Joint Biotechnology Master Program

Palestine Polytechnic University

Deanship of Graduate Studies and Scientific Research

Bethlehem University

Faculty of Science

Novel Mutations for Nonsyndromic Cleft Lip and Palate in Two Consanguineous Palestinian Families

By

Rawan Bilal Abu Geith

In Partial Fulfillment of the Requirements for the Degree
Master of Science

December, 2015

The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

Novel Mutations for Non Syndromic Cleft Lip and Palate in Two Consanguineous Palestinian Families

by

Rawan Bilal Abu Geith

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology.

Graduate Advisory Committee:

Committee Member (Student's Supervisor) — 24/2/2016 Date

Dr. Hashem Shahin, Bethlehem University

Committee Member (Internal Examiner) — 8/3/2016 Date

Dr. Yaqoub Ashhab, Palestine Polytechnic University

Committee Member (External Examiner) — 3.3.16 Date

Dr. Saad Allahham, An- Najah National University

Approved for the Faculties

Dr. Murad Abusubaih
Dean of Graduate Studies and Scientific Research
Palestine Polytechnic University
6/3/2016
Date

Dean of Faculty of Science
Bethlehem University
17/3/2016
Date

Novel Mutations for Nonsyndromic Cleft Lip and Palate in Two Consanguineous Palestinian Families

ABSTRACT

Cleft lip and\or palate (CL/ CP) is the most common congenital malformation that affects the upper lip and the roof of the mouth. It is one of the most frequent congenital anomalies, affecting 1 in every 500 to 1000 births worldwide. In the Middle East, the incidence has variably been reported as 0.3 to 2.19 per 1000 lives. Higher incidence can be attributed to the high percentage of consanguineous marriages and low socioeconomic status (SES). Individuals with CL/ CP may face many problems in breathing, feeding, hearing, language and social integration. They need to undergo many reparative surgeries as well as other non-surgical therapies. CL/ CP may manifest in different forms including: cleft lip, cleft palate only (CPO) and cleft lip with palate (CLP) according to the anatomical structure. The etiology is multifactorial, multiple genes and environmental factors play a central role in the generation of the CL/ CP phenotype.

In this study, next generation exome sequencing was performed to identify the mutations for three consanguineous Palestinian families collected from Ramallah and Hebron. Genotype to phenotype segregation within the families was validated by Sanger sequencing method. Carrier frequency within the healthy population was also determined in at least 200 healthy individuals.

The exome sequencing revealed in CP-AL family a substitution mutation (*BOD1*_R112X, chr5: 173040162 G>A) in the second exon of the *BOD1* gene, which converts the Arginine codon (CGA) to a stop codon (TGA). CP-BM family has a substitution mutation (*IRF6*_R250X, chr1:209964152 G>A). in exon 7 of the *IRF6* gene, which also converts the Arginine codon (CGA) to a stop codon. Insertion mutation (*CCDC141*_I295L, chr2:179809274 ins A) in exon 6 of the *CCDC141* gene has been found in CP-E family which leads to an early stop codon. Those mutations cause premature termination of transcription and release of incomplete, nonfunctional protein molecules.

Validation by Sanger sequencing indicates that the *IRF6* mutation is de-novo and there is no segregation for this mutation through the family. It also indicates that the

BOD1 and *CCDC141* mutations segregate perfectly with the phenotypes in the CP-AL and CP-E families respectively under an autosomal recessive mode of inheritance. Further ascertainment of unaffected individuals from family CP-AL revealed that this mutation can't be the causative one for the clefting phenotype in this family rather it is causing another phenotype in the family. Using 200 Palestinian healthy controls, we could not find any of those three different mutations either in homozygous or heterozygous forms.

Our study revealed that the identified mutations in the Palestinian CL/ CP patients are novel and occurred in two different gens, with zero carrier frequency in 200 healthy people.

Keywords

Cleft lip with or without cleft palate, Non Syndromic Cleft Palate, *IRF6*, *CCDC141*, *BOD1*.

