled a connection between Receptor-interacting protein kinase 4 (*RIPK4*) and *IRF6*, tying different popliteal pterygium syndromes to a shared molecular pathway. *RIPK4* impacts *IRF6* expression via protein kinase C. Mutations in RIPK4 can downregulate RIPK4-mediated activation of *IRF6* translocation (Xu et al., 2020). Serine residues -413 and -424 in *IRF6* play pivotal roles in its activation by *RIPK4*. The AP-2 transcription factor family, specifically AP-2α, plays a role in craniofacial development, and mutations can lead to branchio-oculo-facial syndrome. Another pathway associated with *IRF6* activity is the RhoA pathway (Figure 1.3). *IRF6*-deficient keratinocytes exhibit increased RhoA activity. Blocking Rho-associated protein kinase (*ROCK*) rescues delayed wound healing, indicating the pathway's importance (Kwa et al., 2016).

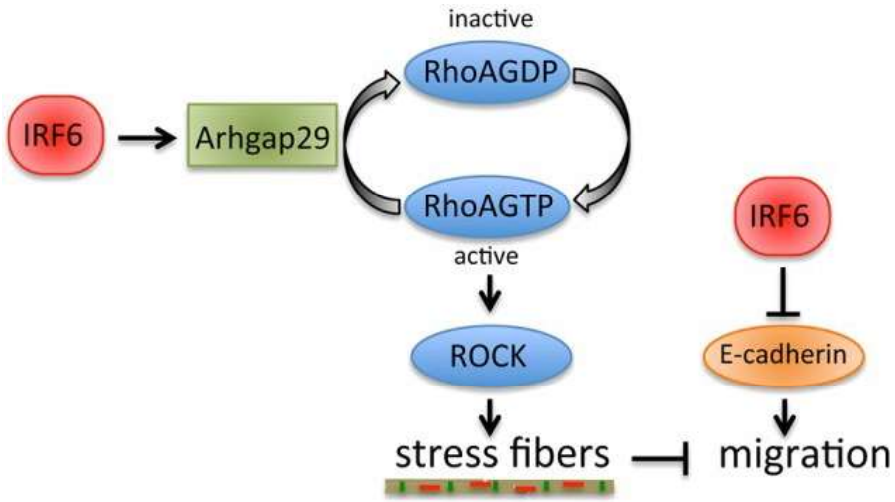


Figure 1.3 Involvement of various factors in NSCLP development

While *IRF6* is a significant player in CLP, it is only one part of the intricate genetic puzzle. The etiology of non-syndromic cleft lip with or without cleft palate (NSCLP) is multifactorial, involving numerous environmental and genetic factors (Girardi et al., 2011). Understanding the precise roles of genes like *CRISPLD2* in NSCLP remains a challenge. Allelic association and genetic linkage studies have highlighted *CRISPLD2*'s

potential involvement in NSCLP, emphasizing its significance in cleft lip palate development (Chiquet et al., 2008). *CRISPLD2*, the gene closest to the D16S3037 marker on chromosome 16q24.1, has been implicated in oropharyngeal and palatal development. Studies have linked *CRISPLD2* to neural crest cell migration and apoptosis, jaw and palatal anomalies, and cleft lip palate development (Yuan et al., 2012). While some polymorphisms like rs4783099, rs16974880, and rs1546124 have been associated with cleft lip, the multifactorial nature of this condition suggests that more markers on *CRISPLD2* and the specific role of SNPs warrant further investigation (Neela et al., 2020). Unravelling the genetic determinants of orofacial clefts, particularly cleft lip and palate, is an ongoing and intricate endeavour. While genes like *IRF6* and *CRISPLD2* have emerged as critical players, the comprehensive gene regulatory network and factors governing their functions continue to be subjects of intense research.

1.2.2 Environmental Factors

In addition to genetic factors, an array of environmental elements has been identified as contributors to the formation of orofacial clefts (Prabhu et al., 2012). These environmental factors play a significant role in the multifactorial etiology of cleft lip and palate. Maternal Smoking and Alcohol Consumption: One well-established environmental risk factor for orofacial clefts is maternal smoking during pregnancy. This habit has consistently been linked to an increased risk of cleft lip and palate. While the evidence on the effects of maternal alcohol consumption during pregnancy has been somewhat inconsistent, emerging research suggests that certain gene variants with reduced enzymatic activity, in combination with maternal alcohol use, could elevate the risk of orofacial clefts (Prabhu et al., 2012). Nutrition during pregnancy is another critical environmental factor. Pregnant women are often advised to take supplements such as folic acid, which has been associated with a reduced risk of cleft lip and palate. However,

studies examining the effects of other nutrients and general multivitamin use have yielded inconclusive results (Prabhu et al., 2012).

1.2.3 Other Factors: The Complex Interplay of Ethnicity, Geography, and Socioeconomics

Orofacial clefts exhibit variations in incidence across different ethnic, racial, geographic, and socioeconomic groups. These factors add complexity to our understanding of cleft lip and palate etiology (Pengelly et al., 2016). Multifactorial Nature of Non-Syndromic Clefts: Non-syndromic cleft lip and palate (NSCLP) is considered a multifactorial condition, characterized by isolated clefts, distinguishing it from monogenic, syndromic forms where clefts are part of a spectrum of phenotypes (Pengelly et al., 2016). NSCLP results from a complex interplay between genetic and environmental risk factors, influencing the likelihood of occurrence. Approximately 70% of all cases of cleft lip and palate and 50% of cleft lip-only cases fall under the non-syndromic category (Pengelly et al., 2016). The etiology of non-syndromic cleft lip and palate is multifaceted, involving both genetic and environmental factors.

1.3 Distribution and Prevention: Understanding, Managing, and Preventing Orofacial Clefts

Orofacial clefts (OFCs) are a significant concern globally, affecting a considerable number of individuals. Understanding their prevalence, the role of cytogenetic factors, and effective prevention and management strategies is crucial for addressing this congenital condition.

1.3.1 Prevalence: A Global Perspective

India, as one of the most populous countries globally, faces a substantial burden when it comes to orofacial clefts. With an estimated annual birth rate of approximately 24.5

million, India witnesses between 27,000 and 33,000 births with clefts each year. This prevalence, however, varies concerning gender and cleft pattern (Mossey and Catilla, 2003). Male predominance is consistently observed in cases of cleft lip with cleft palate (CLP), with a male-to-female sex ratio of 1.81 (CI 95%: 1.75-1.086). On the other hand, isolated cleft palate only counts more females than males, with a sex ratio of 0.93 (95% CI, 0.89-0.96) globally (Mossey & Catilla, 2003). Cleft palate, specifically, has a sex ratio reported as 0.93 (CI 95%: 0.89-0.96).

1.3.2 Cytogenetic Studies

Non-syndromic cleft lip and palate (NSCLP) is a complex disorder marked by etiological heterogeneity, challenging traditional Mendelian inheritance patterns (Chiquet et al., 2007). Beyond genetic and environmental heterogeneity, gene-environment interactions and gene-gene interactions contribute to cleft formation. Early genetic studies on CLP by Fogh–Anderson in 1942 laid the foundation for population-based investigations. Syndromic CLP cases, which account for approximately 30% of cases, exhibit additional chromosomal abnormalities, such as trisomy 13 (Patau syndrome), trisomy 21 (Down syndrome), trisomy 18 (Edward syndrome), deletion of 22q11.21 (DiGeorge syndrome), and autosomal dominant disorders like Teacher Collins syndrome involving chromosome 5q32-q33. Animal studies, such as the one conducted by Zunyi Zhang et al. (2002) in mice, have demonstrated severe craniofacial abnormalities associated with Msx1, a gene that plays a pivotal role in Bmp4 and Bmp2 expression in mesenchyme and Shh in medial epithelium. Research indicates that Msx1 is critical for regulating genetic pathways involved in palatogenesis through BMP and Shh (Zunyi Zhang et al., 2002).

1.3.3 Prevention and Management: Addressing the Challenge

Preventing orofacial clefts necessitates a comprehensive understanding of their multifactorial etiology. Investigating lifestyle and dietary patterns contributing to environmental factors is crucial. The influence of habits, including medication, alcohol, or tobacco, must be thoroughly explored before pregnancy. Notably, folic acid supplements have been recommended as a potential preventive measure (Prabhu et al., 2012). Managing orofacial clefts typically involves surgical procedures aimed at reconstructing or altering the lip or palate. Palatoplasty is a common procedure that enhances speech function, appearance, and breathing (Schönweiler et al., 1999). Effective management necessitates a multidisciplinary approach involving pediatricians, orthodontists, cleft surgeons, speech therapists, and other specialists.

In conclusion, orofacial clefts are a significant global health concern. Understanding their prevalence, genetic underpinnings, and prevention and management strategies is essential. Research focusing on the association between NSCLP, chromosomal abnormalities, and specific genes like *IRF6* and *CRISPLD2* holds promise for addressing the challenges posed by these anomalies.

CHAPTER II: OBJECTIVES AND RELEVANCE OF THE STUDY

2. OBJECTIVES AND RELEVANCE OF THE STUDY

2.1 Objectives

1. To study the cytogenetic variants among the individuals with cleft lip and Palate.

2. To analyze the parental karyotype for inherent familial predisposition if any.

3. To study the presence of molecular genetic level variants rs2235371(*IRF6* gene) and rs8061351 (*CRISPLD2* gene) in non-syndromic cleft lip and palate.

4. To analyze the association of the rs2235371 and rs8061351 polymorphism with non-syndromic cleft lip and palate

2.2 Relevance of The Study

More research about orofacial clefts is needed to make up for the lack of awareness among the general population. There is a need to recognise how orofacial clefts could hinder one's personal growth and impact their quality of life. Proper education of healthcare workers can be beneficial for patients who currently do not receive the best care and treatment. Research could also identify predisposing factors that could help invent preventative measures to be taken early on during developmental stages. There is also the need for proper counseling and tools that could equip persons with the abnormality to cope.

In the recent years, advances in genetics and molecular biology have begun to reveal the basis of craniofacial development. Understanding the reason for clefting disorders is critical for diagnosis, treatment, and assessing recurrence risk in family. Hence there is a need for thorough analysis on epidemiological, phenotypical and genotypical variations in orofacial clefts in the establishment of geographical, environmental, and genetic risks. The aim of this study is to determine the contribution of chromosomal abnormalities and

association of *CRISPLD2* gene and *IRF6* gene to NSCLP in patients. Hundred and thirty individuals with non-syndromic cleft lip and palate patients and hundred and thirty healthy controls will be recruited from the Charles Pinto Centre for cleft lip, palate and craniofacial anomalies at Jubilee Mission Medical College & Research Institute, Kerala. Four ml of peripheral blood samples was collected in EDTA and heparin vacutainers from 130 patients, 130 controls and their parents (if needed) for karyotyping and Single nucleotide polymorphism analysis. The study is preliminary work in the field of orofacial clefts. This study can help in the improvement of quality care and to determine the genetic etiology of clefts.

CHAPTER III: REVIEW OF LITERATURE

3. REVIEW OF LITERATURE

In the field of congenital cleft classification, numerous systems have been proposed over the years, each offering a distinct perspective on the categorization of these conditions. This literature review provides an overview of some of the prominent classification systems developed by various researchers and clinicians.

3.1 Classification

The journey of classifying congenital clefts began in 1922 with Davis and Ritchie, who categorized clefts based on their location relative to the alveolar process. They divided clefts into three groups (Figure 3.1):

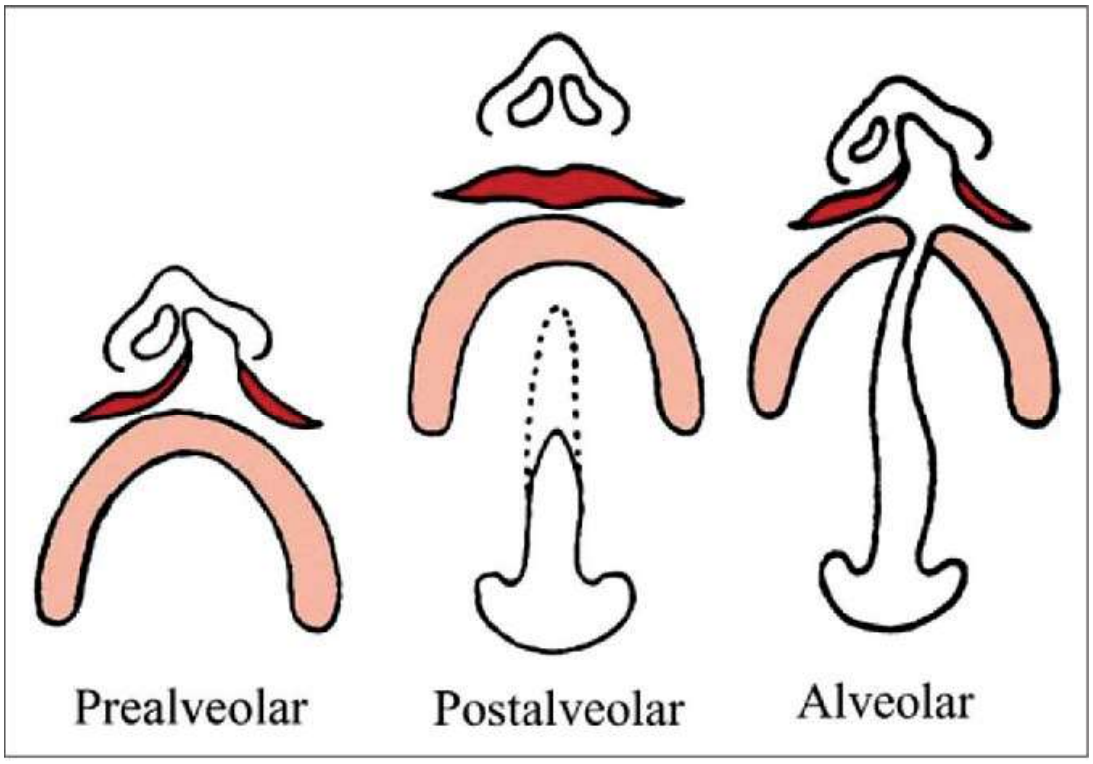


Figure 3.1 Congenital cleft classification

Group I: Pre-alveolar clefts, including unilateral, median, or bilateral clefts.

Group II: Post-alveolar clefts involving the soft palate, the soft and hard palates, or a submucous cleft.

Group III: Alveolar clefts, comprising unilateral, bilateral, or median clefts.

This early classification provided a fundamental framework for further developments.

3.1.1 Veau Classification

Veau introduced an alternative classification system, which categorized clefts into four types (Figure 3.2):

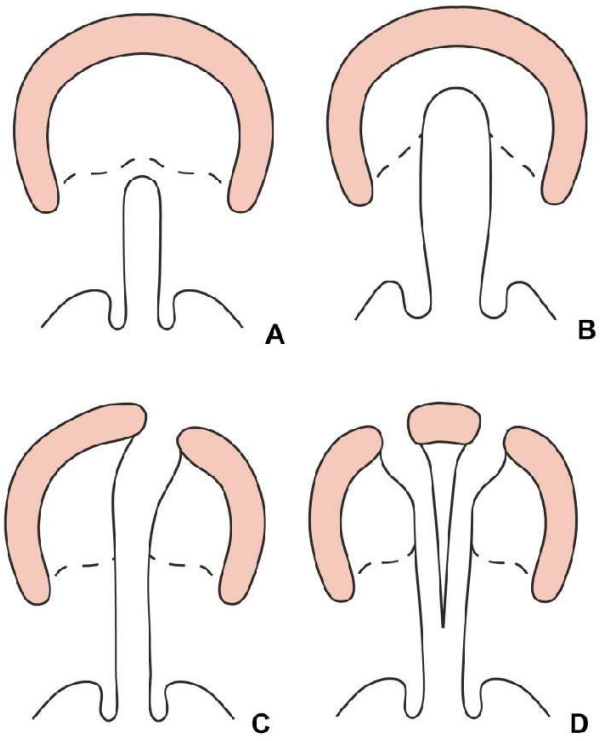


Figure 3. 2 Veau classification

Type 1: Cleft of the soft palate only.

Type 2: Cleft of the soft and hard palate, involving the secondary palate alone.

Type 3: Complete unilateral cleft (lip, soft, hard, and alveolar ridge).

Type 4: Complete bilateral cleft (lip, soft, hard, and alveolar ridge).

This classification system added depth to the understanding of cleft variations.

3.1.2 Fogh-Andersen Classification

Fogh-Andersen introduced a classification system based on embryological development, dividing clefts (Table 3.1) into three groups: clefts of the lip (unilateral or bilateral), clefts of the lip and palate, and clefts of the lip and palate extending up to the incisive foramina.

Table 3.1 The revised system of classification

Cleft of Primary Palate	Cleft Lip • Unilateral: right or left • Bilateral: • Median • Prolabium: small, medium, large • Congenital scar: right, left, median
	Cleft of Alveolar Process • Unilateral: right, left • Bilateral • Median • Submucous • Absent incisor tooth
Cleft of palate	Soft palate
	Hard Palate
Mandibular Process Clefts	Lip

	Mandible
	Lip pits
Naso-ocular	Extending from the narial region toward the medial canthal region.
Oro ocular	Extending from the angle of the mouth toward the palpebral fissure.
Oro aural	Extending from the angle of the mouth toward the auricle.

3.1.3 Kernahan and Stark Classification

Kernahan and Stark emphasized embryology-based classification and categorized clefts into three groups: clefts of the primary palate, clefts of the secondary palate, and combined clefts (Table 3.3) (clefts involving both primary and secondary palates).

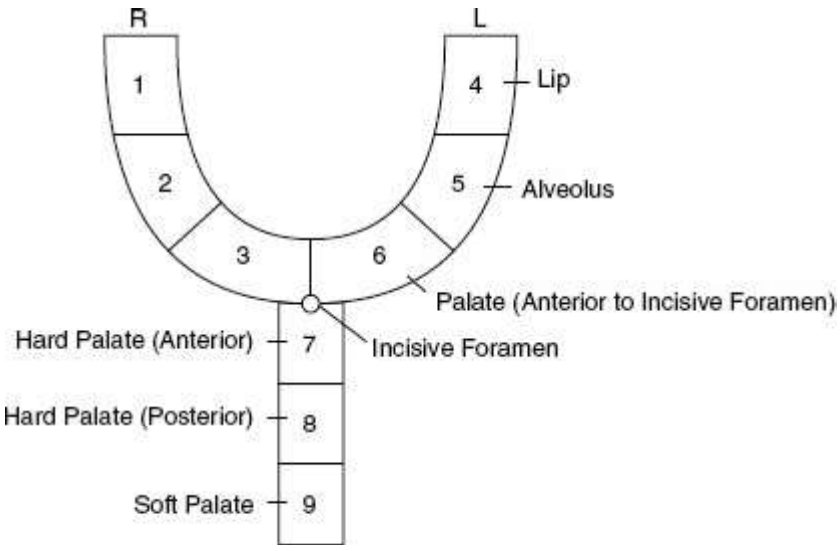


Figure 3.3 Y symbolic classification

3.1.4 Symbolic Representations

Several symbolic representations were introduced to simplify cleft classification. Pfeiffer introduced a pentagonal structure with six rectangles representing different aspects of clefts on top of a triangle, while Kernahan introduced a stripped Y symbolic classification (Figure 3.3 and Figure 3.5) to describe unusual deformities (Figure 3.4).

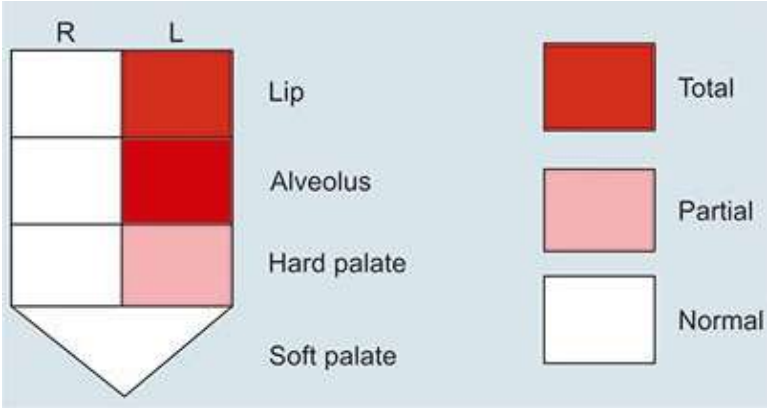


Figure 3.4 Symbolic representation of cleft lip classification

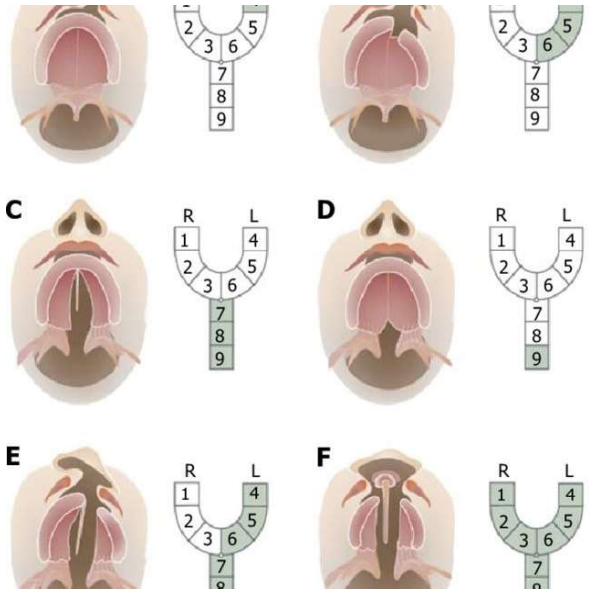


Figure 3.5 Y symbolic classification

3.1.5 Tessier Classification

In 1976, Tessier introduced a clockface analogy (Figure 3.5-3.6). from 0-14 to provide a precise classification of craniofacial clefting. Numbers indicated the location and extent of the cleft, dividing them into four groups: midline, paramedian, orbital, and lateral clefts.

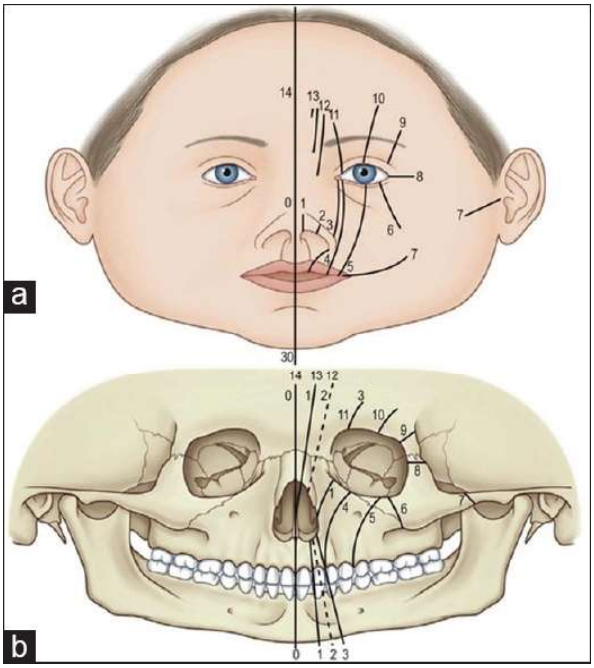


Figure 3.6 Tessier classification

3.1.6 Lahshal System

Kriens introduced the Lahshal system in 1989, which described the site, size, extent, and type of cleft using letters representing Lip, Alveolus, Soft palate, and Hard palate. Right-sided letters indicated left-sided clefts and vice versa. However, this system had limitations in explaining complex clefts.

3.1.7 Smith Modification

In 1998, Smith et al. updated the Kernahan 'Y' classification to provide more detailed descriptions of cleft deformities.

3.1.8 Friedman Modified Classification

Friedman et al. in 1991 combined elements from Elsahy's and Millard's schemes and integrated microforms of clefts, assigning scores based on severity.

3.1.9 LAHSN Classification

Koch and Koch introduced the LAHSN classification in 1995, expanding it to include Vomer and microforms in addition to palate, lip, and alveoli. It considered the complexity of malformation in multiple dimensions.

3.1.10 Mortier's Dual Scale System

In 1997, Mortier introduced a dual scale system consisting of Initial Severity Score (ISS) and Post Operative Result Score (PRS) for objective assessment of surgical procedures.

3.1.11 Tolarova and Cervenka's Nine Major Groups

Tolarova and Cervenka, in 1998, classified facial clefts into nine major groups, encompassing various anomalies, syndromes, and teratogenic effects.

3.1.12 LAPAL System

In 2007, the LAPAL system was introduced, which employed five Arabic numerals to denote the severity of clefts in different anatomical components, including lip, alveolus, hard palate, soft palate, and nose (Table 3.2)

Table 3.2 LAPAL system

Side	Right		Middle	Left	
Anatomic component	Lip	Alveolus and Primary palate	Palate	Alveolus and Primary palate	Lip
Complete	4	4	4	4	4
Larger than half	3	3	3	3	3
Smaller than half	2	2	2	2	2
Subcutaneous or Submucous	1	1	1	1	1
Intact	0	0	0	0	0

Table: Numbering method of LAPAL system illustrated in table.

Example-: A cleft soft palate and submucous cleft: 00200

3.1.13 Ortiz's Numerical Scoring System

In 2009, M. R. Ortiz et al. proposed a detailed numerical scoring system for both primary and secondary cleft palates, considering the complexity of clefts. (Table 3.3)

Table 3.3 Score assigned to the clefts in Primary Palate

Primary palate	Score
Normal	0
Microform 1	1
Incomplete 1/3	3
Incomplete 2/3	6
Complete with contact of segments	12

3.1.14 Lima Clock Diagram

The Lima clock diagram (Figure 3.7), introduced by Percy Rossell-Perry in 2009, offers a circular representation divided into four sections, further subdivided into severity segments, providing a comprehensive classification of clefts.