اكتشاف طفرات جديدة تسبب الشفة الأرنبية و سقف الحلق المفتوح في عائلتين فلسطينيتين

ملخَّص الدِّراسة

تعتبر "الشَّفة الأرنبيّة وسقف الحلق المفتوح" من أكثر العيوب الخِلقيّة شيوعا في العالم والتي تؤثر على الشَّفة العلويّة، وسقف الحلق. وتصل نسبة الإصابة في العالم إلى حالة واحدة من بين كل 700 مولود، فيما تختلف النّسب في الشّرق الأوسط من 0.3-2.19\ 1000 مولود. ويمكن إرجاع ذلك إلى زواج الأقارب والوضع الاجتماعيّ والاقتصاديّ المتدنّي.

ويواجه مصابو الشَّفة الأرنبية مشاكل عديدة في السَّمع والتّغذية والتّكامل الاجتماعيّ واللُّغة والتَّنفُّس. لذا يحتاجون لإجراء العديد من العمليّات الجّراحيّة، بالإضافة إلى علاجات أخرى. وللشَّفة المشقوقة وسقف الحلق المفتوح أشكال مختلفة، قد تكون شَّفة مشقوقة, أوحلقًا مشقوقًا, أو كلايهما، وذلك وفقًا للبنيَّة التَّشريحيَّة. هنالك العديد من مسببات الإصابة بالشَّفة الأرنبيَّة، منها جينات متعددة، وعوامل بيئية كثيرة، قد يكون لها دورًا أساسيًا في المشكلة.

لقد استخدمت هذه الدِّراسة تقنيَّة (Next Generation Sequencing) لتحديد الطّفرات المسؤولة عن الأعراض الظَّاهرة على المصابين في ثلاث عائلات من رام الله والخليل. وكذلك تم استخدام تقنيَّة (SangerSequencing) لتقييم مدى علاقة الطَّفرة المكتشفة بالأعراض الظَّاهرة على أفراد العائلات، وكيفية انتقالها من جيل إلى آخر. بالإضافة إلى اكتشاف نسبة غير المصابين الحاملين لهذه الطَّفرات في 200 فرد سليم من المجتمع الفلسطينيّ.

كشفت تقنيَّة (Next Generation Sequencing) عن وجود طفرة استبدال في عائلة (CP-AL) في الجين (*BOD1*)، وهي عبارة عن تغيُّر في قاعدة نيتروجينيّة واحدة Guanine (G) إلى Adenine (A) في الحمض النوويّ، ما أدّى إلى استبدال الحمض الأمينيّ Arginine (R) إلى كودون وقف. وأظّهرت الدِّراسة وجود طفرة استبدال أخرى في عائلة (CP-BM) في الجين (*IRF6*)، وهي عبارة عن تغيُّر في قاعدة نيتروجينيّة واحدة (G) Guanine إلى Adenine (A)، والتي أدَّت إلى استبدال الحمض الأمينيّ Arginine (R) أيضا إلى كودون وقف. حيث تمّ الكشف عن طفرة جديدة في عائلة (CP-E) في جين (*CCDC141*)، وهي عبارة عن إضافة قاعدة نيتروجينيَّة جديدة Adenine (A)، والَّتي أدَّت إلى كودون وقف مبكر. ما جعل هذا الطَّفرات تؤدِّي إلى وقف مبكِّر للنسخ بالتالي الحصول على بروتين غير كامل وفعَّال.

وبيَّنت تقنيَّة (Sanger Sequencing) أن الطَّفرة في جين (*IRF6*) هي (Denovo) ولا تنتقل عبر العائلة. وأظهرت أن الطَّفرات المتنحِّية التي وجدت في (*BOD1*) و(*CCDC141*)، تنتقل من الآباء الحاملين لها إلى الأبناء المصابين في كلا العائلتين. ووفق الدِّراسة لم نجد أيًّا من الـ200 فرد غير المصابين من المجتمع الفلسطيني يحملون هذه الطَّفرات.