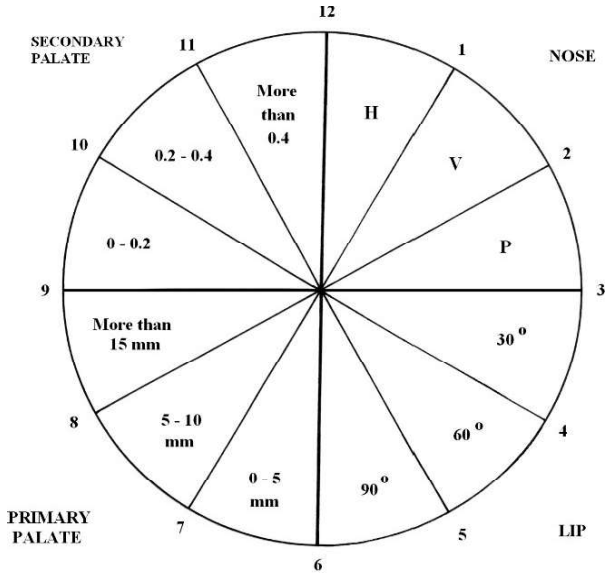


Figure 3.7 Lima clock diagram

3.2 Embryology

Cleft lip and cleft palate represent the most common congenital malformations within the craniofacial region (Cobourne, 2004). These conditions result from the failure of fusion among structures that form various parts of the lip and palate. The development of facial structures commences by the fourth week of pregnancy and is largely completed by the eighth week, with the exception of the palate. This intricate process involves the migration and fusion of cranial neural crest cells (CNC), originating from the anterior neural tube, with mesodermal cells, leading to the formation of facial primordia (Wu et al., 2017). These facial primordia consist of the frontonasal prominence, a pair of

maxillary processes, and a pair of mandibular processes, all of which encircle the primitive oral cavity, forming the basis for facial development.

The occurrence of facial clefts can be attributed to the abnormal fusion of these processes and the subsequent persistence or obliteration of the grooves between the prominences (Wu et al., 2017). Notably, the frontonasal prominence contributes to the development of the forehead and nose. During the fourth week of pregnancy, a nasal placode begins to form within the frontonasal prominence, marked by the thickening of the ectodermal surface on the embryo's lateral aspect. As development progresses (Figure 3.8), the nasal placode divides the frontonasal prominence, giving rise to a paired medial nasal process and a lateral nasal process. These processes contribute to the formation of the nose, with the medial processes forming the nasal septum, premaxilla of the nose, and the philtrum, while the lateral processes contribute to the sides of the nose.

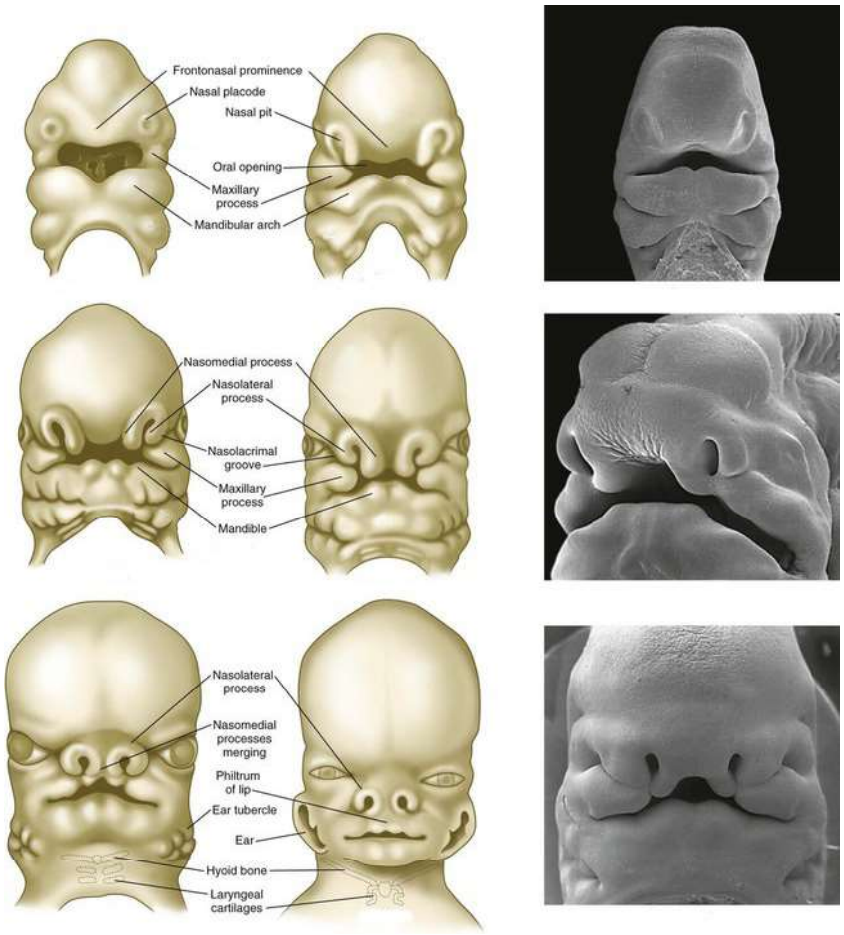


Figure 3.8 Development of nose and face [Courtesy: https://teachmeanatomy.info/the-basics/embryology/head-neck/face-palate/]

The maxillary processes extend toward each other and the paired medial nasal processes, eventually forming the upper lip and the triangular primary palate as they merge with the medial and lateral nasal processes. Although the primary palate comprises only ten percent of the palate, the development of the secondary palate, consisting of both hard and soft palates, is of paramount importance. In humans, secondary palate development occurs between the sixth and eighth weeks of gestation. It involves the creation of the roof of the mouth and the nasal floor. Bilateral extension of palatal shelves from the maxillary processes into the oral cavity is a key feature of this process.

The growth of palatal shelves is driven by the survival and proliferation of mesenchymal cells originating from neural crest (NC) cells and mesodermal cells from the first

pharyngeal arch. Simultaneously, the nasal septum extends downward from the stomodeum's roof, dividing the nasal portion of the oronasal cavity. The palatal shelves are raised to a horizontal position above the advancing tongue, a process facilitated by intrinsic shelf elevation forces, including the hydration of extracellular matrix (ECM) components like glycosaminoglycans (e.g., hyaluronan) in the shelf mesenchyme.

Maintaining the correct structural shape of the palate shelf relies on rapid ECM remodeling. Subsequently, the medial edge epithelium (MEE) of opposite palate shelves forms a midline epithelial seam (MES) through interactions involving cell adhesion molecules and desmosomes. This paves the way for palatal fusion as the MES undergoes rapid degradation through apoptosis, epithelial cell migration, and epithelial-mesenchymal transformation, ensuring mesenchymal continuity and the formation of the secondary palate. The fusion of palate shelves with the primary palate anteriorly divides the oronasal cavity into oral and nasal cavities. The soft palate and uvula form through the fusion of the palatal shelf behind the secondary nasal septum. Palatal shelf elevation and fusion progress in an anterior to posterior direction, with palatogenesis typically completed by the 12th week of development (Wu et al., 2017).

Depending on the timing of intervention during embryonic development, various types of clefts may arise, including typical and atypical forms. Typical facial clefts involve clefts of the lip and/or palate, while atypical clefts encompass median and oblique variations. These clefts may present as unilateral or bilateral, complete or incomplete (Cobourne, 2004). All these factors stimulate and initiate arrays of biological progressions, including cell migration, adhesion, transition, and apoptosis, leading to the formation of the palate.

Cell–cell adhesion plays a crucial role in craniofacial development, with the *CRISPLD2* gene being a significant contributor (Smith et al., 2019). Located on chromosome

16q24.1, *CRISPLD2* spans approximately 8.95 kb and contains 14 exons coding for a 497 amino acid polypeptide. It has been implicated in the migration and differentiation of neural crest cells due to the presence of an LCCL domain, shared with other proteins involved in cellular migration (Smith et al., 2019).

Another pivotal gene involved in the formation and maintenance of oral periderm is the interferon regulatory factor-6 *(IRF6)* (Kondo et al., 2002). Situated on the 'q' arm of chromosome 1, between positions 32.3 and 41, *IRF6* plays a prime role in proper palate adhesion through spatiotemporal regulation (Kondo et al., 2002). Mutations in *IRF6* have been associated with Van der Woude syndrome (VDWS) and popliteal pterygium syndrome (PPS), two common cleft lip syndromes (Kondo et al., 2002). VDWS, the more prevalent of the two, accounts for 2% of the total cleft lip and palate (CLP) population. It is characterized by cleft lip, cleft palate, isolated cleft palate, hypodontia, and mucous cysts. In addition to these common features, PPS presents with syngnathia, popliteal pterygium, genital and urinary organ malformations, and nail or toe abnormalities. Researchers have reported that these two syndromes are caused by different mutations of the same gene, and numerous studies have emphasized the significant role of *IRF6* mutations in the development of cleft lip and palate, contributing to a 12% risk of all common forms of CLP (Kondo et al., 2002).

Another crucial factor involved in cell adhesion during the elevation of palate shelves is *JAG2* (Jagged canonical notch ligand 2) (Bush & Jiang, 2012). It prevents premature adhesion of the palatal shelves to other oral tissues and works in concert with *IRF6* through the same molecular signaling pathways during epithelial differentiation (Bush & Jiang, 2012).

Research data has provided substantial support for the role of *TGFA* (Transforming growth factor alpha) in the development of NSCPO (non-syndromic cleft lip and palate only) (Rahimov et al., 2008). Encoded by a gene mapped at 2p13, *TGFA* functions as a normal epidermal growth factor (EGF)-related growth factor by binding to the epidermal growth factor receptor (EGFR) located in the palate epithelium (Rahimov et al., 2008). Alongside glucocorticoids, it regulates the proliferation, differentiation, and development of epithelial cells in the palate and exerts a negative feedback effect on palate fusion (Rahimov et al., 2008). Mutations in this specific gene affect the timing of expression, leading to the development of cleft lip and palate (Rahimov et al., 2008). Studies have reported an association between *TGFA* genotype and deficiencies in multivitamins containing folic acid, and recent research has also established a connection between cleft lip and palate and *TGFA* Taq 1 polymorphism (Rahimov et al., 2008).

The FOXE1 gene is actively involved in the etiology of NSCLP (non-syndromic cleft lip and palate) (Moreno et al., 2008). It belongs to the forkhead *(FOX)* or winged helix domain transcription factor family and is mapped to the q22 region of chromosome 9 (Moreno et al., 2008). A study conducted by Moreno et al. (2008) reported an association between cleft lip and palate and the *FOXE1* gene. Mutations in this gene can lead to Bamforth-Lazarus syndrome, which is characterized by cleft lip and palate, along with thyroid abnormalities (Moreno et al., 2008). *FOXE1* is expressed in the foregut endoderm during embryogenesis, and its mutation can result in both cleft lip and palate and thyroid.

The homeobox gene *MSX1* (Msh homeobox 1) (Figure 3.9) is a susceptibility gene for facial clefts, mainly non-syndromic, and is actively involved in cell proliferation, differentiation, and apoptosis (Kondo et al., 2002). The human *MSX1* gene is located at 4p16.1 and spans approximately 4.05 kb, consisting of two exons and an intron (Kondo et

al., 2002). Mutations in this gene have been linked to tooth agenesis and non-syndromic clefts (Kondo et al., 2002).

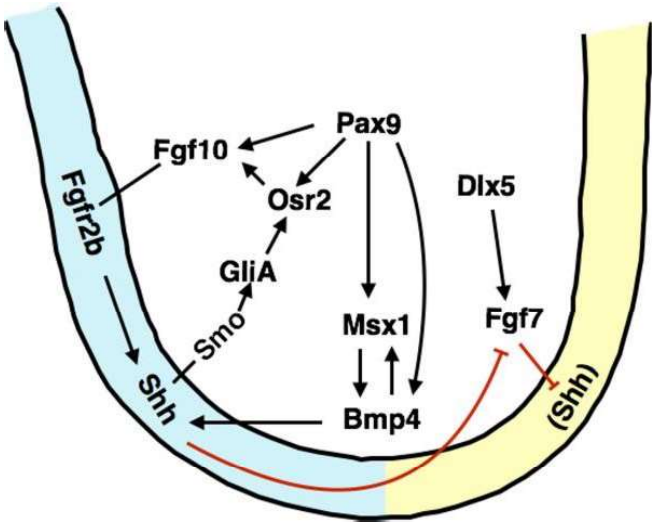


Figure 3.9 Cell proliferation

Another transcription factor, *TBX22* (T box factor 22), also plays a crucial role in mesenchymal proliferation and elevation (Kondo et al., 2002). Mutations in *TBX22* are associated with an X-linked hereditary disorder characterized by cleft lip and tongue tie (ankyloglossia) (Kondo et al., 2002). The expression of *TBX22* is primarily limited to the posterior palatal mesenchyme (Kondo et al., 2002).

The above-mentioned two genes, *MSX1* and *TBX22,* undergo post-transcriptional modification by *SUMO1* (small ubiquitin-like modifier 1) (Rahimov et al., 2008). *SUMO1* is a protein-coding gene involved in various cellular processes (Rahimov et al., 2008). A population study conducted on 383 individuals with non-syndromic cleft lip and palate (NSCLP) reported an association between cleft lip and *SUMO1* (rs3769817) (Rahimov et al., 2008).

Additional transcription factors involved in neural crest formation include TCOF1, a connective gene of Treacher Collins syndrome, and *GRHL3* (Grainyhead Like Transcription Factor 3) (Leslie & Marazita, 2013). *GRHL3* is another transcriptional factor crucial for craniofacial development and neural tube closure (Leslie & Marazita, 2013). It is considered the second candidate gene in Van der Woude syndrome (VWS) (Leslie & Marazita, 2013). De la Garza et al. (2013) reported that *GRHL3* is a target of the *IRF6* gene, further establishing its role in cleft lip and palate (rs41268753) (Leslie & Marazita, 2013).

Animal models lacking Fgf10 or its receptor Fgfrb2 exhibit abnormal palate adhesions with the mandible and tongue (Bush & Jiang, 2012). These models also display maintenance of jagged canonical notch ligand 2 (Jag2) expression, which is essential for maintaining the integrity of the periderm (Bush & Jiang, 2012). Jag2 expression is indirectly regulated by p63 through Fgfr2b (Bush & Jiang, 2012).

A cartilage-specific marker, *COL2A1*, is also involved in epithelial-mesenchymal transition, migration, and differentiation (Kondo et al., 2002). Mutations in *COL2A1* can lead to Pierre-Robin sequence and Stickler syndrome (Kondo et al., 2002).

3.3 Genetic Inferences

3.3.1 Interferon Regulatory factor 6 (*IRF6*)

Non-syndromic oral clefts are recognized as a multifactorial disease with a high prevalence worldwide, largely influenced by genetic factors (Marazita et al., 2002). However, identifying specific genes' exact roles in this context has proven challenging due to genetic heterogeneity, contributing to variations in observed outcomes (Blanton et al., 2005). Genomic screens have revealed overlapping regions potentially harboring clefting susceptibility loci (Prescott et al., 2000; Wyszynski, 2002).

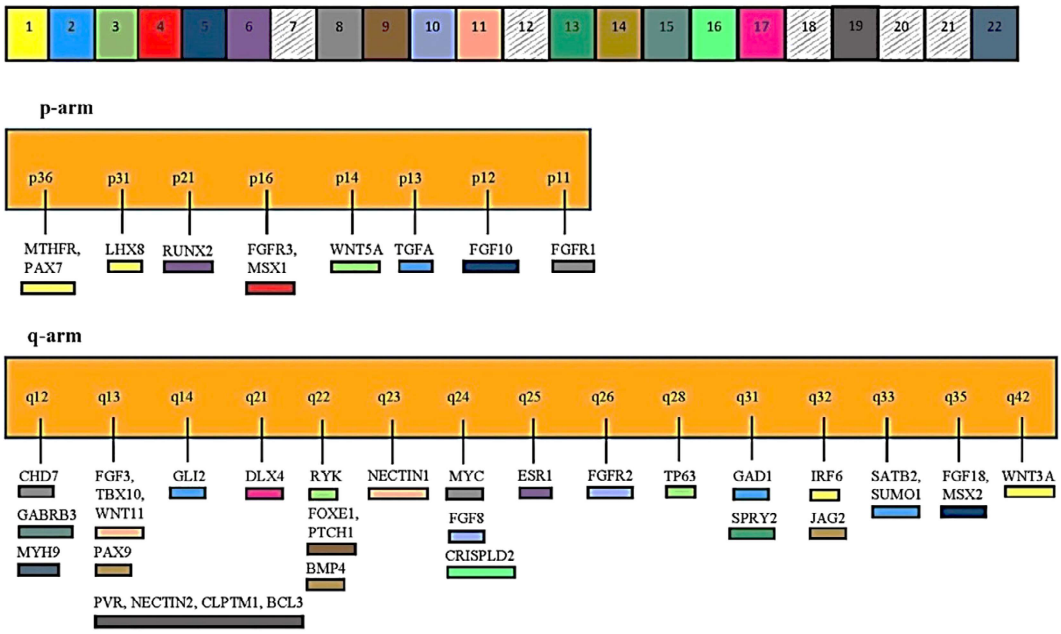


Figure 3. 10 Candidate genes located on different chromosomes involved in the aetiology of Non-syndromic CL/P

Many candidate genes (Figure 3.10) linked to non-syndromic cleft lip (NSCLP), such *as IRF6, FOXE1, BMP4, TGFB3, RARA, MTHFR, PAX7, PVRL1, RUNX2, TBX1, SATB2, BCL3, TGFB2, TGFB1, P63,* and *MSX2,* have been identified in studies worldwide (Funato and Nakamura, 2017; Letra et al., 2010; Mehrotra, 2015; Sull and Turconi, 2008). Among these, Interferon Regulatory Factor 6 *(IRF6)* variants consistently show a role in cleft lip and palate etiology across diverse populations.

IRF6 encodes a transcription factor crucial for ectodermal formation and craniofacial morphogenesis during embryonic development (Kondo et al., 2002; Gritli-Linde, 2008). Its dynamic expression in various embryonic tissues, including ectoderm, periderm, tooth germ, and oral epithelium, highlights its essential role (Kondo et al., 2002; Gritli-Linde, 2008). Loss of *IRF6* expression is implicated in genetic birth defects like Van der Woude (VWS) and Popliteal Pterygium syndromes (PPS) (Kondo et al., 2002). These syndromes

are characterized by cleft lip with or without cleft palate, skin abnormalities, and dental issues.

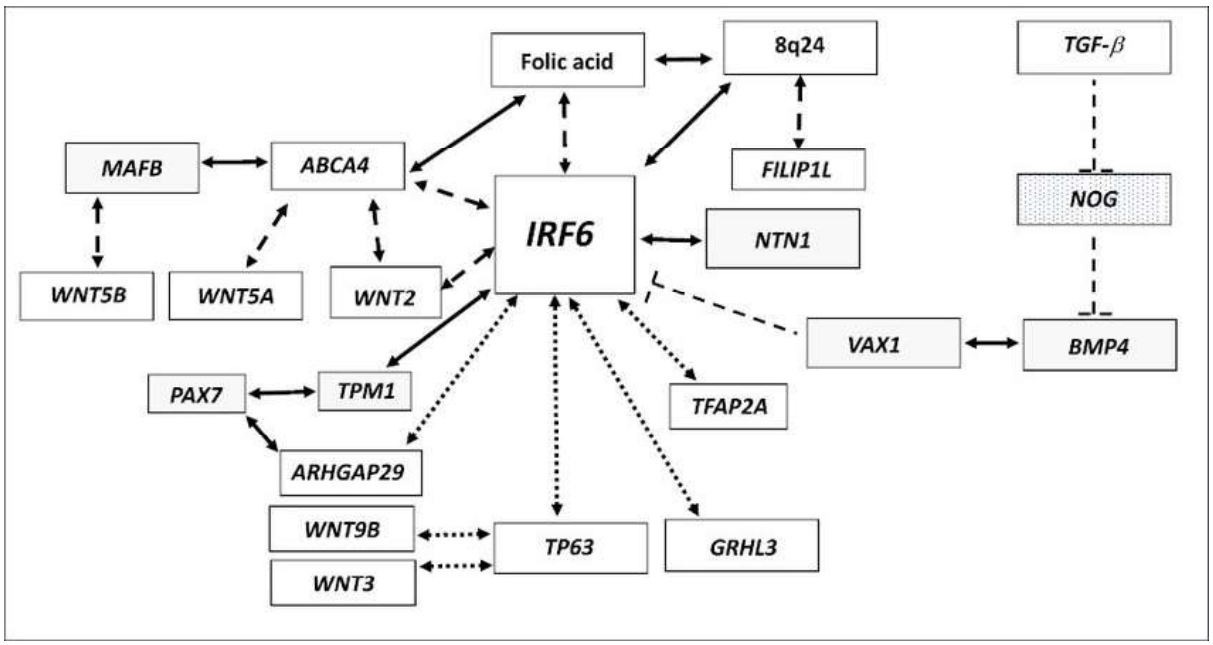


Figure 3.11 Schematic representation of the proposed gene–gene interactions involved in NSCLP

Specific *IRF6* mutations have been observed in families with these disorders, supporting the gene's role in NSCLP development (Kondo et al., 2002). Zucchero et al. (2004) analyzed 36 SNPs in *IRF6* among South American, European, and Asian populations, finding altered SNP transmission in their samples. Similar findings occurred when populations were analyzed separately. Additionally, Scapoli et al. (2005) investigated four SNPs and found altered transmission in the Italian population. Blanton et al. (2005) also confirmed altered transmission of *IRF6* in non-syndromic cleft lip and palate (NSCLP).

While many IRF genes' functions in the human population are understood, the precise role of *IRF6* remains unclear in some aspects. However, studies have shown that a Xenopus gene closely related to mouse IRF-6 is expressed during early development in the posterior mesoderm, contributing to craniofacial growth (Blanton et al., 2005).

Thompson et al. (2019) observed reduced craniofacial bone density, detected by CT, in a Brazilian population with *IRF6* gene influence. Zhao et al. (2018) identified a rare deleterious mutation in *IRF6* contributing to orofacial clefts. Studies across various populations have consistently found significant associations between multiple genes and non-syndromic cleft lip (Machado et al., 2021; Bezerra et al., 2020).

Martinelli et al. (2020) emphasized the importance of complex regulatory processes that control epithelial and mesenchymal cells in embryonic facial development, contributing to craniofacial malformations. Studies by Wu-Chou et al. (2019) and Xing et al. (2019) further confirmed the association between *IRF6* and NSCLP, especially the rs2235371 variant, in Asian populations.

A meta-analysis by Xia et al. (2017) highlighted the significant association between *IRF6* rs2235371 polymorphism and NSCLP risk. Ingraham et al. (2006) conducted studies in mice with *IRF6* gene knockdown, revealing abnormalities in skin, epidermis, limb, and craniofacial development, primarily linked to keratinocyte proliferation–differentiation. Ge et al. (2003) supported the association between non-syndromic cleft lip and palate and *IRF6* gene in the Xinjiang Uyghur population.

Rahimov et al. (2008) reported a strong association between rs2235371 and non-syndromic clefts in international populations. Ibarra-Arce et al. (2015) confirmed the association of *IRF6* polymorphisms with non-syndromic cleft lip in Mexican individuals. However, Paranaíba et al. (2010) found no significant statistical evidence linking *IRF6* rs2235371 polymorphisms to NSCLP development in the Brazilian population.

Jugessur et al. (2008) identified a fetal relative risk associated with NSCLP and the single allele A of rs2235371. Tang et al. (2009) reported an increased risk of NSCLP in Han-Chinese individuals with rs2235371 GG genotype, a missense mutation in exon 7 of

IRF6, shared with Amerindian and Asian populations. Xing et al. (2019) corroborated these findings in the Asian population, including Han Chinese.

In contrast, some studies, such as those by Srichomthong et al. (2005), Hering and Grundmann (2005), and Suazo et al. (2008), have reported a lack of significant linkage between NSCLP and rs2235371 polymorphism in various populations, indicating an unclear role for the gene. Recent research by Wang et al. (2019) in the Taiwanese population found no mutations in the *IRF6* gene.

Pegelow et al. (2008) also found no significant link between NSCLP children and rs2235371 allele frequencies and emphasized the need for larger sample sizes in further studies. Tan et al. (2008) reported no mutations in exons 3, 4, and 7 of *IRF6*, but they did detect polymorphisms near exon 7, not likely to induce complications.

To date, more than 200 mutations in *IRF6* have been identified across various ethnicities, with a concentration in exons 4 and 7. While evidence supports *IRF6's* potential role in NSCLP, more research is needed to explore interacting genes and polymorphisms, especially rs2235371 and rs8061351, in populations with a higher NSCLP incidence, such as Kerala, India. Little literature from this region has explored the role of *IRF6* and *CRISPLD2* in NSCLP, making further studies crucial for a comprehensive understanding of the genetic factors contributing to NSCLP.

3.3.2 Cysteine-rich secretory protein containing LCCL domain 2' gene (*CRISPLD2*)

Certain previous studies (Prescott et al., 2000) have reported that the CLP scan for the first time was carried out with 92 British sib pairs and recognized nine regions with relevant outcomes, containing a location on chromosome 16q. Other studies (Marazita et al., 2004) focusing on the same pattern of genomic scans worldwide, including the Indian region revealed the region of 16q21-24 accomplished prominent statistical significance

toward the linkage with CLP in several investigations. From this point of view in context with the chromosome 16q24.1, it was perceived that the 'cysteine-rich secretory protein containing LCCL domain 2' gene *(CRISPLD2)* has been located on the foresaid chromosome region along with a prominent link with the non-syndromic CLP in Hispanic populations and Caucasian populations (Chiquet et al., 2007).

In addition to the aforementioned inference discussed by them, they also noted *CRISPLD2* expression in the palate, nasopharynx, and mandible and specific areas during craniofacial development and feature recommended *CRISPLD2* gene as a unique candidate for investigating the NSCLP etiology. They have used the Taqman OD assay for the genotyping of four single nucleotide polymorphisms (SNPs) such as rs4783099 [3′ UTR], rs1546124 [5′ UTR], rs2326398 [Intron 8], and rs8061351 [Exon 4] within the *CRISPLD2* gene domain.

Some of the studies discussed the *CRISPLD2* gene polymorphisms rs16974880, rs1546124, and rs4783099 in the Chinese population. Their observations from an Indian perspective were reported by Neela et al. (2020). They also discussed that *CRISPLD2* is an important gene that has a prominent influence on cleft lip palate in the Irish and Chinese populations (Carter et al., 2010; Shen et al., 2011).

A study by Chiquet et al. (2007) witnessed that the rs8061351 in exon 4 showed evidence of a link with NSCLP in the Hispanic simplex population. In agreement with these observations, another investigation also genotyped the following SNPs belonging to the *CRISPLD2* domain to unveil the association perspectives linked with the cohort sample comprising a plethora of inhabitants from South America, Asia, North America, Northern and Eastern Europe; rs4783099, rs1546124, rs2326398, and rs8061351 (Letra et al., 2011). They have employed the applicability of Transmission Disequilibrium Tests (TDT)

to find the association between the complications linked with the clefting and four SNPs such as rs4783099, rs1546124, rs2326398, and rs8061351 in the *CRISPLD2* gene. Among the analyzed four SNPs, two of them such as rs8061351 and rs1546124 were found to be significant for the Caucasian population.

From their findings, it was perceived that they principally replicated the findings of an investigation by Chiquet et al. (2007) that recognized the *CRISPLD2* gene as a prominent candidate for NSCLP studies. The aforesaid gene has a size of approximately 8.95 kb along with 14 exons that are known to code for a 497 amino acid polypeptide. The studies concerning the exact role of the same are found to be not yet known in the current scenario, however, in situ hybridization studies on the above said gene expression in mouse tissues extended that the *CRISPLD2* gene encompassed significant role in the development of mandible, palate, naso- and oropharynx and nasal septum.

Ariadne Letra et al. (2011) in their study validates the effect of a synonymous (C471T, rs8061351) mutation in exon 4 towards association with the Hispanic cohort and the inhabitants from the Brazil region. They also found a strong effect of SNP rs1546124 and SNP rs8061351 on individuals of Caucasian ethnicity and South American origin respectively.

The influence of *CRISPLD2* gene on the development of cleft lip palate in the Irish and Chinese populations was reported by Carter et al. (2010) and Shen et al. (2011). The same perspective on the animal model was explained by Ge et al. (2018). One of the probable explanations for this kind of discrepancy was the presence of different risk alleles in the distinct populations from various parts over the globe.

Carter et al. (2010) studied 31 SNPs including the three SNPs such as rs8061351, rs1546124, and rs4783099 analyzed by Chiquet et al. (2007) in an Irish population consisting of 383 CP case-trios and 509 CL(P).

A study by Brewer et al. (1999a) discussed the probable role of duplications of 16p12-13, translocations of chromosome 16, and trisomy in cleft palate. Based on the findings from many of the studies, it was found that much of the Genetic research concerning the clefts focused on both link analysis and association analysis to find out the genetic determinants behind the same (Neela et al., 2020). For example, as reviewed in the previous strands, such results from the association studies accomplished toward several ethnicities, the populations may have been frequently either conflicting or inconclusive, with numerous candidate loci concerned in the cleft phenotypes (Neela et al., 2020).

From a Chinese scenario, a study by Shen et al. (2011) analyzed the involvement of the *CRISPLD2* gene in NS CL(P). They considered 463 healthy individuals and 444 patients with NSCLP for their study and found a strong association between the studied gene and the disorder. In addition to this, they suggested that the studied *CRISPLD2* gene (SNP rs1546124) strongly pays to the etiology of the disorder in the Northwestern Chinese residents. However, some kinds of controversy were reported from the previous investigations regarding the prominent role of the *CRISPLD2* gene in NSCLP. For instance, a study by Mijiti et al. (2015b) discussed that the allele frequency of T was found to be lesser than that of the allele frequency of C on rs8061351 of the studied gene *CRISPLD2.* However, a strong controversy regarding the aforesaid points was reported by Carter et al. (2010).

In addition to this, a study which was conducted in an Italian population by Girardi et al. (2011) clearly stands firm in their argument that depicts they did not support the various

hypothesis those states that *CRISPLD2* has no specific role in the development of NSCLP malformation in the human population, which justifies the significance of studying the *CRISPLD2* gene in context with the NSCLP. Hence in order to rectify the complications that emerged during such studies, Ge et al. (2018) performed a meta-analysis to validate whether the studied gene *CRISPLD2* is primarily linked with the NSCLP. The results from their meta-analysis strongly support the fact that the *CRISPLD2* gene instigated the chance of development of NSCLP threat in the studied populations. Their findings were also supported by another study (Messetti et al., 2017) conducted in the Brazilian region. According to them, it was evident that the rs8061351 may have a functional role in the NSCLP, however, it is essential to investigate the further aspects of the same for effective output. However, the exact role of the gene was not evident in many of the studies. For example, there exists no association found between the Colombian population, NSCLP and *CRISPLD2* gene, in disparity to the Texas–Hispanic population (Shi et al., 2010). Their finding chains the theory that NSCLP is found to be an etiologically heterogeneous disease and the genetic variation that occurs in different genes triggers the chance of NSCLP in different populations over the globe (Noroozi et al., 2020).

The interaction between the NSCLP and the *CRISPLD2* gene in the individuals belonging to the Xinjiang Uyghur region of China was validated by Mijiti et al. (2015a). They screened about 18 SNPs including the rs8061351 using next-generation sequencing in 200 individuals inhabited the Xinjiang Uyghur region of China and suggested that their preliminary analysis witnessed the genetic variation of the studied gene *CRISPLD2* is allied with NSCLP in the individuals belonging to the studied region. From this point of view, Shi et al. (2010) validate that the NSCLP susceptibility was strongly allied with the *CRISPLD2* in the Chinese population. However, they have only provided maximum consideration towards the following polymorphisms rather than rs8061351; rs16974880,

rs1546124, and rs4783099. As discussed by Messetti et al. (2017); Mijiti et al. (2015a) also exposed the fact that further studies are essential to express the direct functional link between the occurrence of NSCLP and the *CRISPLD2* gene. However, both of the authors were not provided extra consideration towards the rs8061351 with special inference on the NSCLP.

While the exact biological role of the *CRISPLD2* gene is still unclear in the Indian population, based on several previously reviewed studies, it was perceived that this novel gene was proven to be expressed during facial development. The predominant attention paid to these findings was further highlighted by a recent study that unveiled the interaction of *CRISPLD2* with *CRISPLD1* through a folate pathway (Moslemi et al., 2019). The role of mutation in *CRISPLD2* has also been verified in certain studies that address the adverse effect of the same on protein levels or transcription factors allied with the regulation of *CRISPLD2* expression (Gaikwad et al., 2020). Most of such studies were focused on the in situ hybridization at various stages of palatal development.

While considering the gene tracking in response to detecting the prominent role of a specific gene like *CRISPLD2* in non-syndromic clefts, a study by Rajendran et al. (2011) noted a few alleles along with augmented expression frequency in the analyzed group. This has revealed the fact that the corresponding alleles may influence the clefting in individuals. They also suggest that the results from their investigation could help the scientific community for early diagnosis of complications and palling sufficient approaches.

A little bit different way from other discussed points of view, an investigation by Ariadne Letra et al. (2011) slightly changed a common pattern to unveil the link between the incomplete cleft lip/palate and the synonymous mutation in exon 4 of the *CRISPLD2*

gene in the Hispanic families and the Brazilian population. In this point of view, Yuan et al. (2012) knockdown the *CRISPLD2* and observed a prominent range of abnormalities in the jaw and palatal region in a dose-dependent pattern. The loss of the same gene also resulted in abnormal patterning of neural crest cells (NCC) in the studied organism which signifies that the *CRISPLD2* is always essential for the normal formation of neural crest cells (NCC). In addition to the prime role of the *CRISPLD2* gene in NSCLP, certain studies have also investigated the various process allied with other characteristic features with special inference on neonatal lung inflammation, disruption of elastin, and distal airspace enlargement. For example, HU et al. (2020) found that the absence of the *CRISPLD2* gene may usually result in embryonic lethal conditions. The gene may also have a central role in fibroblast cell expansion in fetal lung and epithelial signaling. They also assessed the prominent effects of *CRISPLD2* on protein inflammatory mediators' expression in endogenous and exogenous route in human fetal and adult fibroblasts and epithelial cells.

3.3.3 Cytogenetics and Orofacial Cleft Formation

Orofacial clefts, including cleft lip and palate (CLP) and cleft palate only (CPO), are congenital malformations resulting from complex genetic interactions. Cytogenetic research (Table 3.4) has unveiled crucial connections between chromosomal abnormalities and orofacial clefts. Ingalls' 1963 study in mice demonstrated that administering 6-amino nicotinamide led to cleft palate and chromosomal fragmentation, establishing an early link between chromosomal anomalies and cleft formation. Moreover, specific mouse strains like A/WySn, A/Hej, and A/J have spontaneous clefting tendencies, and clf1 and clf2 genes, particularly those located on chromosome 11qE1, have been associated with cleft phenotypes, highlighting the significance of the Wnt gene family in craniofacial development. Additionally, gene studies using animal models have

revealed the importance of *IRF6, BMP* genes, *SOX* genes, and more, shedding light on the genetic complexities underlying orofacial clefts (Ingalls et al., 1963; Kwa et al., 2016).

Chromosomal anomalies further contribute to orofacial clefts. Brewer et al.'s 1998 chromosome map linked specific bands on chromosomes to cleft lip and palate, emphasizing the role of deletions and duplications in these conditions. Notably, bands 2q32, 4p16-13, and 4q31-35 exhibited strong correlations with cleft palate, while bands 1q21-25, 4p16-15, 4q31-35, and 7q34-35 were significantly associated with cleft lip (Brewer et al., 1998). Furthermore, Shi et al.'s 2009 gene analysis study pinpointed deletions of SUMO1, TBX1, and TFAP2A as potential etiological factors for cleft lip and palate, emphasizing the intricate genetic landscape contributing to orofacial cleft formation (Shi et al., 2009).

Table 3.4 Chromosomal Region and Type of Abnormality

Chromosomal Region	Type of Abnormality
1q21-25	Deletion
2p22.2	Deletion
2q37.1	Deletion
3p26-21	Duplication
4p/tetrasomy 9p	Deletion/Tetrasomy
4p16.3	Deletion
4p16-15	Deletion
4q31-35	Deletion
der(4)t(4;20)(q35;q13.1)	Trisomy
6p24	Deletion
7p15.3	Microdeletion
7q34-35	Deletion
10p15-11	Duplication
11p14-11	Duplication
13q22-34*	Duplication

In conclusion, cytogenetic studies have revealed a complex genetic framework for orofacial cleft formation. Chromosomal abnormalities and genetic mutations, along with the associations between specific genes, have provided critical insights into the etiology of these congenital malformations. This knowledge has profound implications for genetic counseling, personalized treatment strategies, and a deeper understanding of orofacial clefts' underlying genetic factors (Ingalls et al., 1963; Kwa et al., 2016; Brewer et al., 1998; Shi et al., 2009).

3.4 Complications

3.4.1 Common anomalies related to dental in CLP patients

The prevalence of dental anomalies strikingly increased in CLP in children. We get a thorough picture on this when we compare the anomalies in CLP children with the general population (Smith et al., 2010). Permanent and milk teeth can be affected. The cleft side can expect more dental abnormalities.

Based on the severity of the anomaly, the most usual dental abnormalities were supernumerary teeth, missing of maxillary incisors, usually the lateral one, and loss of lower incisors. The commonly affected tooth will be the lateral incisors in the maxilla. Studies revealed that, in a complete CLP, the most commonly lost tooth was the lateral incisors in the maxilla. To compare the dental abnormalities in both UCLP and BCLP patients, a study was conducted. Ninety-six cases were examined, out of which sixty-seven were UCLP and twenty-nine were BCLP. The study revealed that at least one dental anomaly was reported in both UCLP and BCLP cases in the ratio of 93% and 96%. Single and multiple missing teeth, malocclusion of the anterior denture was also reported (Chen et al., 2019).

The medical record review and radiographic review in 146 patients with CLP, by Menezes and Vieira, found that forty-seven patients out of this 146 showed at least one abnormality like non-formation of teeth, rudimentary teeth, impaction, and other structural deformities (Menezes & Vieira, 2020). Complication of anomaly increases in complete CLP patients when compared to incomplete CLP. Taurodontism, ectopic teeth, hypoplasia are other malformations associated with cleft palate and lip. Sexual dimorphism was not exhibited by any of the victims. The anomalies of teeth showed with different types of clefts.

Another study by Alkharboush reported that hypodontia is the commonly provided dental anomaly, which is followed by microdontia, unusual eruption, excess number of teeth, and macrodontia (Alkharboush et al., 2020). It is said that most of the complications can be treated or put a step to if detected in advance.

3.4.2 Psychological issues

In a first look, the adult and the children who face with CLP do not experience many psychological issues, but some specific issues may arrive. Among several problems reported, some of them have remarkable importance in their life. The facial expression, anxiety, disease-related depressions are noticeable among other factors. The facial deformity and the associated speech problem in children lead to depressions and anxiety and it may cause even the drop out from school or sometimes they never start their schooling (Brown & White, 2018).

The communication with others may get hindered by the teasing from different ways. The continuous teasing, they face from others results in impaired communication; sometimes they are not accepted by the teachers also. All these kinds of rejections finally lead to depression. The CLP patients develop low self-esteem and they may communicate only

with their family members. These situations isolate them from society. Strong psychological support is needed to maintain a healthy life in CLP patients.

Parents, teachers, family members, the system prevailing in school are important factors in society that influence self-esteem in CLP children. Interactions from these socially influencing factors can add negative experience in their life. A long duration of speech from their peer group may not occur in cleft lip children.

The family situation is another important factor. When a parent realized that their infant has abnormalities with the face, the total atmosphere of the family changes. Different kinds of emotions may arise. The shock, guilt, anxiety, etc. are very common in such family members. Such dissatisfaction in parents may lead them to behave in an indifferent way and they treat their ill child accordingly. All these factors create a negative impact on affected children. Some of them gradually, socially adapted while some others need extra care, mental support to lead a normal life in society.

3.4.3 Disabilities in communication faced by CLP patients

Communication disorders are another crucial problem faced by the CLP children. In the case of early cleft repair, patients show cleft palate speech (Zeraatkar et al., 2019). In this condition, atypical consonant sounds are produced by the patient, with unusual nasal sounds, impaired nasal airflow, a change in the laryngeal voice, grimaces of the nose and face. Alone the speech therapy can cure the problems. Speech can be assessed perceptually by utilizing different stimuli. Identifying compensatory and obligatory mistakes in articulations has to be identified. Nasoendoscopy or video fluoroscopy can be adopted if velopharyngeal dysfunction is felt. A child born with a cleft lip and palate faces difficulty with feeding also.

3.4.4 Result of cleft on speech

Studies revealed that, even with an early surgery, to repair a cleft palate, it is not helping in fluent speech in preschoolers (Ahmed et al., 2017). They show delayed speech and that also a typical cleft palate speech. The abnormalities in the orofacial structure and growth lead to errors in the production of speech. Cleft-related speech error can be of two types: Obligatory and compensatory (Smith et al., 2021).

3.4.5 Errors in speech and sound production

The abnormalities in the oral and nasal structures result in errors in producing speech in patients with cleft lip and palate. Their psychological development would also be disturbed. Among a wide variety of inaccuracies, the pressure consonants are more afflicted. Peryer et al in their studies concluded the different atypical consonant production which is usually seen in patients with cleft lip and palate (Peryer et al., 2021). Structural abnormalities like malaligned teeth, residual cleft, fistula of oral and nasal cavity lead to obligatory errors. Such errors cannot be rectified through speech therapy unless the structural malformation is corrected. The linguistic level of the patient is also to be checked as it is important and includes the sentence and conversation. Adequate care should be given in children with cleft lip and related anomalies to evolve the controlled single sentence stimulus. This has to be done by giving importance to the pressure consonants.

3.4.6 Unusual nasal resonance and flow of air

Individuals with cleft in lip and palate face a characteristic unusual resonance. The size of the oronasopharyngeal cavities mainly determines the speech resonance. Hyper or hypo nasality can be expected in these patients. But in some cases, both hypo and hyper nasality coexist. An adenoid hypertrophy can also obstruct the nasopharynx and result in

abnormal resonance. Velopharyngeal mislearning is another reason for hypernasality (Park et al., 2019).

Hypernasal resonance along with disrupted nasal airflow can be resulted from large-sized oronasal fistula and defective velopharynx. Velopharyngeal dysfunction (VPD) could be structural or neurological. This condition is due to the lack of tissue or absence of proper movement of walls. The velopharyngeal incompetence is associated with cleft and palate abnormalities (Ahl et al., 2016). An effective and continuous speech is initiated by the VPD.

The nasal resonance can be evaluated by another method called nasometry. This procedure does not allow the practitioner to visualize the VP area as it is a non-invasive method. A numerical value is given out of the instrument, and this output gives an idea regarding the acoustic energy while speaking.

3.4.7 Results of the cleft on sound production

Dysphonia is exhibited by the children with cleft lip and palate (Nagarajan et al., 2009). Breathiness, hoarseness mild intensity of sounds during speech are the main traits of the dysphonia. Usually, this problem arises due to high muscular and respiratory effort and also due to the hyper adducted vocal cord.

CHAPTER IV: MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Study Overview

This research project took place at the Jubilee Center for Medical Research, Jubilee Mission Medical College and Research Institute in Thrissur. The study spanned from June 2016 to June 2022.

4.2 Study Design

The study employed a case-control design and focused on patients with Non-Syndromic Cleft Lip and Palate (NSCLP). It includes only cases presented and diagnosed at Jubilee Mission Medical College and Research Institute and did not include any largescale field-based population survey.

4.3 Selection Criteria

4.3.1 Inclusion Criteria-cases

- Participants had to be clinically diagnosed with cleft lip and palate.
- Cleft lip and palate patients were included only if they did not have any other congenital abnormalities.
- Patients with a family history of cleft lip and palate were eligible to participate.

4.3.2 Inclusion Criteria- Control Samples

- Control participants were required to have no personal or family history of orofacial clefts.

4.3.3 Exclusion Criteria- Cases

- Cleft lip and palate patients with a family history of other genetic diseases were excluded from the study.
- Individuals with any other congenital abnormalities were not included in the study.

4.3.4 Exclusion Criteria-Control Samples

- Individuals with any chronic or active diseases were excluded from the control group.

4.4 Ethical Considerations

Ethical clearance for this study was obtained from the Institutional Ethical Clearance Committee of JMMC & RI under the reference number 38/19/IEC/JMMC&RI. All participants received detailed information about the research project, and their informed consent was obtained before their inclusion in the study. Annexure-1

4.5 Sample Size Calculation

Sample Size is calculated using South Asian Bio project details available at

https://www.ncbi.nlm.nih.gov/snp/rs2235371#frequency_tab

Population size (for finite population correction factor or fpc)(*N*):	1000000
Hypothesized % frequency of outcome factor in the population (*p*):	9%+/-5
Confidence limits as % of 100(absolute +/- %)(*d*):	5%
Design effect (for cluster surveys-*DEFF*):	1

Sample Size(*n*) for Various Confidence Levels

Confidence	Level(%)	Sample Size
80%		54
90%		89
95%		**126**
97%		155
99%		218
99.9%		355
99.99%		496

Sample size $n = [DEFF*Np(1-p)]/ [(d^2/Z^2_{1-\alpha/2}*(N-1)+p*(1-p)]$

Results from OpenEpi, Version 3, open-source calculator-SSPropor Print from the browser with ctrl-P. The sample size has rounded up to 130 (95% Confidence) in each group (for *IRF6* polymorphism -NSCLP and Controls, The *CRISPLD2* polymorphism is not widely studied and population-based reference is not currently available, even though the role of *CRISPLD2* in the formation of orofacial cleft is established animal models, the same samples size will be used for both the polymorphisms)

4.6 Study Population

All patients with orofacial cleft were carefully evaluated, diagnosed independently, and screened for the presence of anomalies and possible associated syndromes by the

clinicians in the Charles pinto centre for cleft lip, palate and craniofacial anomalies, JMMC & RI. A proforma with pedigree, medical history and consent form was filled and obtained from the patients or their guardians. The samples were grouped accordingly to Cleft Lip Only, Cleft Palate Only and Cleft Lip and Palate. The criteria used from CDC 10 is given below

- *ICD10-Q35 Cleft palate*

- Q35.1 Cleft hard palate
- Q35.3 Cleft soft palate
- Q35.5 Cleft hard palate with cleft soft palate
- Q35.7 Cleft uvula

- *ICD10-Q36 Cleft lip*

- Q36.0 Cleft lip, bilateral
- Q36.1 Cleft lip, median
- Q36.9 Cleft lip, unilateral Cleft lip NOS

- *ICD10-Q37 Cleft palate with cleft lip*

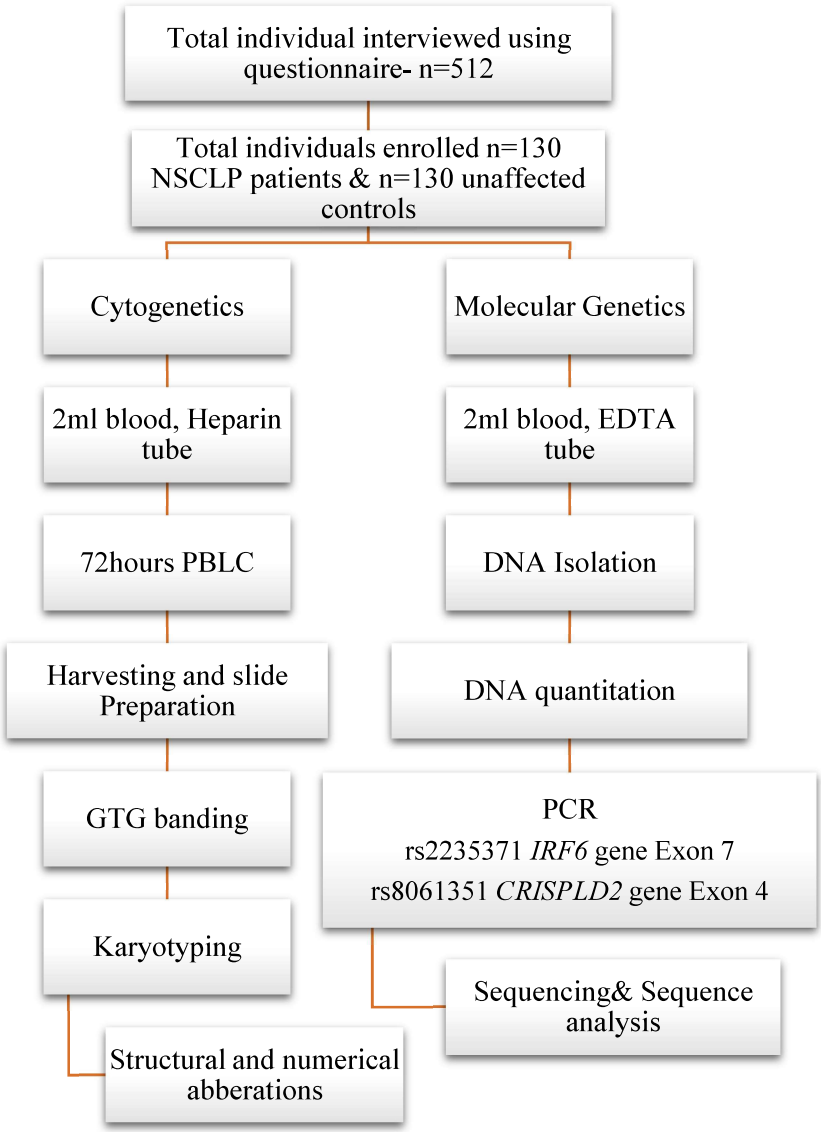
- Q37.0 Cleft hard palate with bilateral cleft lip
- Q37.1 Cleft hard palate with unilateral cleft lip Cleft hard palate with cleft lip NOS
- Q37.2 Cleft soft palate with bilateral cleft lip
- Q37.3 Cleft soft palate with unilateral cleft lip Cleft soft palate with cleft lip NOS
- Q37.4 Cleft hard and soft palate with bilateral cleft lip
- Q37.5 Cleft hard and soft palate with unilateral cleft lip Cleft hard and soft palate with cleft lip NOS

- Q37.8 Unspecified cleft palate with bilateral cleft lip
- Q37.9 Unspecified cleft palate with unilateral cleft lip Cleft palate with cleft lip NOS

4.7 Demography and Epidemiology based data Collection

The Data Collected employing a well-structured questionnaire. The questionnaire is given in Annexure- 2

4.8 Genetic Study Work Plan



4.9 Cytogenetic study

Conventional cytogenetic analysis was carried out in study subjects using peripheral blood lymphocyte (PBLC) samples as per standard protocol. The culture was prepared from peripheral blood and treated with colchicine to arrest the cells at metaphase stage. They were then treated with hypotonic solution to induce lysis of red blood cells such that only lymphocytes remained in the solution. While treated it with a fixative, methanol denature and precipitated proteins of cytoplasmic membrane and acetic acid denatured nucleoproteins. Chromosome analysis requires the following steps;

4.9.1 Peripheral Blood Lymphocyte Culture (PBLC)

Reagents Required:

- Medium – RPMI 1640
- Sample – Blood
- Colchicine
- Potassium chloride solution
- Methanol: Glacial acetic acid (fixative)
- Trypsin
- Phosphate Buffer Saline
- Giemsa stain
- Double distilled water

➢ Hypotonic solution

0.075M KCl is prepared by adding 56 mg of KCl in 10ml of distilled water.