DECLARATION

I declare that the Master Thesis entitled "NOVEL MUTATIONS OF NONSYNDROMIC CLEFT PALATE IN TWO CONSANGUINEOUS PALESTINIAN FAMILIES " is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: Rawan Bilal Abu Geith

Date: Dec, 2015

STATEMENT OF PERMISSION TO USE

This thesis is in partial fulfillment of the requirements for the joint master degree in biotechnology at Palestine Polytechnic University and Bethlehem University; I agree that the library shall make it available to borrowers under rules of the library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation from, reproduction, or publication of this thesis may be granted by my supervisor, or in his absence, by the Dean of science, when in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Signature: Rawan Bilal Abu Geith

Date: 2015

DEDICATION

My humble efforts I dedicate to my family. A special feeling of graduated to my

loving parents

BILAL & MUFIDA

and my husband

MOEEN

***Whose affection, love, encouragement and Prayers of day and night make me able to get such success honor*.**

Rawan Abu Geith

Acknowledgment

I would like to express my sincere appreciation to my supervisor Dr. Hashem Shahin not only for the support he gave me during my research, but most of all for providing the ability to work in a stimulating environment, eager of discovery and continuous learning. I sincerely thank him.

I would like to thank the patients and their families for their trust and cooperation.
I would acknowledge Uraib Sharaha from hereditary research lab (Dr. Hashem's lab) for her help and assistance.

I gratefully acknowledge Tom Walsh and Prof. Mary- Claire King from University of Washington. I would also acknowledge Dr. John Van Aalst from Cincinnati Children's hospital medical center.

I would also thank the coordinators of the biotechnology master program from Bethlehem and Polytechnic Universities.

Special thanks to my dear friends Ms. Lena Qawasmi, Sabreen A.Geith, Fawzyih A.Geith, Naheel A.Geith, Dr. Raed Yacoub and Dr. Nasser Yacoub for the incorporeal support to me and to be with me in achieving my dream of obtaining a master degree.

I convey special acknowledgement to my sister Alaa, also for Ms. Raeda Yacoub and Hind A.Geith for special help and support.

Finally I never forget to thank my caring family, Ms. Haneen, Nabeel, Hussain, Mohammad, Salah and my baby KENAN. Thank you all.

Abbreviations

CL/ CP	Clefts of the lip and/ or palate
CPO	Cleft palate only
CLP	Cleft lip with cleft palate
CL	Cleft lip
NS CL/ CP	Nonsyndromic cleft lip with or without cleft palate
OC	Orofacial Clefts
SES	Socioeconomic Status
ECM	Extracellular Matrix
EMT	Epithelial- mesenchymal transformation
MEE	Medial Edge Epithelia
MES	Midline Edge Seam
EGF	Epidermal Growth Factor
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulfate
PCR	Polymerase chain reaction
KB	Kilobases
Bp	Base pairs
MR	Mental retardation
HGRL	Human Genetics Research Lab

List of Figures

List of Tables

List of Contents

CHAPTER THREE

Materials and Methods

CHAPTER FOUR

Results

CHAPTER FIVE

Discussion and Conclusion

CHAPTER ONE

1.1 Introduction

Orofacial clefts (OC) are the most common craniofacial anomaly in newborn babies (Borno, Hussein et al., 2012). Particularly, cleft lip with or without cleft palate (CL/ CP) and cleft palate only (CPO) represent a major public health problem affecting 1 in every 500 to 1000 births worldwide (Cooper, Ratay et al., 2006).

CL/ CP is a major congenital structure anomaly that has complex etiology also is notable for significant lifelong morbidity(Schutte and Murray, 1999), that also possesses significant medical, social, psychological, and financial implications on the affected individuals and also their families (Allam and Stone, 2014). Currently, treatment includes too many surgeries over the first 18 years of life, in addition to speech, dental therapies, and orthodontic treatment, with an estimated treatment cost of $101,000 per child (Bender, 2000).

The most critical period for face development is between the fifth and seventh weeks of gestation, in particular the sixth week being the most important. During this time, the morphodifferentiation and orientation occur simultaneously of the unpaired frontonasal process, with progressive medial migration and growth of the paired maxillary processes (Hussein-BDS and Abughazaleh,). Incomplete fusion of the processes between the fifth and seventh intrauterine weeks leads to the formation of cleft leaving gab in the affected area, and may extend to the surrounding facial structures resulting in extensive craniofacial deformity. These processes are known to be dependent on a spectrum of signaling molecules, transcriptional factors, enhancers and growth factors (Schutte and Murray, 1999). Mutations in the genes encoding these factors and the molecules may lead to craniofacial deformity.