➢ Fixative

40 ml was prepared by adding 30 ml of methanol and 10 ml of glacial acetic acid (3:1)

- Giemsa stain

  2% of giemsa stain of Himedia was used. Filtered using Whatman filter paper no.1 before staining the slides.

Procedure:

4.9.1.1 Peripheral blood lymphocyte culture (PBLC)

- Five hundred microliters of peripheral blood were added to 4.5 ml of RPMI medium, mixed thoroughly, and placed in an incubator at 37°C for a total of 72 hours. (CO_2 was released every 24 hours until harvesting.)
- At the 69^{th} hour of incubation, 5 microliters of 0.01% colchicine were added, gently shaken, and further incubated.

4.9.1.2 Culture harvesting

- The culture tubes were centrifuged at 1200 rpm for 10 minutes.
- The supernatant was discarded without disturbing the cell pellet.
- The cell pellet was resuspended in 5 ml of pre-warmed (37°C) hypotonic solution, mixed thoroughly, and incubated for 4 minutes and 30 seconds at 37°C.
- The culture tubes were centrifuged again at 1200 rpm for 10 minutes.
- The supernatant was carefully removed.
- 2 ml of freshly prepared methanol-glacial acetic acid fixative were added to the culture, mixed well, and the volume was adjusted to 10 ml using the fixative.

4.9.2 Slide preparation

- Resuspend the cell button with a small volume of freshly prepared fixative depending on pellet quantity.
- Wiped the glass slides which was dipped in old fixative for an hour.

- Dipped slides in 100% ethanol and then in distilled water.
- Dropped the cell suspension in the slide in a zig – zag manner.
- Dried the slides on hot plate at 60°C for 8 hours.
- Checked the spread under microscope for cell density and metaphase spread.

4.9.3 Chromosome Banding

Chromosome banding techniques are employed to differentiate and identify various chromosomes based on their distinctive banding patterns during metaphase. A chromosome band is a discernible section of a chromosome that appears either lighter or darker when subjected to banding techniques, distinguishing it from adjacent segments. The most commonly used chromosome banding methods include G-banding (Giemsa), R-banding (Reverse), C-banding (Centromere), and Q-banding (Quinacrine). Among these, G-banding, specifically the GTG banding technique, is widely utilized for chromosome identification (karyotyping). Therefore, in our study, we utilized GTG banding to identify chromosomal abnormalities in the subjects.

4.9.3.1 GTG banding

G-banding is the predominant chromosome banding technique employed for identifying various chromosomal anomalies, such as abnormalities in chromosome numbers, genetic material translocations between chromosomes, as well as deletions, inversions, or amplifications of chromosomes. G-banding produces a series of dark and light-stained bands on chromosomes. The dark regions typically correspond to late-replicating heterochromatic regions and adenine-thymine (A-T) rich segments, while the light regions correspond to early-replicating euchromatic regions and guanine-cytosine (G-C) rich segments.

Reagents required

- 2% Giemsa stain solution
- Trypsin solution
- Phosphate buffer saline solution
- Distilled water

Procedure

- The prepared slides were positioned on a slide warmer at 60°C and left to age overnight.
- Subsequently, the slides underwent treatment in a trypsin solution (0.024% trypsin in phosphate-buffered saline) for a brief period of 1 to 2 seconds.
- Following this treatment, the slides were given a quick rinse in a phosphate-buffered saline (PBS) solution.
- The next step involved staining the slides in a 2% Giemsa staining solution for a duration of 3 minutes.
- After staining, the slides were thoroughly rinsed in distilled water and then left to dry.

4.9.3.2 C-banding

C-banding is a chromosome banding technique employed to visualize heterochromatin, specifically in the centromeric regions of chromosomes. The procedure involves denaturing chromosomes with a saturated alkaline solution followed by Giemsa staining. C-banding of chromosomes involves the differential solubilization of fragmented DNA from euchromatin by three sequential treatments: 1. Acid - ethanol, 2. Mild base - NaCl, 3. Hot salt $Ba(OH)_2$. Solubilization is affected by 1) depurination, 2) DNA denaturation, 3) chain breakage (by depurination and β elimination) of the depurinated sites

respectively in the three treatments. Here saline sodium citrate (SSC) acts as DNA reassociation reagent.

Reagents Required:

- 95% ethanol
- 0.9% NaCl
- $Ba(OH)_2$
- 2X SSC
- 4% Giemsa
- Distilled water

Procedure

- Slides were kept for aging: 2 days at 60°C.
- Washed slides in 95% ethanol.
- 5-8 times dipped in 0.9% NaCl.
- Filtered $Ba(OH)_2$ for 1minute or 30 seconds.
- Rinsed in 70% ethanol.
- Rinsed in distilled water.
- Rinsed in 0.9% NaCl.
- Slides kept in 2X SSC maintained at 60°C for 1 hour 30 minutes.
- Rinsed the slides in distilled water.
- Cool and air dried the slides.
- Stained with 4% Giemsa for 30 to 40 minutes.
- Washed in distilled water and then air dried the slides.
- Observed the slides under microscope first at 10X and then at 100X.

4.9.4 Identification of chromosomes and karyotyping

The slides were carefully examined for metaphase spreads using a 10X research microscope. These metaphases were then subjected to detailed analysis under a 100X oil immersion objective on a Zeiss Microscope. For each case, thirty well-spread and properly banded metaphases were selected, captured, and analyzed using cytogenetic software (Metasystem Ikaros, Germany). The karyotyping process followed the guidelines outlined in the International System for Human Cytogenetic Nomenclature (ISCN, 2020). Karyotyping involves systematically arranging chromosomes from a cell based on their length, centromere position, and specific banding patterns.

4.10 Molecular Study

4.10.1 Genomic DNA Extraction Method

Genomic DNA extraction is a critical step in molecular biology research, enabling the isolation of high-quality DNA for various downstream applications, including PCR analysis, agarose gel electrophoresis, restriction enzyme digestion, and blotting procedures. This method utilizes the QIAGEN Blood DNA Mini Kit, which employs silica membrane technology and a specialized buffer system for efficient DNA extraction.

Materials required:

- QIAGEN Blood DNA Mini Kit
- Buffer AL
- Buffer AW1
- Buffer AW2
- Proteinase K
- Spin Column CB3
- Collection Tubes (2ml)

- Collection Tubes (1.5ml)
- Sample (e.g., whole blood, plasma, serum, buffy coat, body fluids, or lymphocytes in PBS)

Procedure:

- Pipetted 20 µl of QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.
- Added 200 µl of the sample to the microcentrifuge tube. Ensure that the sample does not exceed 200 µl. Suitable samples include whole blood, plasma, serum, buffy coat, body fluids, or up to 5 x 106 lymphocytes in 200 µl PBS.
- 200 µl of Buffer AL was added to the sample. Mixed the contents by pulse-vortexing for 15 seconds.
- The mixture was then incubated at 56°C for 10 minutes.
- Briefly centrifuged the 1.5 ml microcentrifuge tube to remove any drops from the inside of the lid.
- 200 µl of ethanol (96–100%) was added to the sample, and mixed it again by pulse-vortexing for 15 seconds. After mixing, briefly centrifuged the 1.5 ml microcentrifuge tube to remove any drops from the inside of the lid.
- Carefully applied the mixture from step 6 to the QIAamp Mini spin column (placed in a 2 ml collection tube) without wetting the rim. Closed the cap, and centrifuged at 6000 x g (8000 rpm) for 1 minute. Dispose of the collection tube containing the filtrate.
- Carefully opened the QIAamp Mini spin column and add 500 µl of Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1

minute. Place the QIAamp Mini spin column in a clean 2 ml collection tube and discard the collection tube containing the filtrate.

- Carefully opened the QIAamp Mini spin column and added 500 µl of Buffer AW2 without wetting the rim. Closed the cap and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes.
- Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and centrifuge at full speed for 1 minute.
- Placed the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discarded the collection tube containing the filtrate. Carefully opened the QIAamp Mini spin column and added 200 µl of Buffer AE or distilled water. Incubated at room temperature (15–25°C) for 1 minute, and then centrifuged at 6000 x g (8000 rpm) for 1 minute.

4.10.2 DNA Quantification using Qubit Assays

Quantifying DNA is essential in various molecular biology applications. The Qubit assay offers an accurate and efficient method for DNA quantification. This procedure outlines the steps for DNA quantification using Qubit assays.

Materials required:

- Assay tubes (2 for standards, 3 for protein assay, and 1 for each sample)
- Qubit buffer
- Qubit working solution (200µl per standard and sample)
- Qubit standard (from the kit)
- User samples

Set up two Assay tubes for standards (three for the protein assay) and one tube for each user sample. Prepared the Qubit buffer.

Prepared the working solution (200µl) for each standard and sample as indicated below: (Table 4.1)

Table 4.1 Preparations for DNA quantification

	Standard	User samples
Volume of working solution (From step 2) to	190µl	180µl
Volume of standard (from kit) to add	10µl	-
Volume of user sample to add	-	20µl
Total volume in each Assay tube	200µl	200µl

Procedure

- Prepared the Assay Tubes as per the instructions above.
- Vortexed all tubes for 2 – 3 seconds to ensure proper mixing.
- Incubated the tubes for 2 minutes at room temperature (15 minutes for the Qubit protein assay).
- Inserted the tubes into the Qubit 2.0 fluorometer and record the readings.
- Calculated the stock concentration of the original sample using the dilution calculator feature of the Qubit 2.0 Fluorometer.

4.10.3 Agarose Gel Electrophoresis

Materials required:

- Agarose
- TBE Buffer
- Ethidium Bromide
- Gel Loading dye
- DNA Sample

Procedure

Preparation of 0.7% Agarose Gel for Whole Genome Analysis:

- Dissolved 0.28g of Agarose (Himedia RM 273 – 25G) in 40ml of 0.5X TBE buffer.
- Warmed the agarose-TBE mixture in a microwave until the agarose was completely dissolved.
- Allowed the agarose mixture to cool to 50°C.
- Added 2µl of a 10mg/ml Ethidium Bromide (EtBr) solution to achieve a final concentration of 0.5µg/mL.
- Poured the agarose-EtBr mixture into a gel boat fitted with combs and placed it on a level surface.
- After the gel solidified, carefully removed the combs.
- Submerged the gel boat in a submarine Gel Electrophoresis system filled with 0.5X TBE Buffer (pH 7.5).
- Loaded the DNA samples into the wells and ran the gel at 75V for 3 hours.
- Examined the gel under a UV transilluminator to assess DNA quality.

Preparation of 2% Agarose Gel for PCR Products:

- Dissolved 0.8g of Agarose (Himedia RM 273 – 25G) in 40ml of 0.5X TBE buffer.
- Warmed the agarose-TBE mixture in a microwave until the agarose was completely dissolved.
- Allowed the agarose mixture to cool to 50°C.

- Added 2µl of a 10mg/ml Ethidium Bromide (EtBr) solution to achieve a final concentration of 0.5µg/mL.
- Poured the agarose-EtBr mixture into a gel boat fitted with combs and placed it on a level surface.
- After the gel solidified, carefully removed the combs.
- Submerged the gel boat in a submarine Gel Electrophoresis system filled with 0.5X TBE Buffer (pH 7.5).
- Loaded the PCR product samples into the wells and ran the gel at 75V for 3 hours.
- Examined the gel under a UV transilluminator to assess DNA quality.

4.10.4 SNP Selection, Primer designing, PCR reaction and Sanger Sequencing

The Table 4.2 and Table 4.3 represents the various hotspots of *IRF6* and *CRISPLD2* respectively.

Table 4.2 Hotspots of *IRF6*

Sl. No.	Single nucleotide polymorphism (SNP)	**Population**	**References**
1.	rs642961	**Chinese**	Pan et al., 2010
2.	rs2235371	**Indian**	Rahimov et al., (2008)
3.	rs1546124	**Indian**	Chiquet et al., 2007
4.	rs4783099	**Indian**	
5.	rs16974880	**Indian**	

Table 4.3 Hotspots of *CRISPLD2*

Sl. No.	Single nucleotide polymorphism (SNP)	**Population**	**References**
1.	rs8061351	**Europe**	Chiquet et al., 2007
2.	rs2326398		
3.	rs4783099		
4.	rs1546124		
5.	rs1546124	**North American Caucasian**	
6.	rs2326398		
7.	rs8061351		
8.	rs4783099		
9.	rs1546124	**South/Central American**	
10.	rs8061351		
11.	rs2326398		
12.	rs4783099		
13.	rs1546124	**Hispanic**	
14.	rs2326398		
15.	rs8061351		
16.	rs4783099		
17.	rs1546124	**Asian**	
18.	rs4783099		
19.	rs8061351		
20.	rs2326398		
21.	rs4783099	**ALL Caucasian**	
22.	rs1546124		
23.	rs2326398		
24.	rs8061351		

IRF6 Polymorphism

1. rs2235371 *[Homo sapiens]*

Variant type:	SNV
Alleles:	C>T [Show Flanks]
Chromosome:	1:209790735 (GRCh38) 1:209964080 (GRCh37)
Canonical SPDI:	NC_000001.11:209790734:C:T
Gene:	IRF6 (Varview)
Functional Consequence:	missense_variant,coding_sequence_variant
Clinical significance:	benign
Validated:	by frequency,by alfa,by cluster
MAF:	T=0.029993/9212 (ALFA) T=0.00463/1 (Qatari) T=0.013158/4 (FINRISK) T=0.013492/52 (ALSPAC) T=0.014063/63 (Estonian) T=0.014293/53 (TWINSUK) T=0.014981/8 (MGP) T=0.016032/16 (GoNL) T=0.028333/17 (NorthernSweden) T=0.037683/5285 (GnomAD) T=0.055556/63 (Daghestan) T=0.057214/15144 (TOPMED) T=0.076582/9280 (ExAC) T=0.086693/21729 (GnomAD_exomes) T=0.118059/9291 (PAGE_STUDY) T=0.126483/633 (1000Genomes) T=0.164394/217 (HapMap) T=0.379085/232 (Vietnamese) T=0.387031/1134 (KOREAN) C=0.395161/49 (SGDP_PRJ) T=0.417257/6993 (TOMMO) C=0.5/6 (Siberian) ...less
HGVS:	NC_000001.11:g.209790735C>T, NC_000001.10:g.209964080C>T, NG_007081.2:g.20400G>A, NM_006147.4:c.820G>A, NM_006147.3:c.820G>A, NM_001206696.2:c.535G>A, NM_001206696.1:c.535G>A, NP_006138.1:p.Val274Ile, NP_001193625.1:p.Val179Ile

CRISPLD2 Polymorphism

1. **rs8061351** *[Homo sapiens]*

Variant type:	SNV
Alleles:	C>A,T [Show Flanks]
Chromosome:	16:84849496 (GRCh38) 16:84883102 (GRCh37)
Canonical SPDI:	NC_000016.10:84849495:C:A,NC_000016.10:84849495:C:T
Gene:	CRISPLD2 (Varview)
Functional Consequence:	synonymous_variant,coding_sequence_variant
Validated:	by frequency,by alfa,by cluster
MAF:	C=0.288099/56378 (ALFA) C=0.1/4 (GENOME_DK) C=0.188333/113 (NorthernSweden) C=0.208333/10 (Siberian) C=0.2125/102 (SGDP_PRJ) C=0.24442/1095 (Estonian) C=0.248497/248 (GoNL) C=0.252206/972 (ALSPAC) C=0.272384/1010 (TWINSUK) C=0.272727/18 (PRJEB36033) C=0.281659/516 (Korea1K) C=0.286517/153 (MGP) C=0.291599/4887 (TOMMO) C=0.304736/76568 (GnomAD_exomes) C=0.305573/37066 (ExAC) C=0.305802/896 (KOREAN) C=0.324503/98 (FINRISK) C=0.328704/71 (Qatari) C=0.3457/205 (Vietnamese) C=0.393146/55075 (GnomAD) C=0.395753/5144 (GoESP) C=0.402713/106594 (TOPMED) C=0.419894/2103 (1000Genomes) C=0.434461/822 (HapMap) ...less
HGVS:	NC_000016.10:g.84849496C>A, NC_000016.10:g.84849496C>T, NC_000016.9:g.84883102C>A, NC_000016.9:g.84883102C>T, NM_031476.4:c.471C>A, NM_031476.4:c.471C>T, NM_031476.3:c.471C>A, NM_031476.3:c.471C>T, XM_005256190.2:c.471C>A, XM_005256190.2:c.471C>T, XM_005256190.1:c.471C>A, XM_005256190.1:c.471C>T

Primers specific for exon 7 of the gene *IRF6* were designed using the online Primer Blast software of NCBI. The primers were developed by integrated DNA Technologies (IDT) USA. The PCR experiment targeting the *CRISPLD2* gene utilized two primers, a forward primer and a reverse primer. The forward primer had a sequence of TGTGCAGTCCTGGTATGACG and a length of 20 bases, while the reverse primer had a sequence of TCAGTTTCTGACCTTGCTGGT and a length of 21 bases. The forward primer was designed for the plus strand of the template, while the reverse primer was designed for the minus strand. The melting temperatures (Tm) of the forward and reverse primers were approximately 59.75°C and 59.51°C, respectively, ensuring optimal annealing during PCR. The GC content of the forward primer was 55.00%, while that of the reverse primer was 45.00%. Additionally, both primers exhibited minimal self-complementarity and 3' complementarity, with values of 4.00 and 2.00 for the forward primer and 5.00 and 2.00 for the reverse primer, respectively. The PCR experiment was expected to yield a product with a length of 299 base pairs. The Polymerase chain reaction was performed with 25µl reaction mix containing 4µl genomic DNA, 7.25µl nuclease free water, 0.625 µl forward primer, 0.625µl reverse primer and 12.5µl 2x taq master mix.

These primer details were essential for the successful amplification of the *CRISPLD2* gene in the PCR experiment.

For the exon 7 of the *IRF6* gene, specific primers were designed to facilitate PCR amplification. The forward primer, with the sequence TTCCAGAGAGTGATTCCCACGA, was 22 nucleotides long and aligned with the plus strand of the template. The forward primer had a melting temperature (Tm) of 60.82°C and a GC% content of 50%. Self-complementarity was observed at 3.00, and there was 3.00 self 3' complementarity. In contrast, the reverse primer, with the

sequence TGAAGCAGGACTCTCACTGTC, spanned 21 nucleotides and aligned with the minus strand of the template. This reverse primer had a melting temperature (Tm) of 59.38°C and a GC% content of 52.38%. Self-complementarity for the reverse primer was S.00, with 3.00 self 3' complementarity. The designed primers were intended for the amplification of a DNA fragment with a length of 476 base pairs within exon 7 of the *IRF6* gene. The Polymerase chain reaction was performed with 25µl reaction mix containing 4µl genomic DNA, 7.25µl nuclease free water, 0.625 µl forward primer, 0.625µl reverse primer and 12.5µl 2x taq master mix. PCR product is outsourced for sequencing (Barcode biosciences, Bangalore).

4.10.5 Statistical Data analysis

We analyzed epidemiological data using simple and easy-to-understand statistics. To show the geographical distribution, we used a user-friendly open-access software called QGIS (available at https://www.qgis.org/en/site/). For analyzing karyotypes, we used a tool called IKAROS from Metasystem. To perform cross-tabulation analysis, we used GraphPad Prism 9.3.1 statistical software, and we used the chi-squared test to check the Hardy-Weinberg equilibrium (HWE). To compare genotype and allele frequencies between groups, we employed an open statistical analysis tool called SISA, which can be accessed at http://www.Quantitativeskills.com/sisa/. We also conducted stratified analysis to confirm genetic associations based on gender and types of NSCLP by categorizing the samples accordingly. Using genotype data, we estimated linkage disequilibrium (LD) using the SNPstats web-based tool, available at SNPstats (https://www.snpstats.net/snpstats/). Additionally, we performed haplotype analysis to compare the most common haplotype frequencies between controls and NSCLP using SNPstats, selecting haplotypes with frequencies above 1%. Any p-value less than 0.05 was considered statistically significant.

CHAPTER V: RESULTS

5. RESULTS

5.1 Epidemiology and Geographical distribution

In this study out of 130 cases 77 cases (59.23%) were females and 53 cases (40.77%) were males. In large series, 46% with cleft lip and palate, 21% of patients presented with isolated cleft lip, and 33% with cleft of the secondary palate only. In this study out of 130 cases of non-syndromic cleft lip and cleft palate studied, 88 cases (68%) had cleft lip along with cleft palate, 18 cases (14%) had isolated cleft lip and 24 cases (18%) had isolated cleft palate. Moreover, out of the 130 patients 33 (25.38%) were having bilateral cleft, 53 (40.76%) were having unilateral left cleft and 20 (15.38%) had unilateral right cleft lip.

Table 5. 1 Different types of clefting in the patients with respect to gender

Type of Cleft	Bilateral			Unilateral		
	Male (%)	Female (%)	Total (%)	Male (%)	Female (%)	Total (%)
CL	0 (0.0)	4 (3.1)	4 (3.1)	7 (5.4)	7 (5.4)	14 (10.8)
CLP	14 (10.8)	15 (11.5)	29 (22.3)	23 (17.7)	36 (27.7)	59 (45.4)
CP	-		-		-	
Total	14 (10.8)	19 (14.6)	33 (25.4)	30 (23.1)	43 (33.1)	73 (56.2)

Note : CP is mentioned as 0% as it does not come under the laterality differentiation

Table 5.1, provides a breakdown of different types of cleft conditions categorized by gender. It aims to depict the distribution of cleft conditions among male and female patients, taking into account both bilateral and unilateral cases. ***Type of Cleft:*** *This column indicates the specific type of cleft condition being analyzed, including "CL" for Cleft Lip, "CLP" for Cleft Lip and Palate, and "CP" for Cleft Palate.* ***Bilateral:*** *Under*

this section, the table displays the percentage distribution of male and female patients with bilateral cases of the respective cleft condition. For example, for "CL," there are no male patients (0.0%) with bilateral clefts, while there are 4 female patients (3.1%) with bilateral "CL." Similarly, for "CLP," 10.8% of male patients and 11.5% of female patients have bilateral cases. ***Unilateral:*** *This part of the table provides the percentage distribution of male and female patients with unilateral cases of the respective cleft condition. For "CL," 5.4% of male and 5.4% of female patients have unilateral clefts. For "CLP," the distribution shows that 17.7% of male patients and 27.7% of female patients have unilateral clefts.* ***CP:*** *The table omits data for "CP" (Cleft Palate) as it does not come under the laterality differentiation criteria (bilateral or unilateral). Therefore, there are no entries in this row.* ***Total:*** *The "Total" row sums up the distribution, showcasing the overall percentage of male and female patients with each cleft condition. For instance, 10.8% of patients in the study have "CL," with 14.6% being female, while 22.3% have "CLP," with 45.4% being female.*

Table 5. 2 Different types unilateral clefting in the patients with respect to symmetry

Type of Cleft	Unilateral Left			Unilateral Right		
	Male (%)	Female (%)	Total	Male (%)	Female (%)	Total
CL	6 (4.6)	2 (1.5)	8 (6.1)	1 (0.7)	5 (3.8)	6 (4.6)
CLP	19 (14.6)	26 (20)	45 (34.6)	4 (3)	10 (7.7)	14(10.8)
CP	-		-		-	
Total	25 (19)	28 (21.5)	53 (40.7)	5 (3.8)	15 (11.5)	20 (15.5)

Note : CP is mentioned as 0% as it does not come under the laterality differentiation

The table 5.2 presents data on different types of unilateral cleft conditions categorized by gender and the affected side (left or right). The aim is to illustrate the distribution of unilateral cleft conditions among male and female patients for both left and right sides. The cleft conditions considered are "CL" (Cleft Lip) and "CLP" (Cleft Lip and Palate). ***Type of Cleft:*** *This column specifies the type of unilateral cleft condition being analyzed, including "CL" for Cleft Lip and "CLP" for Cleft Lip and Palate.* ***Unilateral Left:*** *In this section, the table displays the percentage distribution of male and female patients with unilateral cleft conditions on the left side. For example, for "CL," 4.6% of male patients and 1.5% of female patients have a unilateral cleft on the left side. Similarly, for "CLP," 14.6% of male patients and 20% of female patients have a unilateral cleft on the left side.* ***Unilateral Right:*** *This part of the table provides the percentage distribution of male and female patients with unilateral cleft conditions on the right side. For "CL," 0.7% of male patients and 3.8% of female patients have a unilateral cleft on the right side. For "CLP," 3% of male patients and 7.7% of female patients have a unilateral cleft on the right side.* ***CP:*** *The table omits data for "CP" (Cleft Palate) as it does not come under the laterality differentiation criteria (left or right). Therefore, there are no entries in this row.* ***Total:*** *The "Total" row sums up the distribution, showcasing the overall percentage of male and female patients with each type of unilateral cleft condition. For instance, in the study, 19% of patients have unilateral left "CL," with 21.5% being female, while 34.6% have unilateral left "CLP," with 10.8% being female.*

Geographical distribution of the whole data (all cases presented) is given in the Figure 5.1

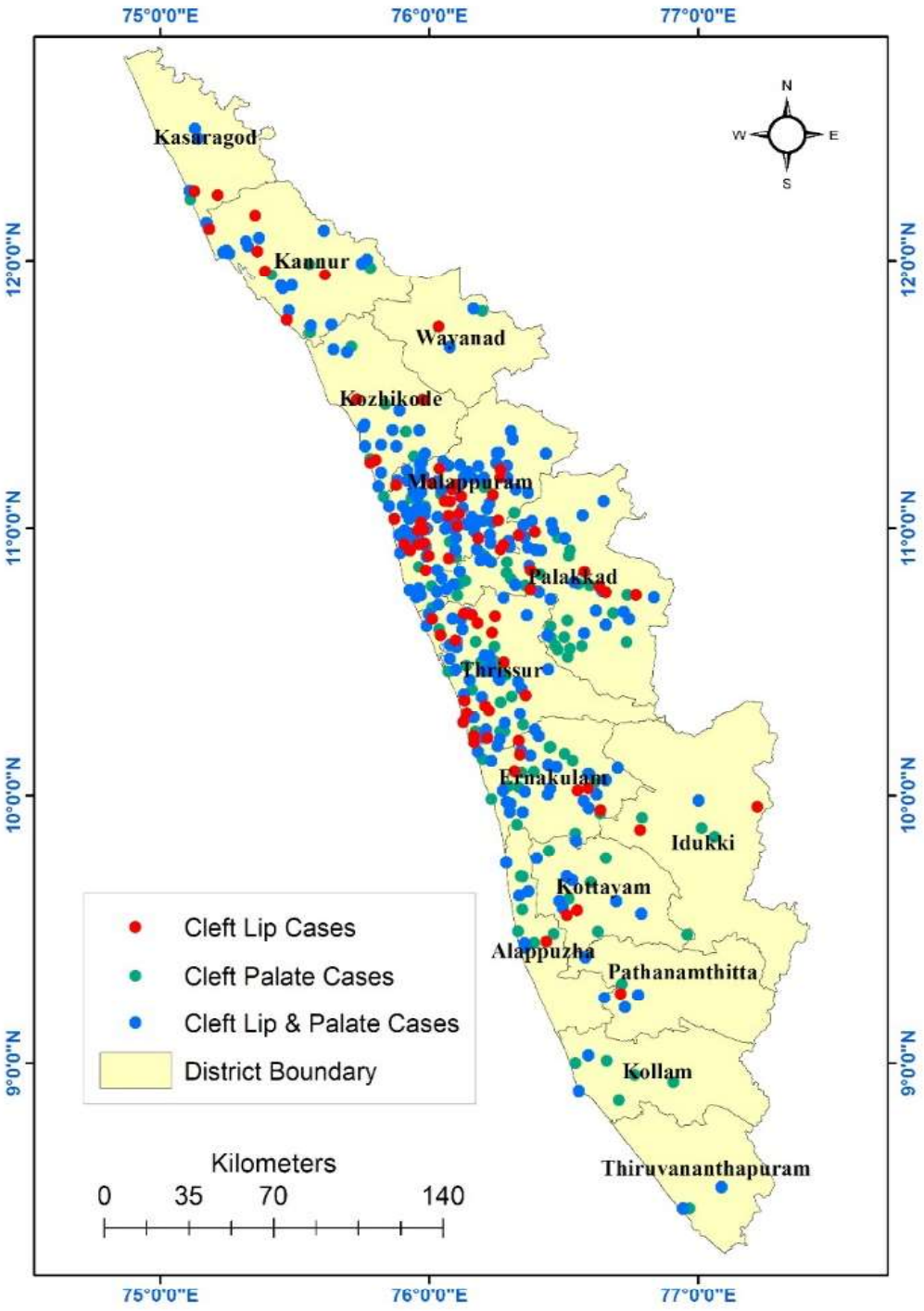


Figure 5.1 Geographical distribution of the orofacial cleft patients included in the current study

5.2 Cytogenetic Analysis

Cytogenetic analysis (Figure 5.2-5.3) explored the applicability of C banding to find any kind of heterochromatic variation in the patients. Based on the results obtained from the C banding analysis, it was found that five of the patients screened in this study have prominently reported with heterochromatic variation in the aforementioned region. Inversion 9 is found in 3 cases of NSCLP, 2 male patients with CLP and one in male patients with isolated cleft lip only (Figure 5.4). Typically, the variations have been found in chromosome 9 are as follows; 9ph+, 9qh-, 9cenh+, 9qh+, or inv(9)(p11q13). The parents' karyotypes showed that all of the cases were de novo heterozygous inv(9)(p11q13). The major findings of the cytogenetics clearly revealed that, the polymorphic variation 9qh+ was observed in male patient with the unilateral cleft lip & palate and 9qh- variation was observed in a male patient with an isolated missing palate. The present investigation can clearly assume the fact that the heterochromatin variations have been noted as one of the predominant probable factors that illustrate its probable role in the development of the disorders allied with the cleft lip and palate. Table 5.4 Out lined the major cases and their cytogenetic observations.

Table 5. 3 Cytogenetic abnormalities found in the study

Case No	NSCLP	Type of cleft	Side	Gender	Chromosome Abnormality	Parents*
1	CLP	Unilateral	Left	Male	46,XY,9qh+	Normal
2	CP	NA		Male	46,XY,9qh-	Normal
3	CLP	Unilateral	Left	Male	46,XY,inv(9)(p11q13)	Normal
4	CLP	Bilateral	Both	Male	46,XY,inv(9)(p11q13)	Normal
5	CL	Unilateral	Left	Male	46,XY,inv(9)(p11q13)	Normal

*Karyotyping of both parents was conducted. NA-Not applicable.

The table 5.3 provides information about different cases within the study, each associated with specific details related to cleft conditions and chromosome abnormalities. Here's an explanation of each column: ***Case No.:*** *This column indicates the numerical identification assigned to each case within the study, helping to distinguish between them. NSCLP: The term "NSCLP" stands for "Non-Syndromic Cleft Lip and Palate," denoting the type of cleft condition observed in each case.* ***Type of Cleft:*** *This column specifies the specific type of cleft condition for each case. The types include "CLP" (Cleft Lip and Palate), "CP" (Cleft Palate), and "CL" (Cleft Lip).* ***Side:*** *This column indicates the side of the face affected by the cleft condition. For example, "Unilateral Left" signifies that the cleft is present on the left side of the face.* ***Gender:*** *This column identifies the gender of the patient associated with each case, with "Male" indicating male patients.* ***Chromosome Abnormality:*** *This section provides information about any chromosome abnormalities detected in each case. For instance, "46,XY,9qh+" indicates that Case No. 1, a male patient with unilateral left CLP, has an additional segment on chromosome 9. Similarly, Case No. 2, a male patient with CP, exhibits a chromosome abnormality denoted as "46,XY,9qh-," suggesting a missing segment on chromosome 9. Cases 3, 4, and 5, all male patients with CLP and CL conditions, share a karyotype of "46,XY" but display variations involving chromosome 9 in the 9p11q13 region, indicating inversions.* ***Parents:*** *This column mentions the parental karyotype status. In this context, "Normal" suggests that the karyotyping of both parents was conducted and did not reveal any significant chromosomal abnormalities.*

Table 5.4 Out line on the major cases and their cytogenetic observations

Case	Observation	Cytogenetic diagnosis
Case I	A 22-year-old male with left unilateral cleft lip and palate.	A male karyotype with a heterochromatin variant in chromosome 9 in all the twenty metaphases analysed. [46,XY,9qh-]
Case II	A one-year-old baby with left unilateral cleft lip and palate.	A male karyotype with a heterochromatin variant in chromosome 9 in all the twenty metaphases analysed. [46,XY,9qh+]
Case III	A nine- year-old male with left unilateral cleft lip and palate.	A male karyotype showing a pericentric inversion in chromosome 9 at p11q13 region in all the twenty metaphases analysed. [46,XY,inv(9)(p11q13)]
Case IV	A six-year-old male with bilateral cleft lip and palate.	A male karyotype with a heterochromatin variant in chromosome 9 in all the twenty metaphases analysed. [46,XY,inv(9)(p11q13)]
Case V	A one- year-old male baby with left unilateral cleft lip.	A male karyotype with a heterochromatin variant in chromosome 9 in all the twenty metaphases analysed. [46,XY,inv(9)(p11q13)]

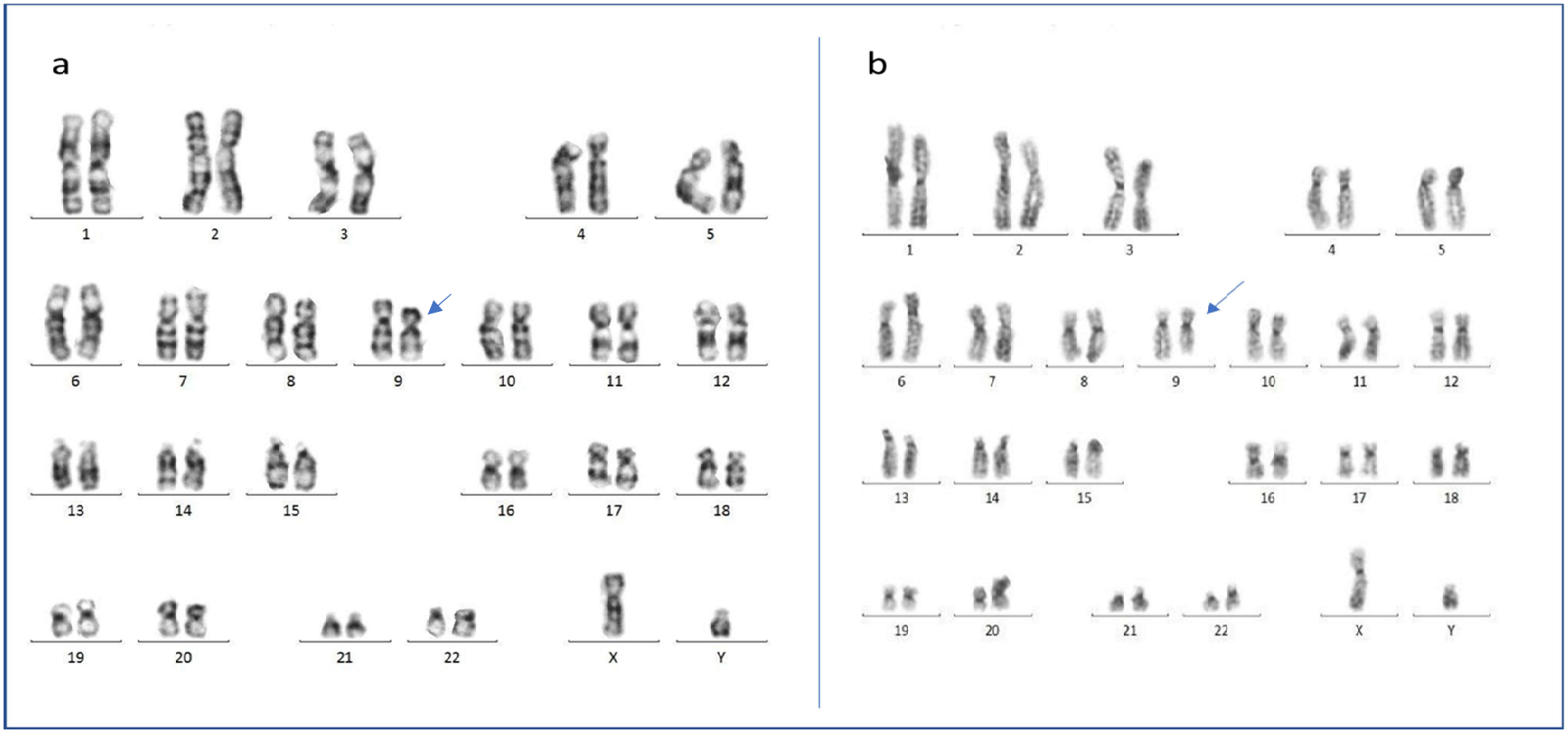


Figure 5. 2 GTG banding analysis of case 1: a)showing 9qh- in GTG banding; b) showing c banding to confirm 9qh-

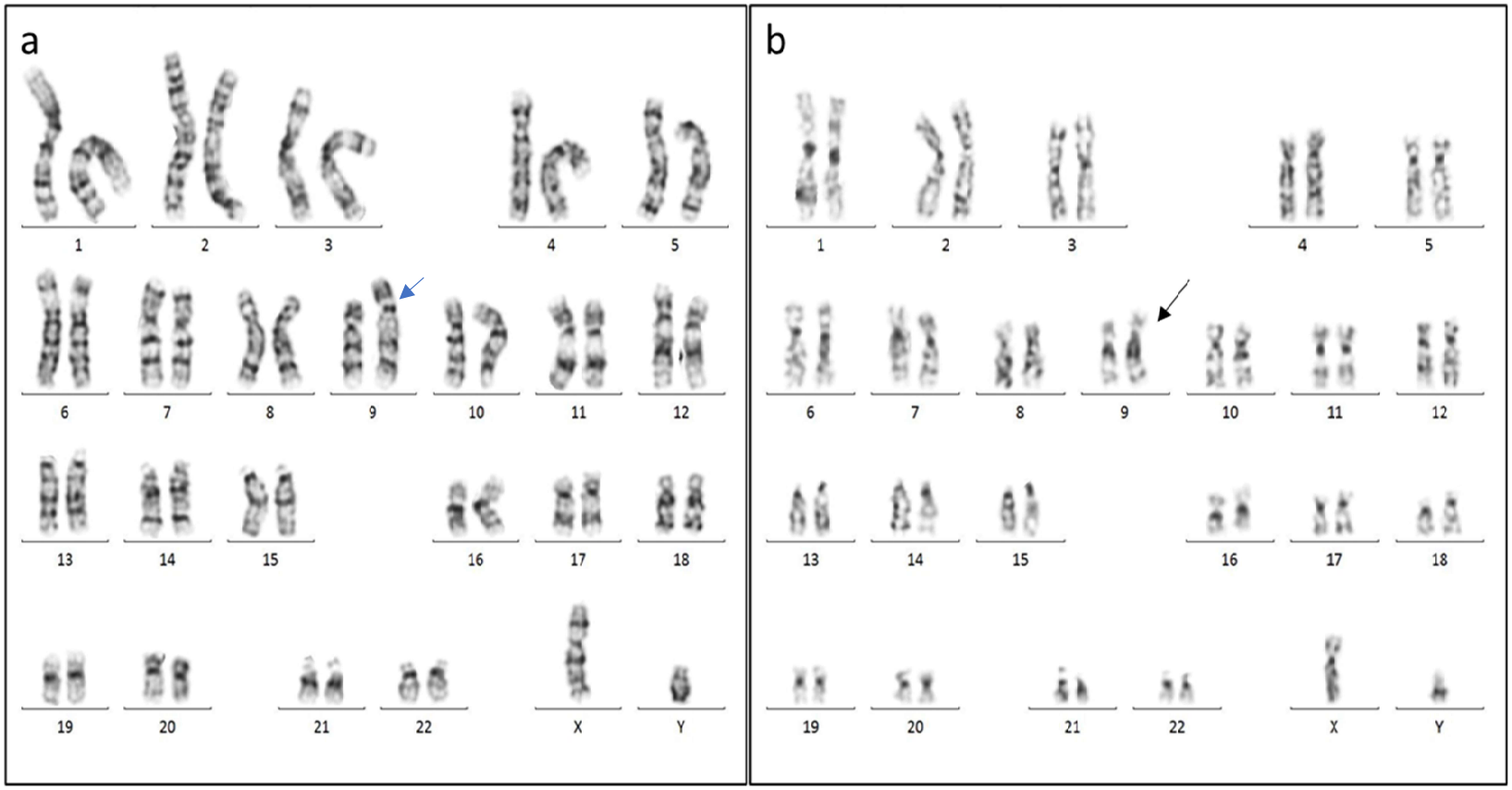


Figure 5.3 GTG banding analysis of case 2: a: showing 9qh+ in GTG banding; b: showing c banding to confirm 9qh+

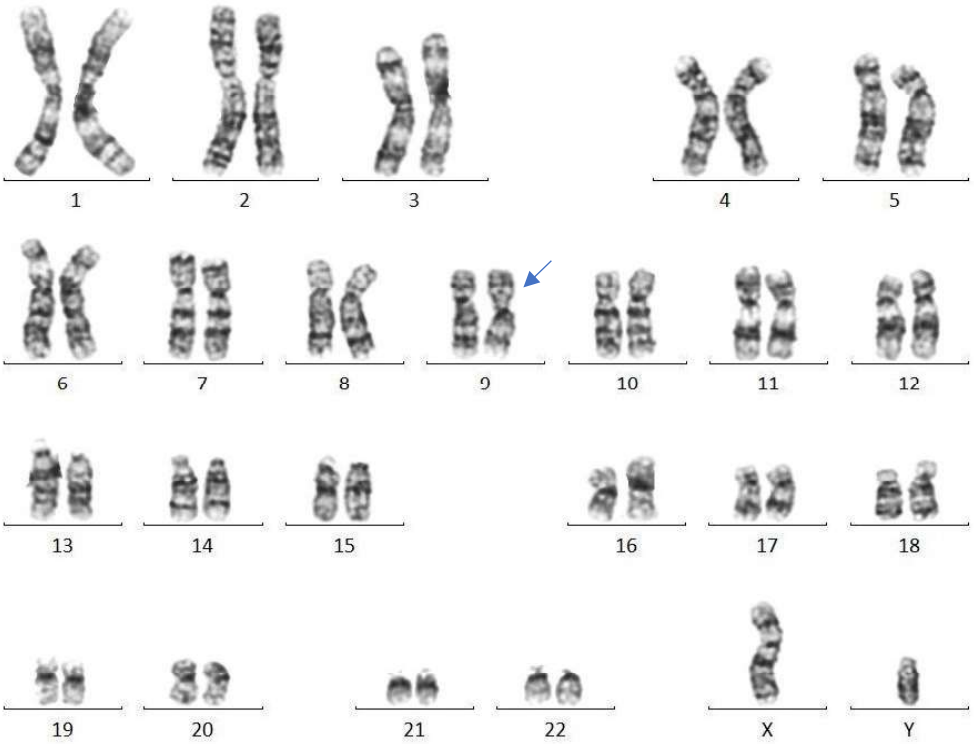


Figure 5.4 Representative image of the G banded karyotype showing heterochromatic variation in chromosome 9 [46,XY,inv(9)(p11q13)]. It appeared in case number 3, 4 and 5 (Refer Table 5.3)

5.3 Molecular Analysis

5.3.1 Interferon Regulatory Factor 6 (*IRF6*) Gene

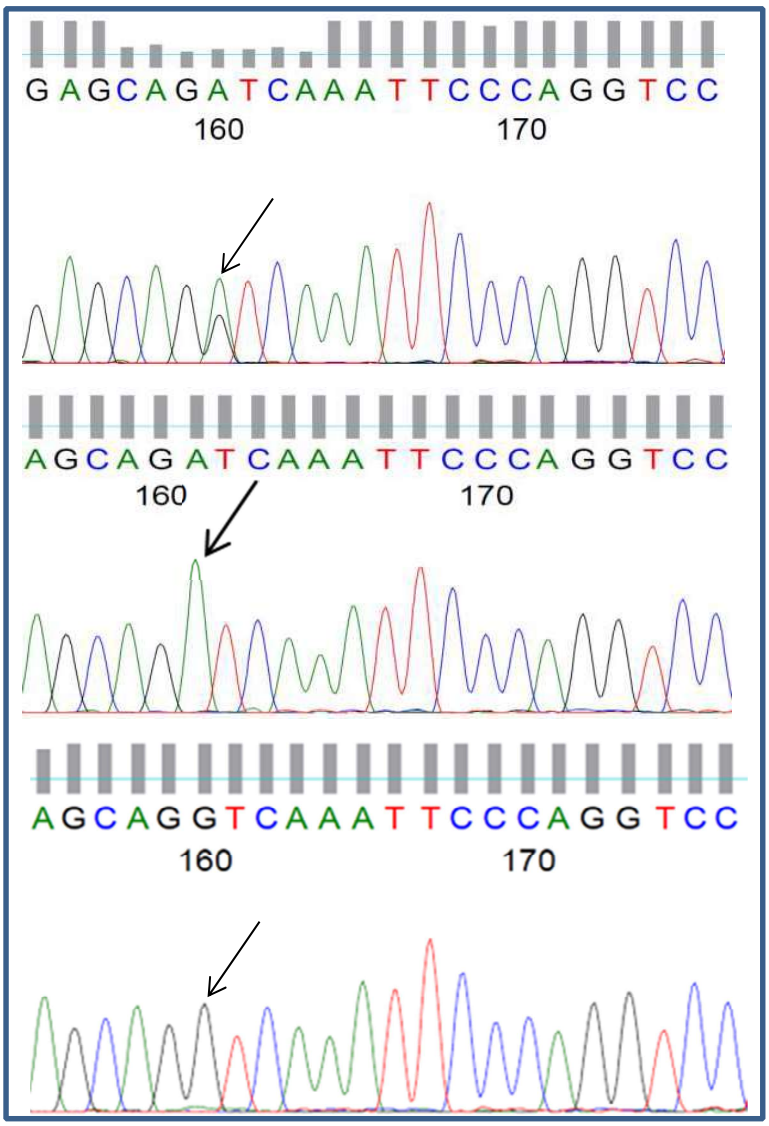


Figure 5.5. Sanger Sequenced Chromatogram of the rs2235371included sequence

The sequences strand is minus strand instead of C/T we mention G/A in the sequenced data

Top Chromatogram (Heterozygous G/A) **(**Figure 5.5**):** *The top chromatogram displays a heterozygous genotype (G/A) for the rs2235371 polymorphism. In this chromatogram, the green peak represents the presence of the A allele, while the black peak represents the presence of the G allele.* ***Middle Chromatogram (Homozygous A):*** *The middle*

chromatogram represents a homozygous A genotype, where the green peak exclusively indicates the presence of the A allele. ***Bottom Chromatogram (Homozygous G):*** *The bottom chromatogram illustrates a homozygous G genotype, with the black peak indicating the presence of the G allele. These chromatograms provide visual information about the genetic variations at the rs2235371 locus, including heterozygosity and homozygosity for the A and G alleles.*

5.3.1.1 Allele Frequencies in the Study Population (n=260)

Allele frequency data for the studied population, with a focus on control samples and individuals with Non-Syndromic Cleft Lip and Palate (NSCLP).

Table 5.5 rs2235371 allele frequencies (n=260)

	All subjects		Control Samples		NSCLP	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
G	466	0.9	244	0.94	222	0.85
A	54	0.1	16	0.06	38	0.15

Allele G: In the entire study population, allele G (Table 5.5) is observed with a frequency of 466, accounting for 90% of the alleles. Among the control samples, allele G is prevalent, with a frequency of 244, making up 94% of the alleles. In the NSCLP group, allele G is also predominant, with a frequency of 222, representing 85% of the alleles.

Allele A: In the entire study population, allele A is observed with a frequency of 54, constituting 10% of the alleles. Among the control samples, allele A is less common, with a frequency of 16, representing 6% of the alleles. In the NSCLP group, allele A is observed with a frequency of 38, accounting for 15% of the alleles.

These allele frequency distributions provide valuable genetic insights into the studied population, particularly in the context of NSCLP. The predominance of allele G is notable, while allele A exhibits variations between the control and NSCLP groups. This summary provides a clear overview of the allele frequencies for alleles G and A within the entire study population, control samples, and the NSCLP group, emphasizing the differences observed in allele A frequencies between the control and NSCLP subsets.

5.3.1.2 Genotype Frequencies (n=260)

The genotype frequencies for the studied population, distinguishing between control samples and cases (individuals with Non-Syndromic Cleft Lip and Palate, NSCLP).

Table 5. 6 rs2235371 genotype frequencies (n=260)

	All subjects		Control Samples		NSCLP	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	4	0.02	0	0	4	0.03
G/A	46	0.18	16	0.12	30	0.23
G/G	210	0.81	114	0.88	96	0.74

Genotype A/A: In the entire study population, the A/A genotype (Table 5.6) is observed in 4 individuals, representing 2% of the total genotypes. Among the control samples, there are no individuals with the A/A genotype. In the NSCLP group, 4 individuals (3%) have the A/A genotype.

Genotype G/A: In the entire study population, the G/A genotype is observed in 46 individuals, constituting 18% of the total genotypes. Among the control samples, 16

individuals (12%) have the G/A genotype. In the NSCLP group, 30 individuals (23%) exhibit the G/A genotype.

Genotype G/G: In the entire study population, the G/G genotype is the most prevalent, with 210 individuals (81%) having this genotype. Among the control samples, 114 individuals (88%) possess the G/G genotype. In the NSCLP group, 96 individuals (74%) display the G/G genotype.