CL/ CP varies in severity, from small notches in the lip to clefts that extend through the alveolar ridge and involve the floor of the palate (Bender, 2000). It is subdivided into CL/ CP, and CPO. However, recent studies have subdivided clefts into three categories: cleft lip (CL), cleft lip with cleft palate (CLP), and CPO, which due to differences concerning embryologic development, prevalence, risk factors, and associations with other congenital anomalies (Maarse, Rozendaal et al., 2012).

Generally CL/ CP and CPO are genetically distinct phenotypes in terms of their inheritance patterns. CPO is less common, with a prevalence of approximately 1/1500–2000 births in Caucasians, while CL/ CP is more common, 1–2/1000 births (Lidral, Moreno et al., 2008). It has been reported also that CLP more common in males, while the sex bias is reversed for CPO, which is more common in females (Stanier and Moore, 2004). The prevalence of CPO does not vary in different racial backgrounds, but the prevalence of CL/ CP varies, with Asian and American Indians having the highest rate and Africans the lowest (Vanderas, 1987). Clefts may be unilateral, occurring on one side, or bilateral occurring on both sides. Unilateral clefts are nine times common than bilateral clefts, which occur twice on the left side than on the right (Hopper, Cutting et al., 2007).

OC have also been categorized into syndromic and non-syndromic (isolated) clefts, according to the presence of other physical and developmental anomalies. Syndromic forms of CL/ CP often have simple Mendelian inheritance patterns which is more suitable for conventional genetic mapping strategies (Lidral, Moreno et al., 2008), usually associated with other anomalies in different organs. The syndromic cases can be subdivided into chromosomal syndromes which includes more than 350 Mendelian disorders, teratogens and uncategorized syndromes (Murray, 2002). Syndromic types are associated with other malformations, including the Pierre-Robin Sequence, Treacher - Collins Malformation, Trisomies 13 and 18, Apert's Syndrome, Stickler's Syndrome and Waardenburg's Syndrome (Muhamad, Azzaldeen et al., 2014).

Nonsyndromic CL/ CP (NS CL/ CP) genetically is a complex trait which have no physical or development anomalies except the CL/ CP with no known teratogenic exposures that may cause CL/ CP (Bender, 2000). The majority of the cases the evaluation of inheritance patterns have not revealed a simple Mendelian mode of inheritance also with no positive family history and in the familial cases has (Lidral, Moreno et al., 2008).

NS CPO is less common, with a prevalence of approximately 1 per 1500 to 2000 births in most racial backgrounds (Nikopensius, Jagomägi et al., 2010), but with exceptions for some geographic areas like Finland which has higher frequencies, most likely due to founder effects or environmental triggers (Nikopensius, Jagomägi et al., 2010). Approximately 70% of CL/ CP cases are nonsyndromic unassociated with any

recognizable anomalies, and 30% are syndromic cases association with deficits or structural abnormalities occurring in other parts of the body (Murthy and Bhaskar, 2009).

Epidemiologic studies support a role for environmental factors in clefting, especially in regions of low socioeconomic status (SES) (Murray, 2002). It has revealed an increased risk for CL/ CP with alcohol and smoking exposure during pregnancy, in addition recognized teratogens also cause clefts include rare exposures, such as phenytoin, valproic acid and Thalidomide. Furthermore, some studies suggest that some supplement has a protective effect against CL/ CP such as periconceptional folate or multivitamin supplementation (Lidral, Moreno et al., 2008). From 18% to 50% decreasing in CL/ CP risk occurring with supplements containing folic acid in humans and from 69% to 76% in experimental animals (Allam and Stone, 2014).In other hand studies reported that the CL\CP increases with Low maternal B6 and B12 levels measured after pregnancy was reported to increase the risk of CL/ CP especially in cases associated with low serum folate (Allam and Stone, 2014).