5.3.1.3 Exact Test for Hardy-Weinberg Equilibrium (n=260)

An exact test for Hardy-Weinberg equilibrium was conducted to assess the genetic equilibrium within the study population. The test results are as follows:

Table 5.7 rs2235371 exact test for Hardy-Weinberg equilibrium (n=260)

	N11 [G/G]	N12 [G/A]	N22 [A/A]	N1 [G]	N2 [A]	p-value
All subjects	210	46	4	466	54	0.5
Control Samples	114	16	0	244	16	1
NSCLP	96	30	4	222	38	0.47

*The table 5.7 showing **N11 [G/G]:** This column represents the number of individuals in the sample with the genotype G/G. **N12 [G/A]:** This column represents the number of individuals with the genotype G/A. **N22 [A/A]:** This column represents the number of individuals with the genotype A/A. **N1 [G]:** This column represents the total number of G alleles in the population (sum of G/G and G/A genotypes). **N2 [A]:** This column represents the total number of A alleles in the population (sum of G/A and A/A genotypes).*

P-value: *This column displays the p-value resulting from the exact test for Hardy-Weinberg equilibrium.*

In All subjects (n=260) there are 210 individuals with the G/G genotype, 46 individuals with the G/A genotype, and 4 individuals with the A/A genotype. The total count of G alleles (N1 [G]) is 466, and the total count of A alleles (N2 [A]) is 54. The p-value for the HWE test in this entire population is 0.5. This suggests that, based on the observed genotype frequencies, there is no strong evidence to reject the assumption that the population is in Hardy-Weinberg equilibrium. In the control group, there are 114 individuals with the G/G genotype, 16 individuals with the G/A genotype, and no individuals with the A/A genotype. The total count of G alleles in the control group (N1 [G]) is 244, and the total count of A alleles (N2 [A]) is 16. The p-value for the HWE test in the control group is 1. This indicates that, within the control group, the genotype frequencies are in strong agreement with the expectations of Hardy-Weinberg equilibrium. In the NSCLP group, there are 96 individuals with the G/G genotype, 30 individuals with the G/A genotype, and 4 individuals with the A/A genotype. The total count of G alleles in the NSCLP group (N1 [G]) is 222, and the total count of A alleles (N2 [A]) is 38. The p-value for the HWE test in the NSCLP group is 0.47. This suggests that, within the NSCLP group, the genotype frequencies are not significantly different from the expectations of Hardy-Weinberg equilibrium.

5.3.1.4 Association of rs2235371 with Orofacial Clefts (n=260, Crude Analysis)

This analysis examines the association between the rs2235371 genotype and the occurrence of orofacial clefts, with a focus on control samples and individuals with Non-Syndromic Cleft Lip and Palate (NSCLP).

Table 5.8 rs2235371 association with orofacial clefts (n=260, crude analysis)

Model	Genotype	Control Samples	NSCLP	OR (95% CI)	P-value
Codominant	G/G	114 (87.7%)	96 (73.8%)	1 (Reference)	0.0033
	G/A	16 (12.3%)	30 (23.1%)	2.23 (1.15-4.33)	
	A/A	0 (0%)	4 (3.1%)	NA (0.00-NA)	
Dominant	G/G	114 (87.7%)	96 (73.8%)	1 (Reference)	0.0043
	G/A-A/A	16 (12.3%)	34 (26.1%)	2.52 (1.31-4.85)	
Recessive	G/G-G/A	130 (100%)	126 (96.9%)	1 (Reference)	0.018
	A/A	0 (0%)	4 (3.1%)	NA (0.00-NA)	
Overdominant	G/G-A/A	114 (87.7%)	100 (76.9%)	1 (Reference)	0.022
	G/A	16 (12.3%)	30 (23.1%)	2.14 (1.10-4.15)	
Log-additive	---	---	---	2.55 (1.38-4.71)	0.0017

The table 5.8 displays results from the analysis of the association between rs2235371 and orofacial clefts in a sample of 260 individuals. The term "crude analysis" suggests that

this is a basic or preliminary analysis. ***Model:*** *This column represents the genetic models used to assess the association. Different models are used to categorize individuals based on their genotypes and then calculate odds ratios (OR) to determine the risk of orofacial clefts compared to a reference group.* ***Genotype:*** *This column lists the genotypes of rs2235371 that are being compared in each genetic model.* ***Control Samples:*** *This column provides information about the distribution of genotypes in the control group (individuals without orofacial clefts).* ***NSCLP (Non-syndromic Cleft Lip and Palate):*** *This column provides information about the distribution of genotypes in the NSCLP group (individuals with orofacial clefts).* ***OR (95% CI):*** *This column displays the odds ratio along with a 95% confidence interval (CI). The odds ratio represents the likelihood of having orofacial clefts in one genotype group compared to a reference group (often G/G in this case). The confidence interval provides a range of values within which the true odds ratio is likely to fall.* ***p-value:*** *This column displays the p-value associated with the odds ratio, which indicates the statistical significance of the association. A lower p-value suggests a more significant association.*

Codominant Model: Genotype G/G is the reference category, observed in 114 control samples (87.7%) and 96 NSCLP cases (73.8%). Genotype G/A is associated with an odds ratio (OR) of 2.23 (95% CI: 1.15-4.33) compared to G/G, with 16 control samples (12.3%) and 30 NSCLP cases (23.1%). Genotype A/A is compared to G/G, with no occurrences in control samples and 4 cases (3.1%) in NSCLP.

Dominant Model: Genotype G/G is the reference category, observed in 114 control samples (87.7%) and 96 NSCLP cases (73.8%). Genotype G/A-A/A combined is associated with an OR of 2.52 (95% CI: 1.31-4.85) compared to G/G, with 16 control samples (12.3%) and 34 NSCLP cases (26.1%).

Recessive Model: Genotype G/G-G/A combined is the reference category, observed in 130 control samples (100%) and 126 NSCLP cases (96.9%). Genotype A/A is associated with an OR of "NA" (not applicable) compared to G/G-G/A, with no occurrences in control samples and 4 cases (3.1%) in NSCLP.

Overdominant Model: Genotype G/G-A/A combined is the reference category, observed in 114 control samples (87.7%) and 100 NSCLP cases (76.9%). Genotype G/A is associated with an OR of 2.14 (95% CI: 1.10-4.15) compared to G/G-A/A, with 16 control samples (12.3%) and 30 NSCLP cases (23.1%).

Log-Additive Model: This model doesn't distinguish between specific genotypes but examines the additive effect of the A allele. The analysis reveals an OR of 2.55 (95% CI: 1.38-4.71) for the log-additive model, with a p-value of 0.0017, indicating a significant association between the A allele and orofacial clefts.

These results suggest that the rs2235371 genotype in the Exon 7 of *IRF6* gene is associated with an increased risk of orofacial clefts, particularly in individuals carrying the A allele. The various genetic models provide insights into different aspects of this association, highlighting its significance in the development of orofacial clefts. This summary provides a comprehensive overview of the association analysis results, including different genetic models and their implications for the relationship between rs2235371 genotype and orofacial clefts.

5.3.2 Cysteine Rich Secretory Protein LCCL Domain Containing 2 (*CRISPLD2*)

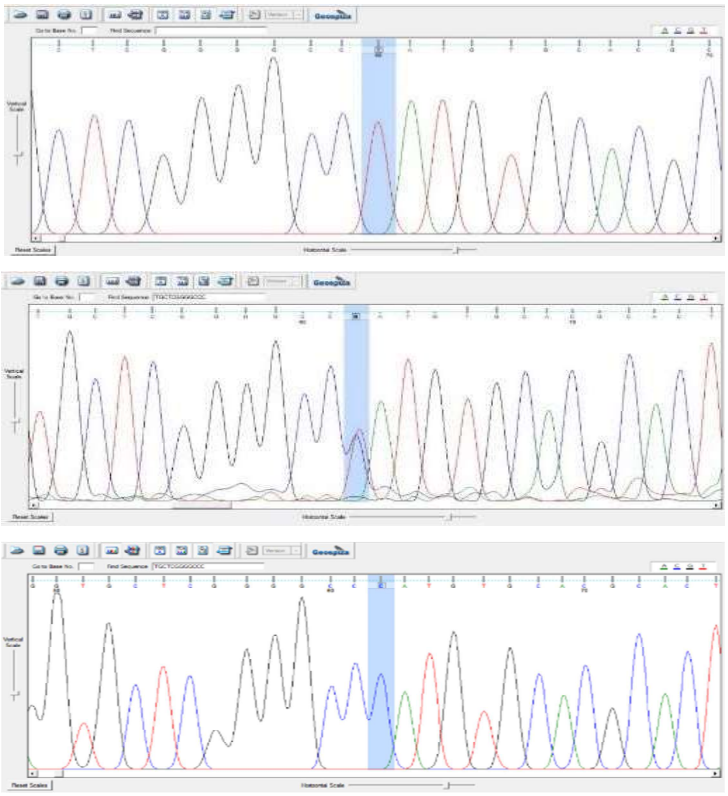


Figure 5.6 Sanger Sequenced Chromatogram of the rs8061351 included sequence

Top Chromatogram (Homozygous T/T) (Figure 5.6)***:*** *The top chromatogram represents a homozygous genotype (T/T) for the rs8061351 polymorphism. In this chromatogram, the red peak exclusively indicates the presence of the T allele.* ***Middle Chromatogram (Heterozygous T/C):*** *The middle chromatogram depicts a heterozygous genotype (T/C) for rs8061351. In this chromatogram, the red peak represents the presence of the T allele, while the blue peak indicates the presence of the C allele.* ***Bottom Chromatogram (Homozygous C/C):*** *The bottom chromatogram illustrates a homozygous genotype (C/C) for rs8061351, with the blue peak exclusively indicating the presence of the C allele. These chromatograms visually convey the genetic variations at the rs8061351 locus, including homozygosity and heterozygosity for the T and C alleles.*

5.3.2.1 Allele Frequencies (n=260)

Allele frequencies for the *CRISPLD2*.rs8061351 variant in the studied population, distinguishing between all subjects, control samples, and individuals with Non-Syndromic Cleft Lip and Palate (NSCLP).

Table 5.9 rs8061351 allele frequencies (n=260)

	All subjects		Control Sample		NSCLP	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
T	425	0.82	225	0.87	200	0.77
C	95	0.18	35	0.13	60	0.23

The table 5.9 presents data on the allele frequencies of rs8061351 in a sample of 260 individuals. ***Allele:*** *This column specifies the two alleles for the rs8061351 genetic variant, namely T and C.* ***Count:*** *This column shows the number of each allele (T or C) observed in the respective groups.* ***Proportion:*** *This column displays the proportion of each allele (T or C) in the total number of alleles for the group. It is calculated by dividing the count of a specific allele by the total count of alleles in the group.*

Allele T: In the entire study population, the T allele is observed in 425 individuals, constituting 82% of the total alleles. Among the control samples, 225 individuals (87%) possess the T allele. In the NSCLP group, 200 individuals (77%) exhibit the T allele.

Allele C: In the entire study population, the C allele is observed in 95 individuals, representing 18% of the total alleles. Among the control samples, 35 individuals (13%) have the C allele. In the NSCLP group, 60 individuals (23%) display the C allele.

5.3.2.2 Genotype Frequencies (n=260)

Table 5.10 rs8061351 genotype frequencies (n=260)

	All subjects		Control Sample		NSCLP	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
C/C	4	0.02	0	0	4	0.03
T/C	87	0.33	35	0.27	52	0.4
T/T	169	0.65	95	0.73	74	0.57

The table 5.10 presents data on the genotype frequencies of rs8061351 in a sample of 260 individuals. ***Genotype:*** *This column specifies the different genotypes that can result from the rs8061351 genetic variant, namely C/C, T/C, and T/T.* ***Count:*** *This column shows the number of individuals with each genotype in the respective groups.* ***Proportion:*** *This column displays the proportion of individuals with each genotype in the total number of individuals for the group. It is calculated by dividing the count of a specific genotype by the total count of individuals in the group.*

Genotype C/C: In the entire study population, the C/C genotype is observed in 4 individuals, representing 2% of the total genotypes. Among the control samples, there are no individuals with the C/C genotype. In the NSCLP group, 4 individuals (3%) have the C/C genotype.

Genotype C/T: In the entire study population, the C/T genotype is observed in 87 individuals, constituting 33% of the total genotypes. Among the control samples, 35 individuals (27%) have the C/T genotype. In the NSCLP group, 52 individuals (40%) exhibit the C/T genotype.

Genotype T/T: In the entire study population, the T/T genotype is the most prevalent, with 169 individuals (65%) having this genotype. Among the control samples, 95 individuals (73%) possess the T/T genotype. In the NSCLP group, 74 individuals (57%) display the T/T genotype.

These data provide insights into the allele and genotype frequencies of rs8061351 in the studied population, with variations observed between the control samples and individuals with NSCLP. This summary offers a clear overview of the allele and genotype frequencies for the rs8061351 variant, emphasizing the differences between the studied groups.

5.3.2.3 Exact Test for Hardy-Weinberg Equilibrium (n=260)

An exact test for Hardy-Weinberg equilibrium was conducted to assess the genetic equilibrium within the study population. The test results are as follows:

Table 5.11. rs8061351 exact test for Hardy-Weinberg equilibrium (n=260)

	N11 [T/T]	N12 [T/C]	N22 [C/C]	N1[T]	N2 [T]	p-value
All subjects	169	87	4	425	95	0.061
Control Samples	95	35	0	225	35	0.13
NSCLP	74	52	4	200	60	0.22

The table 5.11 presents results from the HWE test for rs8061351 in a sample of 260 individuals. ***N11 [T/T]:*** *This column represents the number of individuals in the sample with the T/T genotype.* ***N12 [T/C]:*** *This column represents the number of individuals with the T/C genotype.* ***N22 [C/C]:*** *This column represents the number of individuals with the C/C genotype.* ***N1 [T]:*** *This column represents the total count of T alleles in the*

population (sum of T/T and T/C genotypes). ***N2 [T]:*** *This column represents the total count of C alleles in the population (sum of T/C and C/C genotypes). p-value: This column displays the p-value resulting from the exact test for Hardy-Weinberg equilibrium.*

In All subjects there are 169 individuals with the T/T genotype, 87 individuals with the T/C genotype, and 4 individuals with the C/C genotype. The total count of T alleles (N1 [T]) in the entire population is 425, and the total count of C alleles (N2 [T]) is 95. The p-value for the HWE test in this entire population is 0.061. This suggests that, based on the observed genotype frequencies, there is a slight departure from the expectations of the Hardy-Weinberg equilibrium, but not statistically significant. In the control group (individuals without orofacial clefts), there are 95 individuals with the T/T genotype, 35 individuals with the T/C genotype, and no individuals with the C/C genotype. The total count of T alleles in the control group (N1 [T]) is 225, and the total count of C alleles (N2 [T]) is 35. The p-value for the HWE test in the control group is 0.13. This indicates that, within the control group, the genotype frequencies are in good agreement with the expectations of Hardy-Weinberg equilibrium. In the NSCLP group (individuals with orofacial clefts), there are 74 individuals with the T/T genotype, 52 individuals with the T/C genotype, and 4 individuals with the C/C genotype. The total count of T alleles in the NSCLP group (N1 [T]) is 200, and the total count of C alleles (N2 [T]) is 60. The p-value for the HWE test in the NSCLP group is 0.22. This again suggests that, within the NSCLP group also, the genotype frequencies are not significantly different from the expectations of Hardy-Weinberg equilibrium.

In summary, the HWE test results for rs8061351 show that all the subjects, control samples and NSCLP genotype frequencies are in good agreement with the expectations of Hardy-Weinberg equilibrium (p-value of 0.13), indicating the quality of the genetic data.

5.3.2.4 Association of rs8061351 with Orofacial Clefts (Crude Analysis)

The data provided in the table below presents the results of a crude analysis of the association between genetic variant rs8061351 and orofacial clefts (OFC) involving 260 individuals. This type of analysis is essential in genetic research to assess whether a specific genetic variant is associated with a particular trait or disease. In this case, the focus is on the relationship between rs8061351 genotypes and the occurrence of orofacial clefts.

Table 5. 12 rs8061351 association with orofacial clefts (n=260, crude analysis)

Model	Genotype	Control Samples	NSCLP	OR (95% CI)	p-value
Codominant	T/T	95 (73.1%)	74 (56.9%)	1 (Reference)	0.0032
	C/T	35 (26.9%)	52 (40%)	1.91 (1.13-3.23)	
	C/C	0 (0%)	4 (3.1%)	NA (0.00-NA)	
Dominant	T/T	95 (73.1%)	74 (56.9%)	1 (Reference)	0.0061
	C/T-C/C	35 (26.9%)	56 (43.1%)	2.05 (1.22-3.46)	
Recessive	T/T-C/T	130 (100%)	126 (96.9%)	1 (Reference)	0.018
	C/C	0 (0%)	4 (3.1%)	NA (0.00-NA)	
Overdominant	T/T-C/C	95 (73.1%)	78 (60%)	1 (Reference)	0.025
	C/T	35 (26.9%)	52 (40%)	1.81 (1.07-3.05)	
Log-additive	---	---	---	2.13 (1.30-3.49)	0.0023

The table 5.12 presents results from the analysis of the association between rs8061351 and orofacial clefts in a sample of 260 individuals. The term "crude analysis" suggests that this is a basic or preliminary analysis. ***Model:*** *This column represents the genetic models used to assess the association. Different models are used to categorize individuals based on their genotypes and then calculate odds ratios (OR) to determine the risk of orofacial clefts compared to a reference group (often T/T in this case).* ***Genotype:*** *This column lists the genotypes of rs8061351 that are being compared in each genetic model.* ***Control Samples:*** *This column provides information about the distribution of genotypes in the control group (individuals without orofacial clefts).* ***NSCLP (Non-syndromic Cleft Lip and Palate):*** *This column provides information about the distribution of genotypes in the NSCLP group (individuals with orofacial clefts).* ***OR (95% CI):*** *This column displays the odds ratio along with a 95% confidence interval (CI). The odds ratio represents the likelihood of having orofacial clefts in one genotype group compared to a reference group (often T/T in this case). The confidence interval provides a range of values within which the true odds ratio is likely to fall.* ***P-value:*** *This column displays the p-value associated with the odds ratio, which indicates the statistical significance of the association. A lower p-value suggests a more significant association.*

Codominant Model: Genotype T/T: 95 individuals (73.1%) in the control group, 74 individuals (56.9%) in the NSCLP group Genotype C/T: 35 individuals (26.9%) in the control group, 52 individuals (40%) in the NSCLP group Genotype C/C: 0 individuals (0%) in the control group, 4 individuals (3.1%) in the NSCLP group. The codominant model analysis indicates that individuals with the C/T genotype have an odds ratio of 1.91 for having orofacial clefts compared to those with the T/T genotype. This result is statistically significant ($p = 0.0032$), suggesting that the C/T genotype may be associated with an increased risk of orofacial clefts.

Dominant Model: Genotype T/T: 95 individuals (73.1%) in the control group, 74 individuals (56.9%) in the NSCLP group Genotype C/T-C/C: 35 individuals (26.9%) in the control group, 56 individuals (43.1%) in the NSCLP group. In the dominant model analysis, individuals with either the C/T or C/C genotype have an odds ratio of 2.05 for having orofacial clefts compared to those with the T/T genotype. This result is also statistically significant (p = 0.0061), suggesting that the presence of the C allele (C/T or C/C genotype) is associated with an increased risk of orofacial clefts.

Recessive Model: Genotype T/T-C/T: 130 individuals (100%) in the control group, 126 individuals (96.9%) in the NSCLP group Genotype C/C: 0 individuals (0%) in the control group, 4 individuals (3.1%) in the NSCLP group**.** In the recessive model analysis, individuals with the C/C genotype do not show a statistically significant association with orofacial clefts compared to those with the T/T-C/T genotype as the odds ratio compared with the reference showed NA (0.00-NA), even though the p = 0.018. However, it is important to note that the small number of individuals with the C/C (n=4) genotype may limit the statistical power of this analysis.

Overdominant Model: Genotype T/T-C/C: 95 individuals (73.1%) in the control group, 78 individuals (60%) in the NSCLP group Genotype C/T: 35 individuals (26.9%) in the control group, 52 individuals (40%) in the NSCLP group**.** In the overdominant model analysis, individuals with either the T/T or C/C genotype have an odds ratio of 1.81 for having orofacial clefts compared to those with the C/T genotype. This result is statistically significant (p = 0.025).

Log-additive Model: In the log-additive model analysis, each additional copy of the C allele is associated with an odds ratio of 2.13 for having orofacial clefts. This result is

statistically significant (p = 0.0023), indicating a dose-response relationship between the number of C alleles and the risk of orofacial clefts.

In summary, the analysis suggests that the rs8061351 genetic variant is associated with an increased risk of orofacial clefts, particularly in individuals carrying the C allele (C/T or C/C genotypes). These findings may have implications for understanding the genetic basis of orofacial clefts and their potential clinical relevance. Further research and replication studies may be needed to confirm these associations and explore their underlying mechanisms.

5.4 Highlights of the molecular Study

From the genetic study several important findings and insights can be highlighted:

***IRF6* Gene:** In the studied population, the C (in Minus Strand G) allele of rs2235371 is more common than the T (in Minus Strand A) allele (the chromatogram in the Figure 5.6, shows the sequencing results of the minus strand and the result section mention G and A as alleles) both in the control samples and the NSCLP group. The predominance of allele C is notable, while allele T shows variations between the control and NSCLP groups. The C/C genotype is the most prevalent in the entire population, the control samples, and the NSCLP group. However, there is a difference in genotype frequencies between the control and NSCLP groups, indicating a potential association between genotypes and orofacial clefts. The HWE tests show that the genotype frequencies in the control group agree with Hardy-Weinberg equilibrium, suggesting the quality of the genetic data. The entire population and the NSCLP group show slightly higher p-values, indicating a minor departure from HWE but not significantly so. Various genetic models (Codominant, Dominant, Recessive, over dominant, Log-additive) have

been applied to assess the association between rs2235371 genotypes and orofacial clefts. In the Codominant model, the C/T genotype is associated with a significantly increased risk of orofacial clefts compared to the C/C genotype. The Dominant model also shows an increased risk associated with the presence of the T allele (C/T or T/T genotype). In the Over dominant model, having C/T genotype is associated with an increased risk of orofacial clefts compared to the C/C and T/T genotype. The Log-additive model suggests a dose-response relationship, where each additional copy of the T allele increases the risk of orofacial clefts. In summary, these findings indicate that the rs2235371 genetic variant in the *IRF6* gene may play a role in the risk of orofacial clefts, particularly in individuals carrying the T allele. The association is observed in multiple genetic models, suggesting robust evidence of an association. Further research and replication studies are warranted to validate these findings and better understand the genetic basis of orofacial clefts.

***CRISPLD2* Gene:** Allele frequencies and genotype frequencies for rs8061351 in the *CRISPLD2* gene have been provided, showing differences between the control samples and NSCLP group. The HWE test results for rs8061351 indicate that the control group's genotype frequencies are in good agreement with the expectations of Hardy-Weinberg equilibrium, supporting the quality of the genetic data. Similar to the analysis for rs2235371, various genetic models have been applied to assess the association between rs8061351 genotypes and orofacial clefts. In the Codominant model, the C/T genotype is associated with a significantly increased risk of orofacial clefts compared to the T/T genotype. The Dominant model also shows an increased risk associated with the presence of the C allele (C/T or C/C genotype). In the Overdominant model, having C/T genotype is associated with an

increased risk of orofacial clefts compared to the T/T and C/C genotype. The Log-additive model suggests a dose-response relationship, where each additional copy of the C allele increases the risk of orofacial clefts. In summary, similar to the *IRF6* gene, the rs8061351 genetic variant in the *CRISPLD2* gene is also associated with an increased risk of orofacial clefts, particularly in individuals carrying the C allele. These findings highlight the potential significance of this genetic variant in the development of orofacial clefts and warrant further investigation and replication studies.

CHAPTER VI: DISCUSSION

6. DISCUSSION

The epidemiological and genetic data presented in this study shed light on the occurrence of non-syndromic cleft lip and palate (NSCLP) and its association with genetic variations, specifically in the *IRF6* and *CRISPLD2* genes. Here, we discuss the key findings and their implications:

Epidemiology and Geographical Distribution: In this study of 130 NSCLP cases, a notable gender distribution was observed, with 59.23% of cases being females and 40.77% males. This gender distribution is consistent with previous research, which has indicated varying prevalence rates between males and females in NSCLP cases. Furthermore, the distribution of cleft types within the NSCLP cases was analyzed, revealing that 68% of cases had both cleft lip and palate, 14% had isolated cleft lip, and 18% had isolated cleft palate. These findings align with existing literature, which has reported similar proportions of different cleft types in NSCLP populations. Cleft lip is reported to be more common in males at a 2:1 male to female ratio, whereas a cleft palate is more common in females with a ratio of 1:2 male to female (Wyszynski);(Al Omari and Al-Omari).

The geographical distribution of cases was assessed using QGIS software, emphasizing the importance of geographic epidemiology in understanding the prevalence and distribution of NSCLP cases. Geographical factors can play a role in the incidence of cleft lip and palate, and this analysis could provide valuable insights for future studies.

Cytogenetic Analysis: Cytogenetic analysis focused on the identification of heterochromatic variations in patients with NSCLP. The study identified five cases with prominent heterochromatic variations, including 9ph+, 9qh-, 9cenh+, 9qh+, or inv(9)(p11q13). Notably, all cases exhibited de novo heterozygous inv(9)(p11q13). These

findings suggest a potential role for heterochromatic variations in the development of cleft lip and palate disorders. This insight may open avenues for further research into the genetic mechanisms underlying NSCLP. According to the findings of (Kaiser, 1984), it was perceived that many of the frequent pericentric inversion observed in the human karyotype is found to be the inversion of the heterochromatic region of chromosome inv(9)(p11q13), 9 (inv(9)), or inv(9)(p12q13). In this regard, the current study was explored the applicability of C banding to find any kind of heterochromatic variation in the patients. Based on the results obtained from the C banding analysis, it was found that about five of the patients screened in this study had heterochromatic variation in the aforementioned region. According to the findings by Brewer et al., 1999a, the incidence of inversion 9 has been determined to be 3.57% in different peoples through several approaches including the antenatal cytogenetic and peripheral blood karyotype analysis. The present study strongly corroborated with these findings but, the percentage for the incidence of inversion 9 was reported to be 2.3%. In agreement with the findings of this chapter and (Jeong et al., 2010, Babu et al., 2006), authenticated that the inversion 9 is primarily linked to CL and the palate in a 7-year-old girl, which justifies the importance of analysing the role of inversion 9 based inferences towards disorders associated with CL. While interpreting the findings of this chapter in context with the above discussed inferences, the following outline has been detected; it is found in 3 cases of NSCLP, 2 male patients with CLP and one in male patients with isolated cleft lip only.

Typically, the variations have been found in chromosome 9 are as follows; 9ph+, 9qh-, 9cenh+, 9qh+, or inv(9)(p11q13). The probable link between the heterochromatin variant of chromosome 9 and the cleft lip and palate was verified by (Kosyakova et al., 2013) and, their findings principally unveiled that the chromosome 9 displays the maximum number of phenotypical variants amid the non-acrocentric human chromosomes research.

The parents' karyotypes showed that all of the cases were *de novo* heterozygous inv(9)(p11q13). The breakage and re-insertion during inversion may also result to a loss or suppression of the euchromatic region that might cause an abnormality. Molecular cytogenetic probes can be applied for the better understanding of the breakpoints and its effects. Along with chromosome abnormality, two cases were reported with polymorphic variations. Although there is a disagreement over polymorphic variations, its origin and results cannot be ignored. Long arms of the 1, 9, 16, and distal long arm of the Y chromosome all have polymorphic variations often (Kosyakova et al., 2013). The major findings of this study clearly revealed that, the polymorphic variation 9qh+ was observed in male patient with the unilateral cleft lip & palate and 9qh- variation was observed in a male patient with an isolated missing palate. Given the above discussed facts, the present investigation can clearly assume the fact that the heterochromatin variations have been noted as one of the predominant probable factors that illustrate its probable role in the development of the disorders allied with the cleft lip and palate.

Molecular Analysis - *IRF6* Gene: The molecular analysis of the *IRF6* gene variant (rs2235371) revealed significant associations with NSCLP risk. The allele frequencies indicated that allele G was predominant in both the controls and NSCLP cases, while allele A showed variations between them. Various genetic models, including codominant, dominant, recessive, overdominant, and log-additive models, were applied to assess the association between rs2235371 genotypes and NSCLP risk. The results consistently suggested that the presence of the A allele (G/A or G/G genotypes) is associated with an increased risk of NSCLP. These findings underscore the potential importance of the rs2235371 variant in NSCLP susceptibility.

The genetic variant rs2235371, located within the *IRF6* (Interferon Regulatory Factor 6) gene, has been the subject of extensive research regarding its association with non-

syndromic cleft lip and palate (NSCLP). Several studies have investigated this variant, and their findings collectively underscore the significance of rs2235371 in NSCLP susceptibility. Rahimov et al. (2008) identified a genetic variant within the *IRF6* enhancer region associated with cleft lip. The variant disrupts an AP-2α binding site, emphasizing the functional significance of rs2235371. Their findings suggest that rs2235371 variant disruption is implicated in cleft lip pathogenesis. Zucchero et al. (2004) conducted a pioneering study that established the link between *IRF6* gene variants, including rs2235371, and isolated cleft lip or palate. Their research identified rs2235371 as a risk variant for isolated cleft lip or palate. Park et al. (2007) conducted a multi-population analysis that corroborated the association between rs2235371 and non-syndromic cleft lip with or without cleft palate. Their findings consistently indicated that rs2235371 associates with cleft lip and palate across diverse populations. Mangold et al. (2016) performed a genome-wide association study that identified rs2235371 as one of the susceptibility loci for non-syndromic cleft lip with or without cleft palate. Birnbaum et al. (2009) extended the evidence of rs2235371's association with non-syndromic cleft lip with or without cleft palate, focusing on Central European patients. Their research further supported the link between rs2235371 and cleft lip and palate susceptibility in Central European populations. In summary, these studies collectively provide compelling evidence supporting the role of rs2235371 within the *IRF6* gene as a significant genetic determinant of NSCLP risk. The variant's disruption of regulatory elements and its consistent association across diverse populations highlight its importance in the complex genetic architecture of orofacial clefts (Rahimov et al., 2008; Zucchero et al., 2004; Park et al., 2007; Mangold et al., 2016; Birnbaum et al., 2009). Further research may delve into the functional mechanisms underlying this association, potentially shedding light on novel therapeutic targets or preventive strategies for NSCLP.

Molecular Analysis - *CRISPLD2* Gene: The molecular analysis of the *CRISPLD2* gene variant (rs8061351) revealed allele frequencies and genotype distributions within the study population. Allele T was predominant in both control population and NSCLP cases, while allele C exhibited variations between them, so it can be considered as a risk allele. The exact test for Hardy-Weinberg equilibrium indicated subtle deviations from equilibrium in each group, while these deviations were not statistically highly significant. They further warrant importance of investigation to understand potential genetic associations or population dynamics related to rs8061351.

The genetic variant rs8061351, located within the *CRISPLD2* (Cysteine Rich Secretory Protein LCCL Domain Containing 2) gene, has been a subject of investigation regarding its potential association with non-syndromic cleft lip and palate (NSCLP). Several studies have explored this variant, shedding light on its role in NSCLP susceptibility. Here, we discuss the results of these studies with respect to rs8061351. Leslie et al. (2012) conducted expression and mutation analyses that implicated *ARHGAP29* as the etiologic gene for orofacial cleft (OFC), identified by genome-wide association on chromosome 1p22. While not directly related to rs8061351, their research underscores the genetic complexity of OFC and the importance of exploring multiple loci. Vieira et al. (2007) examined *IRF6* in individuals originating in South America and its association with oral-facial cleft. Although their study focused on *IRF6,* it highlights the broader interest in genetic factors contributing to OFC. Srichomthong et al. (2005) explored the significant association between *IRF6* 820G->A and non-syndromic cleft lip with or without cleft palate in the Thai population. Their findings emphasize the involvement of *IRF6* variants in OFC risk.

In summary, while specific research directly linking rs8061351 with orofacial clefts is limited, the studies mentioned above highlight the broader genetic context in which

variants like rs8061351 may play a role. OFC is a complex condition influenced by multiple genetic factors, and future research may uncover the specific contributions of rs8061351 within the *CRISPLD2* gene to this complex trait.

Association of Genetic Variants with Orofacial Clefts: Both genetic variants, rs2235371 (*IRF6*) and rs8061351 (*CRISPLD2*), were found to be associated with an increased risk of orofacial clefts, particularly in individuals carrying specific alleles or genotypes. These associations were observed across multiple genetic models, emphasizing the robustness of the findings. These results contribute to our understanding of the complex genetic underpinnings of orofacial clefts and may have implications for future research and clinical applications in cleft lip and palate management and prevention. Further studies are needed to confirm and expand upon these findings, ultimately advancing our knowledge of NSCLP etiology.

CHAPTER VII: SUMMARY AND CONCLUSION

7. SUMMARY AND CONCLUSION

This study investigated non-syndromic cleft lip and palate (NSCLP), exploring both epidemiological and genetic aspects. Among the 130 NSCLP cases studied, a slight gender bias was observed, with 59.23% females and 40.77% males affected. Cleft lip and palate together were the most common presentation, occurring in 68% of cases. Geographical distribution analysis was performed to understand regional prevalence patterns. Cytogenetic analysis focused on heterochromatic variations, revealing prominent heterochromatic changes in five cases, suggesting their potential role in NSCLP development.

Molecular analysis scrutinized two genetic variants, rs2235371 (*IRF6*) and rs8061351 (*CRISPLD2*). In the *IRF6* gene, allele G was prevalent in both the entire study population (90%) and NSCLP cases (85%). Allele A exhibited variations between control samples (6%) and NSCLP cases (15%). Genetic models consistently associated the A allele (G/A or G/G genotypes) with an increased NSCLP risk. The *CRISPLD2* gene variant, rs8061351, showed allele T predominance in the entire study population (82%) and NSCLP cases (77%), with allele C variations between control samples (13%) and NSCLP cases (23%).

In conclusion, this study enhances our understanding of NSCLP epidemiology and genetic factors, highlighting the significance of rs2235371 (*IRF6*) and rs8061351 (*CRISPLD2*) variants in NSCLP susceptibility. These findings, consistent across diverse genetic models, may have implications for future research, advancing cleft lip and palate management and prevention strategies. Additional investigations are warranted to validate

and build upon these insights, potentially shaping the landscape of cleft research and patient care.

CHAPTER VIII: RECOMMENDATIONS

8. RECOMMENDATIONS

Cytogenetic variations

Further Characterization of Heterochromatic Variations:

- Conduct a more in-depth analysis of the heterochromatic variations observed in chromosome 9, such as 9ph+, 9qh-, 9cenh+, 9qh+, or inv(9)(p11q13).
- Investigate whether these variations are specific to certain subtypes of cleft lip and palate disorders or if they occur across different phenotypes.

IRF6 Gene:

- Allele C of rs2235371 is more common than allele T in both control and NSCLP groups.
- The C/C genotype is the most prevalent in the entire population, control samples, and NSCLP group, which suggest the presence of rs2235371 polymorphism in our entire population.
- Genotype frequencies differ between control and NSCLP groups, suggesting a potential association with orofacial clefts. The study can be extended to understand the protein level changes if any as the reported SNP considered as a missense variant yet it points to Valine to Isoleucine change.
- Hardy-Weinberg equilibrium (HWE) tests support the genetic data quality, with minor deviations in the entire population and NSCLP group.

- Various genetic models (Codominant, Dominant, Recessive, Overdominant, Log-additive) indicate an increased risk of orofacial clefts associated with the T allele.
- The association is robust across multiple genetic models, suggesting a significant role of rs2235371 in orofacial cleft risk.
- Further research and replication studies are needed to confirm these findings and understand the genetic basis of orofacial clefts.

CRISPLD2 Gene:

- Allele frequencies for rs8061351 in the *CRISPLD2* gene differ between control samples and the NSCLP group.
- Genotype frequencies in the control group align with Hardy-Weinberg equilibrium, indicating data quality.
- Like the *IRF6* gene, various genetic models (Codominant, Dominant, Overdominant, Log-additive) suggest an increased risk of orofacial clefts associated with the C allele.
- The association is consistent across multiple genetic models, highlighting the significance of rs8061351 in orofacial cleft development.
- These findings underscore the potential importance of the *CRISPLD2* gene variant in orofacial clefts and warrant further research and replication studies.

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PUBLICATIONS

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Sl No	Authors in order andTitle of Publication*	Journal Name, Volume, Number,Year & Digital Object Identifier (DOI) Number	International/ National**	Publisher with ISSN	Web Address of the Journal	Indexed by	Impact factor if any
1.	**Soumya Raj, Leyon Varghese** Narayanan PV Suresh Kumar R Varghese PR Alex George Title: Identification of heterochromatic variations in nonsyndromic cleft lip and palate	© 2023 Journal of Orofacial Sciences January-June 2023 - Volume 15 - Issue 1 DOI: 10.4103/jofs.jofs_136_23 |	International	Published by Wolters Kluwer - Medknow 0975-8844	www.jofs.in	Scimago Journal Ranking, SCOPUS	0.45
2	Madhuri Menon **Soumya Raj** Achuthan C Raghavamenon, **Leyon Varghese** Evaluation of the tumor reduction potentials of Pleurolobus gangeticus using in vitro as well as in vivo models	Revista Brasileira de Farmacognosia. Volume 33 Issue 5, October 2023	International	Springer Nature 1981-528X	https://www.springer.com/journal/43450	Scimago Journal Ranking, SCOPUS	2.3

PAPER PRESENTATIONS

PAPER PRESENTATIONS

SlNo	Authors in order andTitle	Seminar name	International/ National**	organizer
1.	Association of rs8061351 in *CRISPLD2* gene and non-syndromic cleft lip and palate: A case-control study. **Soumya Raj** **Leyon Varghese** P.V Narayanan P.R Varghese Alex George	Advances in Genetic Diagnosis in the Era of OMICS (3rd prize)	International Conference	Institute of Genetics and Hospital for Genetic Diseases, Osmania University June 2022.
2.	Genetics of cleft lip and palate patients from Kerala: A pilot study. **Soumya Raj** **Leyon Varghese** Narayanan PV Suresh Kumar R Varghese PR Alex George	8th annual conference of Indian academy of biomedical sciences (Best oral presentation award)	International conference	CSIR-NIIST, Trivandrum 2019

PARTICIPATIONS AND WORKSHOPS ATTENDED

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- Completed certification course 'Genetics and Society: A Course for Educators by American Museum of Natural History, 2020.
- Completed certificate online course on Research to publication by British Medical Journal 2020.
- Oral presentation done on the topic 'Variations in the Karyotypes of Patients with Non-Syndromic Cleft Lip and Palate' Jubilee Research Day 2023 held on May 20th 2023.
- Attended three days Hands on Training on 'DNA Barcoding & Phylogenetic Analysis' in association with National Facility for Biopharmaceuticals, Mumbai was conducted from 18th November 2019 to 20th November 2019.
- Presented a paper in the 8th annual conference of Indian academy of biomedical sciences held at CSIR-NIIST, Trivandrum 2019, Topic: Genetics of cleft lip and palate patients from Kerala: A pilot study.
- Presented a paper in the International Conference on Advances in Genetic Diagnosis in the Era of OMICS, held at Institute of Genetics & Hospital for Genetic Diseases, Osmania University, from 17th -18th June 2022.
- Participated in 'Salisbury 19', a workshop on SPSS conducted by department of Community medicine, JMMC& RI, Thrissur on 2018 September 23rd and 24th.
- Presented a paper in National Symposium conducted on 2017 September 15, 16 in JMMC & RI "Genetics in clinical medicine" Chromosomal aberrations and *IRF6* gene polymorphism in orofacial clefts 54 – 58

Appendix-1

JUBILEE MISSION MEDICAL COLLEGE & RESEARCH INSTITUTE, THRISSUR-5

Patient Record

PART ONE : PATIENT INFORMATION

I . GENERAL INFORMATION

Patient Record Number : ……..……. Smile train Number : ……..

Did the patient's guardian sign the Guardian Consent form?

☐ Yes ☐ No

Name: ……...……

Date of Birth : ……..……/…….……/…..…… (dd/mm/yyyy)

Is this patient sponsored by Smile Train: (pick the one that applies)

☐ Yes ☐ No ☐ Unknown

If yes, how did the patient hear about The Smile Train? (pick the one that applies)

☐ Charity Organization ☐ Hospital/Physician

☐ Newspaper and TV ☐ Internet

☐ Friends and relatives ☐ other

If not which of the intervention were NOT under Smile Train?

Address :

Village / town / city :

Pin code :

Contact number :

Age :

Education :

Gender : (pick the one that applies)

☐ Male ☐ Female ☐ Unknown

Religion : Hindu / Christian / Muslim

Community :

Birth weight :

Height : Weight : BMI :

Blood Group :

Cleft lip / Cleft palate / CLP Unilatereal/ bilateral Right / left side
complete/ partial

Syndromic / Non Syndromic Which Syndrome:

Any other Difficulty other than CLP? Yes/ No

Cardio vascular/ Renal/ Pulmonary / Limb / Ophthalmic/ Auditory / Hepatic / Spleenic / Speech / MR

II . FAMILY HISTORY

❖ **MOTHER**

Mother's name : Age:

Blood group :

Education :

Occupation :

Consanguineous marriage : Yes / No Relation:

Age of mother at the time of patient's birth :

Number of children :

Para (No. Of Delivery) : gravida (No. Of Pregnancy):

Birth order of patient :

Health status of other children :

Any family history of cleft lip /palate : maternal / paternal / unknown

Occupation at the time of gestation :

Age of menarche : menopause :

Any miscarriages :

If yes, which month ?

Method of MTP :

Use of contraceptives :

Any treatment taken for infertility ? :

If yes , ayurveda / allopathy / homeo / IVF / IUI / Hormonal

Delivery type : normal / c section / vaccum / forceps

Any comorbidity present during pregnancy period : Thyroid / PCOD/ Asthma / Diabetes / Renal failure/ Cardiac disease

Have you undergone any treatment during the pregnancy period :

Any history of disability / genetic disorders/ MR / Anomalies in your family ? Yes/no

maternal /paternal relation with you :

❖ **FATHER**

Father's name : Age :

Blood group :

Education :

Occupation :

Age of father at the time of patient's birth :

Any comorbidity : Thyroid / Asthma / Diabetes / Renal failure/ Cardiac disease

If yes , Any treatment going on :

Any history of disability / genetic disorders/ MR / Anomalies in your family ? Yes/ no/ unknown

If yes, maternal or paternal : relation with you :

III. LIFESTYLE

	MOTHER	FATHER
Diet VEG/NON VEG		
Homily Food		
Commonly used cereals		
Use of Canned food		
Alcohol consumption		
Regular/frequent/occasional		
Brand name		
Aerated drinks/beer/wine		
Others		

Smoking		
Regular/frequent/occasional		
Brand name		
bidi/cigar/rolled pipe		
Chewing		
Tobacco/ betels/ hans/ panparag Regular/frequent/occasional		

IV . GESTATIONAL PERIOD - INFORMATIONS

- **Medications / drugs**
- Antibiotics :
- Steroids :
- Vitamin A supplement :
- Folic acid :
- Sodium valproate/ anti epileptic medications
- Pain killers :
- Sleeping pills :
- Any other :

- **Chemical exposure**
- Lithium :
- DDT :
- Sodium hypochlorite :
- Pesticides / chemical fertilizer
- Factory near by :
- Any history of radiation :
- Others :

- **Medical consultation**

- Mode of detection of pregnancy : self / lab
- In which month antenatal consultation started ?
- Consulted Gynaecologist :
- Hospital name : govt./private :
- Before consulting doctor have you taken any medicines or vitamin supplements by self ?
- Any infections during gestation ? Viral / bacterial / parasite
- Whether you were aware about cleft lip /palate before delivery ?
- Are you planning for next child ?

❖ **Occupation**

- Mother :
- Father :

V. SOCIOECONOMIC STATUS

- ✓ Annual income :
- ✓ Type of house : Area :
- ✓ Material used - roof : floor : wall :
- ✓ Type of family :
- ✓ Number of people staying at your house :
- ✓ Number of rooms :
- ✓ Water facility : pipeline / borewell / well
- ✓ Source of electricity :
- ✓ Toilet access :
- ✓ Type of toilet :
- ✓ Drainage facility :
- ✓ Fuel used for cooking : fuel / firewood / induction
- ✓ Use of electronic gadgets : TV/fridge/washing machine/inverter/computer/ laptop/AC
- ✓ Type of mobile phone :

✓ Internet access :

✓ Use of vehicle : two wheeler/ three wheeler/ four wheeler

PART TWO: INTERVENTION INFORMATION:

FORM IV- SURGICAL TREATMENT:

Type of Operation : (pick the one that applies)

Primary Lip/Nose unilateral repair ☐ Partial ☐ Complete

Primary Lip/Nose bilateral repair ☐ Partial ☐ Complete

Partial Cleft Palate Repair ☐

Secondary cleft Palate (Velopharyngeal) Repair ☐

Lip/Nose Revision ☐

Fistula Repair ☐

Alveolar Bone Graft ☐

Millard's forked flap to lengthen columella ☐

'Whole – in one ' or ' Combi' lip + Palate repair ☐

Type of Repairs : (Pick the one that applies)

☐ Rotation – Advancement Variant ☐ Trangular Varient

☐ Veau- Wardill V-Y Procedure ☐ Delaire Procedure

☐ Pinto Modification of Wardill Procedure ☐ Others

Where there any complications, injury or patient mortality?

☐ Yes ☐ No

If yes, please go on and complete the Event Report Form (Part Four)

Additional Comments on Intervention (Optional :

……...

……

……..

Any postponement of surgery : ☐ yes ☐ No

If yes: Reason

Duration of postponement

Are there additional craniofacial deformities? (Pick the one that applies)

☐ Yes ☐ No ☐ Don't know

Does this patient have velopharyngeal insuffiency following prior cleft palate repair?

☐ Yes ☐ No ☐ Don't know

Does this patient have abnormalities in any on the following areas?(check all that may apply)

Heart ☐ Yes ☐ No ☐ Don't know

Urinary System ☐ Yes ☐ No ☐ Don't know

Eyes ☐ Yes ☐ No ☐ Don't know

Nose ☐ Yes ☐ No ☐ Don't know

Ears ☐ Yes ☐ No ☐ Don't know

Limbs(Arms/Legs) ☐ Yes ☐ No ☐ Don't know

Fingers or Toes ☐ Yes ☐ No ☐ Don't know

Skin ☐ Yes ☐ No ☐ Don't know

Tongue Yes ☐ No ☐ Don't know

Skull ☐ Yes ☐ No ☐ Don't know

Mandible ☐ Yes ☐ No ☐ Don't know

Speech ☐ Yes ☐ No ☐ Don't know

Retarded Growth ☐ Yes ☐ No ☐ Don't know

Mental Retardation ☐ Yes ☐ No ☐ Don't know

Others ☐ Yes ☐ No ☐ Don't know

If yes what:

Allergies ☐ Yes ☐ No ☐ Don't know

If YES Please list all mediation allergies :

……..……

Other allergies :

…..……

Syndromic Y/N (if yes what syndrome

.....

Other Health Problems :

……...…….

……

……

If congental anomaly present : ☐ Yes ☐ No

If yes : Detected at birth Yes/ No

Prenatal Yes / No

If No, at what age :

Genetic study done : Yes / No

If yes : report :

Reason for study :

Preoperative Orthodontic : ☐ Yes ☐ No

If Yes : NAM/ LATHAMS

NAM : 1st Visit to hospital

1st visit to Orthodontics

Commencement of NAM

Commencement of nasal stent

Completed Yes / No

If No reason :_____

If yes How many Months/ weeks:_____

Unsuccessful NAM : Yes/ No

If yes – reason :_____

LATHAM'S

Direct surgery,

LATHAMS: 1st Visit to hospital

1st visit to Orthodontics

Commencement of LATHAMS

Insertion of Device : Any Complication_____

Was it, Failed NAM. Yes/ No

Fixed Dentition orthodontic Yes/ No:_____

Done JMH Yes/ No

Complete Yes/ No

Duration of treatment : _____

Preoperative Investigation

X-ray Chest :

Echo Cardiogram :

CT Scan Head

Others

MRI brain

Other

Blood Investigation : HB

Blood Group :

Abnormal Clotting : Yes/ No

If yes : details.

Hearing Assessment OAE : Yes/No

BERA : Yes/ No

Tympanometry : Yes/ No

Piere Robin Sequence : Yes/ No

Any intervention performed Yes/ No

If Yes- Oro gastric tube

Nasopharyngeal Airway

Tongue lip Adhesion

Distraction

Tracheostomy

ENT Consultation Yes/ No

Surgical intervention (ENT) Yes/ No

Tympanostomy

Mastoidectomy

Tympanoplasty

Others

Follow UP

Photos : Yes/ No

If yes date of photo

Speech : Yes/ No

If yes: Dates

Speech Report :

Preoperative paediatric Assessment : Yes/ No

More than Once : Yes/ No

Any other significant details:

VPI : Yes/ No

If yes Nasality : Yes/ No

Finding of Nasoendoscopy :

Closure: Concentric

Sagittal

Coronal

Asymmetric

Palate Movements :

Lateral wall Movement :

Posterior pharyngeal wall movement :

Adenoid enlarged : Yes/ No

If Yes- uniform central or on one side.

Asymmetrical

Details

Speech assessment : details

Surgical intervention : Yes/ No

If No- Why :

If yes- Procedure :

Redo palate

Furlow procedure

Bilateral Buccal flap

Orticochea sphincter pharyngoplasty

Pharyngeal flap

Hogans

Fulow Chen

Others

Other :

Post op speech assessment

Details :

Nasoendoscopy :

Appendix-2

JUBILEE MISSION MEDICAL COLLEGE & RESEARCH INSTITUTE, THRISSUR – 5

Research in MD/MS/DM/MCh Course
Informed consent

Institution : Jubilee Centre for Medical Research
Speciality : Human Genetics
Name of Investigator : Soumya Raj
Phone No : 9633220561
Title of project : Genetic studies in cleft lip and palate patients from Kerala

Nature of research with specific role of the participant : Four ml of peripheral blood samples will be collected from patients for molecular study. A proforma with consent form will be filled and obtained from patients or their guardians.

In addition to the details, cited above, I have been informed as follows :

a) I am physically fit to be a participant for the above study.

b) This study has direct relation with my illness, treatment and follow up/or that there is no tionship with my illness.

c) There is no additional investigations or intervention for the study purpose and hence there is additional financial commitment on my part.

If at all there is any additional tests required, the expenses will be met by the Institution.

d) There is no direct benefit for me out of this study. The society may be benefitted in future of this scientific study. It is also understood that I will not get incentives in any form for purpose of ticipation in this study.

e) I understand that the personal data collected will be kept confidential and that the clinical, oratory and Radiological data used for the study may be even published for future scientific elopment.

Appendix-3

INSTITUTIONAL ETHICS COMMITTEE
JUBILEE MISSION MEDICAL COLLEGE & RESEARCH INSTITUTE, THRISSUR
Reg. No: ECR/835/KL/Inst/2016
P.B No.737, Bishop Alapatt Road, Jubilee Mission P.O. East Fort,Thrissur 680005
Ph: 0487-2432200, 0487-2461000, 2462000. E-mail: ethicsjubilee@gmail.com

Date: 07.09.2019

Ms. Soumya Raj
Research Assisstant
Jubilee Centre for Medical Research
Jubilee Mission Medical College & Research Institute
Thrissur -5

IEC Study Ref.No :38 /19/IEC/JMMC&RI

Dear Student,

Sub: Approval of Research Proposal

I wish to inform you that your Research Proposal entitled "Genetic studies in cleft lip and palate patients from Kerala" has been approved by the Institutional Ethics committee, JMMC&RI on 07.09.2019 and has decided to give permission to proceed with the study / publish the article.