The malformation threshold in the developing embryos may be shifted to the extent by introducing into the maternal diet of a strain mice, human teratogenic which lead to 100% of the offspring are born with the expected deformity (Muhamad, Azzaldeen et al., 2014). However, not all mothers who drink or smoke have children with CL/ CP, nor do all mothers taking multivitamins have normal children, this is due to genes and environmental factors interaction.

Multiple genes and environmental factors play a role to modulate the cleft formation. "Causes linked to environment, genetics and gene-environment interaction are known, although there is still a lot to do, especially in clarifying the role of genetics in producing susceptibility to the environment" (Bianchi, Calzolari et al., 1999).

"The molecular events that underlie the formation of orofacial structures are under the strict control of an array of genes that includes the fibroblast growth factors (*Fgfs*), sonic hedgehog (*Shh*), bone morphogenetic proteins (*Bmps*), members of the transforming growth factor β (*Tgf-β*) superfamily, and transcription factors such as *Dlx, Pitx, Hox, Gli* and *T-box* families" (Jugessur and Murray, 2005). Additional growth and signaling factors include *JAGGED1*, Patched, *CREB* binding protein, *GLI3 FGFR1, CASK*, Treacle, and *FGFR2*. Other transcriptional factors include

DLX5/6 and *PAX3* (Schutte and Murray, 1999). ECM protein such as *Col2A1*, *Col1A2*, *Col11A2*, *PIGA*, integrin, glypican3, fibrillin and aggrecan are essential as well (Schutte and Murray, 1999).

Several genes playing a role in the etiology of isolated clefts as also known to underlie Mendelian syndromic forms of CL/ CP which include *IRF6*, *MSX1*, *PVRL1*, *TBX22* and *FGFR1* (Jugessur and Murray, 2005).

DNA-sequence variants associated with *IRF6* are major contributors to CL/ CP (Zucchero, Cooper et al., 2004) which confer a significant attributable risk for nonsyndromic CL (Thomason, Zhou et al., 2010). Mutations in the *IRF6* gene have been shown to be the cause of Van der Woude syndrome that has CL/ CP as a common feature (Scapoli, Palmieri et al., 2005).

Recent research has been highly successful in identifying the genetic mutations underlying syndromic forms CLP. For example, "mutations in *TBX22* cause X-linked cleft palate with ankyloglossia; in *FOXE1* mutation cause Bamforth-Lazarus syndrome; in *PVRL1*, cleft lip and palate-ectodermal dysplasia syndrome; in *MSX1*, CLP with tooth agenesis; in *FLNA*, otopalatodigital syndromes types 1 and 2; in *FGFR1*, autosomal-dominant Kallmann syndrome; and in *TFAP2A*, branchio - oculo-facial syndrome" (Thomason, Zhou et al., 2010).

1.2 Problem Statement and Objectives

CL/ CP is one of the most common birth defects, and the second most common congenital anomaly that affects the upper lip and the roof of the mouth. The etiology is complex and multifactorial, multiple genes and environmental factors play a central role in the generation of CL/ CP. The etiology and complexity of the clefting have been investigated for many years, which lead to continuous investigation to explain the causes of clefting.

Clefting presents major public health problems affecting 1 in every 500 to 1000 births worldwide (Cooper, Ratay et al., 2006). Currently, the incidence of clefting in Palestinians living in the territories had not reported in the literature . Reports from Palestinian populations in Israel and Jordan infer an incidence of 1.39 per 1000 live births (Borno, Hussein et al., 2014). This incidence can be attributed to the high percentage of consanguineous marriages and SES.

Children with CL/ CP face a variety of challenges, depending on the type and severity of the cleft. They may face problems in hearing; feeding, social integration, language and breathing. They need to undergo many reparative surgeries over the first 18 years of life, in addition to speech therapy, dental treatment, and orthodontic treatment, plastic surgery, maxillofacial surgery, audiology, psychological and genetic counseling, with an estimated treatment cost of $101,000 per children.