Name of Guide: Dr. Leyon Varghese

You must inform the IEC of the following:

1. The occurrence of Serious Adverse Events (SAE)/AE/ and/or Death, during the study period, in the IEC specified format, as per DCGI regulations.
2. Protocol violations/Protocol amendment
3. Discontinuation/Abandonment stating the reasons.
4. It is mandatory that, during and on completion of the above Research Project, the Principal Investigator is responsible for submitting an Annual report and a brief summary of the results obtained, to the Member Secretary of the Institutional Ethics Committee.

Secretary
SECRETARY
ETHICS COMMITTEE
JMMC & RI, THRISSUR-5
Dr. M P Raphael

Chairperson
CHAIRPERSON
ETHICS COMMITTEE
JMI, C & RI, THRISSUR
Dr. K M Francis

'Service with Love'

GIVE BLOOD * GIFT LIFE * GIVE EYES * GIFT SIGHT

Identification of heterochromatic variations in nonsyndromic cleft lip and palate

Soumya Raj[1,2], Leyon Varghese[2], Puthucode Viswanathan Narayanan[3], Suresh Kumar Raveendran[1], Pulikkottil Raphael Varghese[1], Alex George[1]

[1]Jubilee Centre for Medical Research, JMMC & RI, Thrissur 680005, Kerala, India, [2]Department of Zoology, Christ College, Irinjalakuda, Thrissur 680125, Kerala, India, [3]The Charles Pinto Centre for Cleft Lip, Palate and Craniofacial Anomalies, Jubilee Mission Medical College & Research Institute, Thrissur 680005, India

Abstract

Introduction: Orofacial cleft (OFC) has been one of the major common congenital anomalies exhibiting prominent ramifications allied with the medical, social, psychological, and economic strands. Most OFC occurrences do not have additional features, so they are categorized as nonsyndromic. The classification of the aforesaid complication has been directed toward the following categories: cleft lip (CL) with cleft palate, isolated CL, and finally the isolated cleft palate. The recent research concerning the aforementioned anomalies always searches for advanced novel inferences linked with the chromosomal perspectives since some of the specific genes are probably known to produce significant effects over the anomalies. **Materials and Methods:** Karyotyping was performed for all 130 cases of nonsyndromic cleft lip and palate (NSCLP). Aseptic collection of peripheral blood lymphocyte culture (PBLC) was performed from the patients using heparin vacutainers, and C-banding was done to confirm heterochromatic variations. **Results:** A total of 130 patients known to have the NSCLP were recruited for this study of which 88 cases (68%) had CL along with cleft palate, 18 cases (14%) had isolated CL and 24 cases (18%) had isolated cleft palate. Cytogenetic analysis by G-banding by Trypsin and Giemsa (GTG) banding in these patients revealed five cases (3.84%) with abnormal karyotype where a higher frequency of pericentric inversion in the analyzed region, specifically the chromosome 9, inv(9)(p11p13) was observed. **Conclusion:** The heteromorphisms or structural rearrangements involving the centromere were confirmed by centromere banding in two cases. Understanding the etiology with special inference on the above-said perspectives is significant to develop an effective strategy for the prevention and treatment of the individuals affected with the anomalies.

Keywords: *Centromeric banding, Heterochromatin variation, Inversion, Karyotyping, Orofacial cleft*

Introduction

The cleft lip (CL) and palate have been principally recognized as a common congenital defect documented at the birth stage, generally resulting from failure of fusion of palatal shelves or maxillary processes together with prominent psychological, medical, economic, and social complications. The various factors including the cleft type, race, and gender may determine the intensity and incidence of the complication.

The statistics concerning the incidence of CL and palate over the globe in the recent years was reported to be in the following pattern: 1 in 700 live births and the major fact is that from an Indian perspective, it is reported to be 1 in 500 live births.[1],[2] Development of the face starts in the fourth week of gestation with the migration of neural crest cells and the sixth to seventh weeks of gestation have witnessed a condition in which the MxP (paired maxillary processes) merges with the LNP (lateral nasal processes) and fuses with MNP (medial nasal processes) to develop the primary palate as well as the upper lip. Any failure in any of these could result in the development of an OFC in the upper lip, alveolus, and the primary palate.[3],[4] The influence of various genetic and environmental factors toward the complex molecular signaling pathways that are known to be essential for cellular processes regulation and palate morphogenesis has been reported by previous investigations.[5]

OFCs are recognized as either syndromic or nonsyndromic depending upon the presence

Received: 25 April 2023 **Revised:** 26 May 2023
Accepted: 1 June 2023 **Published:** 8-August-2023

How to cite this article: Raj S, Varghese L, Narayanan PV, Raveendran SK, Varghese PR, George A. Identification of heterochromatic variations in nonsyndromic cleft lip and palate. J Orofac Sci 2023;15:55-60.

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Access this article online
Website: www.jofs.in
DOI: 10.4103/jofs.jofs_136_23
Quick Response Code:

or absence of associated physical or cognitive anomalies. Syndromic CL and palate are generally associated with chromosomal anomalies such as trisomy 13 (Patau syndrome), trisomy 21 (Down syndrome), trisomy 18 (Edward syndrome), 22q11.2 deletion syndrome (Di George syndrome), and Treacher Collins syndrome where chromosome 5q32-q33 region is involved. The origin of syndromic clefting can be chromosomal, Mendelian, or sporadic. There are no <400 syndromes identified so far which have clefting as a primary feature.[6] These are usually instigated by certain mutations that happen in a single genetic locus, teratogens, or by chromosomal abnormalities. Based on the previous studies, among the major classes of OFC, it was perceived that CL with cleft palate is twice as frequent in males than in females, whereas isolated CL is found to be more frequent in females.[7,8]

Materials and Methods

Patient Recruitment and Ethical Clearance

The patients involved in this study were opted from The Charles Pinto Centre for Cleft Lip, Palate and Craniofacial Anomalies, Jubilee Mission Medical College and Research Institute (JMMC & RI), Thrissur, Kerala. A total of 130 patients having complications (NSCLP) were selected with the help of clinicians and detailed family background and medical history were recorded in a standard proforma. Detailed information regarding the clinical history of patients along with their personal data such as age, gender, relevant medical history, family history, and type of cleft were noted. The present investigation obtained written consent from the respective individuals participating in the present study. Ethical approval for this study (Ethical Committee No. 38/19/IEC/JMMC&RI) was provided by the Ethics Committee IEC of Jubilee Mission Medical Centre & Research Institute, on 07 September 2019.

Cytogenetic Analysis

Karyotyping was performed for all 130 cases of NSCLP. The PBLC was made from the patients' blood. GTG banding was performed according to the standard procedure.[9] Briefly, 72-hour Phytohemagglutinin (PHA)-stimulated PBLC was treated with colchicine to obtain metaphase chromosomes and used for the GTG.[10] At least 30 well-spread and banded metaphase spreads from each case were collected and analyzed using MetaSystem, Ikaros, Germany and karyotyped according to the guidelines prescribed by International System for Human Cytogenetic Nomenclature (ISCN) (McGowan-Jordan *et al.*).[11]

C-banding Analysis

The C-banding approach was used to unveil the heterochromatin variations in chromosomes. It is an effective complementary method to identify chromosomal heteromorphic or structural rearrangements involving the centromere.[12] C-banding using a milder 5% BaOH for 5–15 minutes at 50°C followed by incubation for 1 hour at 60°C performed to provide denaturation. The air-dried slides were then stained with 4% Giemsa and analyzed under Microscope.[9]

Results

There exists a prominent difference in the frequency of occurrence of CL and palate with regard to gender and the side of clefting. CL is reported to be more predominant in males (with a male-to-female ratio of 2:1), whereas the cleft palate is extensively predominant in females (with a male-to-female ratio of 1:2).[13,14] According to Bellis and Wohlgemuth, it was clear that the incidence of infants born with the above said complications, specifically the CL and palate anomaly in 502 cases in which 291 (58%) were males and 211 (42%) were females.[15] In this study, out of 130 cases, 77 cases (59.23%) were females and 53 cases (40.77%) were males. In large series, 46%, 26%, and 21% presented with CL and cleft palate, isolated CL, and cleft of the secondary palate only, respectively.[16] In this study, among the 130 studied cases, 88 cases (68%) had CL along with cleft palate, 18 cases (14%) had isolated CL, and 24 cases (18%) had isolated cleft palate [Table 1]. In our case, out of the 130 patients, 33 (25.38%) were having bilateral cleft, 53 (40.76%) were having unilateral left cleft, and 20 (15.38%) were having unilateral right CL [Table 2]. In this study, cytogenetic analysis by GTG banding was conducted in all the 130 NSCLP patients and revealed abnormal karyotype in 5 cases, pericentric inversion in the chromosome, which is usually designated as 9 (inv[9] [p11p13]) in 3 patients, a patient with 9qh–, and a patient with 9qh+ [Figs. 1 and 2].

Discussion

Recent studies from various fields, especially the combination of various perspectives arising from the epidemiology studies in context with strong genetic support would be greatly acknowledged by the scientific community in many circumstances since such studies can offer valuable knowledge regarding the role of candidate process and genes along with risk factors. Such perspectives

Table 1: Different Types of Clefting in the Patients with Respect to Gender.

Type of Cleft	Bilateral			Unilateral		
	Male (%)	Female (%)	Total (%)	Male (%)	Female (%)	Total (%)
CL	0 (0.0)	4 (3.1)	4 (3.1)	7 (5.4)	7 (5.4)	14 (10.8)
CLP	14 (10.8)	15 (11.5)	29 (22.3)	23 (17.7)	36 (27.7)	59 (45.4)
CP	-	-	-	-	-	-
Total	14 (10.8)	19 (14.6)	33 (25.4)	30 (23.1)	43 (33.1)	73 (56.2)

CP is mentioned as 0% as it does not come under the laterality differentiation. CL = cleft lip, CLP = cleft lip with cleft palate, CP = cleft palate.

can also unveil the prominent probable factors that are responsible for the disorder, specifically the CL and cleft palate. In this regard, this investigation has aimed to unveil the probable genetic factors that contribute prominently to emerging complications in affected individuals. From the findings of previous investigations, it was evident that the heterochromatin variations were usually found in the 1st, 9th, 16th, and Y chromosome. In addition to this, it was also evident that the chromosomal regions such as 1q25, 3p21, 4p15, 4q32, and 10p15 have been implicated as causative loci for clefts. Deletions in 4p16–14 and 4q31–35, duplications in bands 3p26, 3p24–23, 8q21, 3q23–25, 7q22–32, 14q11–21, 10p15–11, 16p12–13, and 22q12–13 were also found to be prominently allied with cleft palate.[17] The incidence of variation in the number and the structure of chromosomes in the studied patients with OFC was reported to be 3.6% in the previous studies,[18] and in the current study, it was 3.85%.

Table 2: Different Types Unilateral Clefting in the Patients with Respect to Symmetry.

	Unilateral Left			Unilateral Right		
Type of Cleft	Male (%)	Female (%)	Total (%)	Male (%)	Female (%)	Total (%)
CL	6 (4.6)	2 (1.5)	8 (6.1)	1 (0.7)	5 (3.8)	6 (4.6)
CLP	19 (14.6)	26 (20)	45 (34.6)	4 (3)	10 (7.7)	14 (10.8)
CP	–	-	-	-	-	-
Total	25 (19)	28 (21.5)	53 (40.7)	5 (3.8)	15 (11.5)	20 (15.5)

CP is mentioned as 0% as it does not come under the laterality differentiation. CL = cleft lip, CLP = cleft lip with cleft palate, CP = cleft palate

The previous inferences by many of the authors concerning the role of heterochromatic variants clearly validated the fact that the aforesaid variations in chromosome 9 have a critical influence over a plethora of clinical perspectives, specifically toward the CL and palate. But the fact is that a detailed study to reveal the above-said perspectives along with a strong genetic support has been found to be scarce in the current scenario from an Indian context. From this point of line, the present investigation has analyzed and validated the strong link between heterochromatic variation and disorders. Precisely, the current study has found about three cases of structural chromosomal abnormality, and all of them involved pericentric inversion in chromosome 9 [Fig. 3]. In agreement with our findings concerning the strong genetic influence toward the disorders, an investigation by Kosyakova *et al.*, detected 17 different variants and a majority of the detected variants were belonging to the pericentric inversions, as found in the current investigation.[19] One of the major factors that has been found in such studies is that the results concerning the heterochromatic variants and the disorder have been strongly influenced by the sample size of the population. For instance, several studies have reported to have a strong link between the disorder and the heterochromatic variation, but only within a limited range of populations, even if they have considered about a large population size for their study. But the fact is that the genetic data concerning the individuals that are reported to have the disorder with heterochromatic variation in the specific location would definitely be noted as

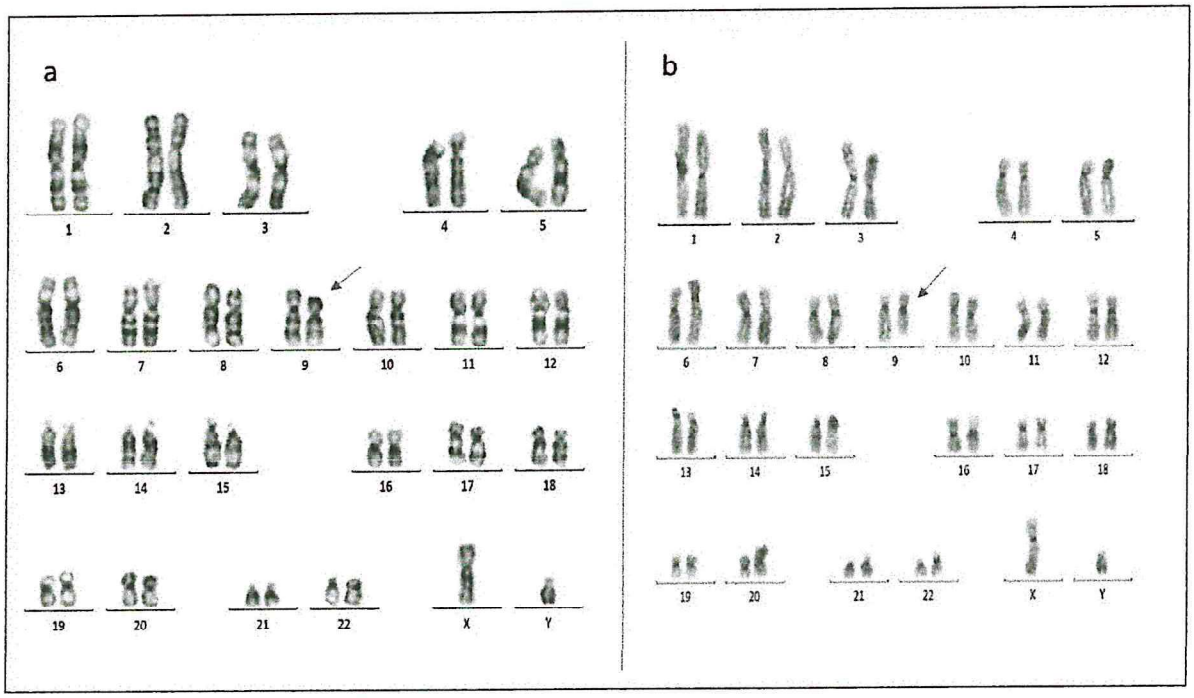

Figure 1: G-banding by Trypsin and Giemsa (GTG) banding analysis of case 1. (a) 9qh− in GTG banding; (b) C banding to confirm 9qh−.

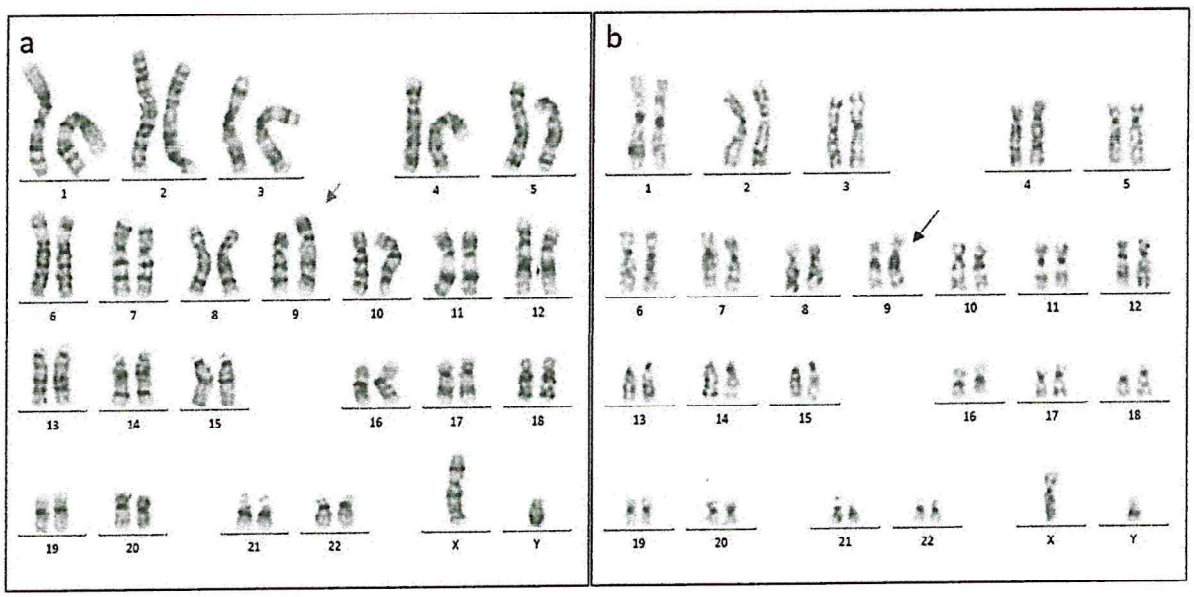

Figure 2: G-banding by Trypsin and Giemsa (GTG) banding analysis of case 2. (a) 9qh+ in GTG banding; (b) C banding to confirm 9qh+.

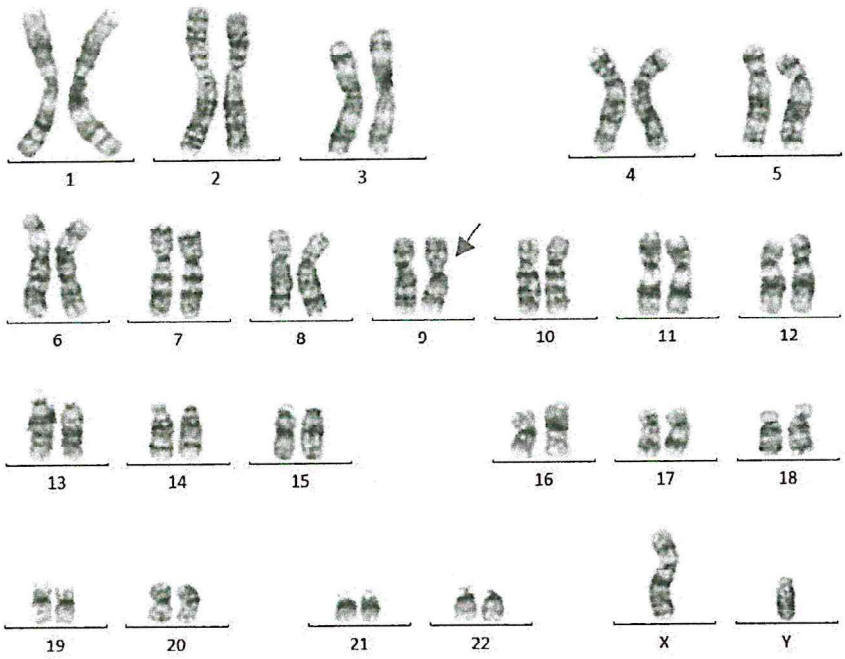

Figure 3: Representative image of the G-banded karyotype showing heterochromatic variation in chromosome 9, 46,XY,inv(9)(p11q13). It appeared in case number 3, 4, and 5 [Refer Table 3].

an important and critical contribution toward the scientific community since the studies concerning aforesaid aspect from all over the world, especially in the Indian context was found to be very rare and scanty in the current scenario; indicating the fact that, any data that are obtained in this regard can be explored to unveil the various unreported and unidentified link between the disorder and the heterochromatic variation found in the chromosome 9.[20]

According to a previous study by Kaiser, it was evident that the most frequent pericentric inversion discovered in the human karyotype is the inversion of the heterochromatic region of chromosome inv(9)(p11q13), 9 (inv(9)), or inv (9)(p12q13). In this regard, the present investigation has employed the applicability of C-banding to detect any kind of heterochromatic variation in the patients and found that about five individuals in this investigation exhibited

Table 3: Cytogenetic Abnormalities Found in the Study.

Case No.	NSCLP	Type of Cleft	Side	Gender	Chromosome Abnormality	Parents*
1	CLP	Unilateral	Left	Male	46,XY,9qh+	Normal
2	CP	NA		Male	46,XY,9qh–	Normal
3	CLP	Unilateral	Left	Male	46,XY,inv(9)(p11q13)	Normal
4	CLP	Bilateral	Both	Male	46,XY,inv(9)(p11q13)	Normal
5	CL	Unilateral	Left	Male	46,XY,inv(9)(p11q13)	Normal

*Karyotyping of both parents was conducted. CL = cleft lip, CLP = cleft lip with cleft palate, CP = cleft palate, NA = not applicable, NSCLP = nonsyndromic cleft lip and palate

heterochromatic variation in the 9th chromosome. According to the findings by Jeong *et al.*, it was perceived that incidence of inversion 9 has been calculated to be 3.57% in different populations through various analyses including antenatal cytogenetic and peripheral blood karyotype analysis. In addition to the above said findings, an investigation by Babu *et al.* unveiled the possible pathways and the link between the chromosome and certain specific genetic diseases, including the CL palate.[21] The high frequency of the same in children were also known to be the predominant factor responsible for the complications allied with the CL. In agreement with this, an investigation representing the international population, it is exciting to note that the specific gene *SLC31A1*, which is known to locate near the SPRY domain and B-box, has been strongly influenced and increased the chance of emergence of the CL-associated complications.[22] The present investigation has reported the same perspective as reported by Jeong *et al.*; however, the percentage for the same was reported to be 2.3% in the current study.[23]

A study by Rao *et al.* validated that inversion 9 is connected to CL and the palate in a 7-year-old girl justifying the significance of investigating the role of inversion 9–based inferences toward disorders allied with CL.[24] While considering the findings of this investigation in context with the aforesaid perspective, the following pattern was observed; it is found in three cases of NSCLP, two male patients with cleft lip with cleft palate (CLP), and one in male patients with isolated CL only. Usually, the variations have been found in chromosome 9 are 9ph+, 9qh–, 9cenh+, 9qh+, or inv (9) (p11q13).[19]

The probable link between the heterochromatin variant of chromosome 9 and the CL and palate was verified by Dong *et al.*, and they reported that chromosome 9 shows the highest number of phenotypical variants amid the nonacrocentric human chromosomes research.[25] The parents' karyotypes showed that all of the cases were *de novo* heterozygous inv(9)(p11q13). The breakage and reinsertion during inversion may result in a loss or suppression of the euchromatic region that might cause an abnormality. Molecular cytogenetic probes can be applied for a better understanding of the breakpoints and their effects.[26],[27] Along with chromosome abnormality, polymorphic variations were seen in two cases of NSCLP. Although there is a disagreement over polymorphic variations, its origin and results cannot be ignored. Long arms of the 1, 9, 16, and the distal long arm of the Y chromosome all have polymorphic variations often.[28] In the present study, polymorphic variation 9qh+ was observed in a male patient with unilateral CL and palate and 9qh– variation was observed in a male patient with an isolated missing palate [Table 3]. Given the above-discussed facts, the present investigation can clearly assume the fact that the heterochromatin variations have been noted as one of the predominant probable factors that illustrate its probable role in the development of the disorders allied with the CL and palate.

Conclusions

Cytogenetic studies are recognized to be useful to determine the involvement of chromosome abnormality on OFCs. This gives some conclusive evidence for patient management and offers genetic counseling to the patients at risk, and it helps for the future pregnancies. Further evaluation is needed on pericentric inversion of chromosome 9, particularly the study of the breakpoint region to find the association between chromosomal abnormalities and NSCLP which helps to delineate the association of structural abnormality with the incidence of palate and CL.

Acknowledgements

The authors would like to thank the late Dr Hirji Sorab Adenwalla for his inspirational advices for this research work and Smile Train India for providing patients and fund to conduct the research work. The authors like to thank the Management of JMMC & RI, Thrissur, India for providing all necessary support.

Declaration of Conflict of Interests

The author(s) declared no potential conflicts of interest.

Funding

Not applicable

Ethical Approval

The informed written consent was obtained from all the human study subjects. The ethical clearance for this study was obtained from the Institutional Ethical Clearance Committee of JMMC & RI (38/19/IEC/JMMC&RI).

Competing Interests

No financial interests.

Consent to Participate

Informed consent was obtained from all individual participants included in the study. Written informed consent was obtained from the parents.

Consent to Publish

The authors affirm that human research participants provided informed consent for the publication of the images in Figure(s) 1, 2, and 3.

Availability of Data and Materials

Not applicable

Authors Contributions

All authors have read and approved the manuscript

S.R. Conduct of study, validation of protocols, drafting of manuscript

L.V. Design of manuscript, critical reading of manuscript

N.P.V. Recruitment of subjects for the study, critical reading of manuscript

S.K.R. Validation of protocols, critical reading of manuscript

V.P.R. Patient recruitment, ethical clearance, critical reading of manuscript

A.G. Design of the study, finalizing the manuscript

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