The ultimate objective and prerequisite of this aim is prevention, which may be facilitated by complete understanding of the concepts regarding the genetic and environmental factors contributing to OC and the interaction between them. It also, by complete understanding of the etiology of this condition. Detection of the mutations may help in reducing the clefting incidence, especially in communities which have high percentage of consanguineous marriages in particular, Palestinian community. Identification of genetic causes may help in many things, including assisting in better counseling and diagnosis, and also leading to interventions for those at high risk for having a child with an OC.

The purpose of this study is to identify the genetic causes of NS CL/ CP, and to determine the mutation responsible for cleft palate phenotype in three Palestinian

families affected with NS CL/ CP. Phenotypically affected members of those three families have CLP or CLO.

1.2.1 Specific objectives

- Whole exome sequencing on DNA samples from the affected individuals of CP-AL, CP-E and CP-BM families to detect the mutations responsible for the NS CL/ CP phenotype.
- Results validated by Sanger Sequencing for all CP-AL, CP-E and CP-BM family members to determine segregation of the mutations with the CL/ CP phenotype.
- Genotyping of 200 healthy, unaffected Palestinian controls from the West Bank.

CHAPTER TWO

Review of Literature

Facial clefting, which is the most common congenital facial anomaly and the second most common congenital anomaly, accounts for 13% of all congenital malformations. About half of the affected individuals have both cleft lip and cleft palate, and of the remainder, about half have isolated cleft lip and half isolated cleft palate (Jaber, Nahmani et al., 2002).

2.1 Orofacial Embryogenesis

Craniofacial development is a regulated process that involves many genetic and environmental factors that play a central role in facial development, including interaction of cell growth, growth factors and receptors, the fusion of the facial and palatal processes, apoptosis, and adequate nutrient supply.

2.1.1 Normal craniofacial development

Development of the head and face during embryonic development represent one of the most complex events, coordinated by a network of transcription factors and signaling molecules together with proteins conferring cell polarity and cell – cell interactions (Stanier and Moore, 2004). The development of craniofacial structures from the originating oropharyngeal membrane coordinated by many processes involving cell migration, growth, differentiation and apoptosis (Jugessur and Murray, 2005).

Normal facial development begins with migrating neural crest cells that combine with mesodermal cells to establish the five facial primordial (Schutte and Murray, 1999), which formed by migration of the neural crest cells from the dorsal area of the anterior neural tube (Chitturi, Reddy et al., 2014). Neural crest cells proliferate and migrate during the third week of gestation into the frontonasal and visceral arch region (Bender, 2000). "It arises within the peripheral neural ectoderm early in development and subsequently migrates into the presumptive facial primordial" (Francis-West, Ladher et al., 1998).

Around the primitive mouth there is series of small buds of tissue forms called the facial primordia, which consisting of a neural crest cells and mesodermally-derived mesenchymal core covered by an epithelial layer of ectoderm and endoderm that originate from the cranial crest (Muhamad, Azzaldeen et al., 2014, Francis-West, Ladher et al., 1998). The primordia consist of the frontonasal prominence, two maxillary prominences, and two mandibular prominences (Figure 2.1). The frontonasal prominence forms the forehead and the nose. The maxillary prominences are bilateral and form the lateral stomodeum the primitive mouth. The mandibular prominences are also bilateral and are responsible for the caudal growth of the stomodeum (Bender, 2000).

By day 32, due to a thickening of the surface ectoderm of the frontonasal prominence the nasal placodes arise and form. Subsequently, the lateral and medial nasal processes forms with the nasal pit in the center when the prominence bulges out around the placode. (Meng, Bian et al., 2009). During this time, the unpaired frontonasal process going to morphodifferentiation and orientation which occur simultaneously with progressive medial migration and growth of the paired maxillary processes (Hussein-BDS and Abughazaleh, 2012). The maxillary prominences enlarge and grow towards each other pushing the lateral nasal process, thus allowing the maxillary process to fuse with both the lateral and medial nasal process. The medial nasal prominences and the bilateral maxillary processes merge with each other to form the intermaxillary segment resulting in both the philitrum and primary palate (Jugessur and Murray, 2005) (Figure 2.1). The labial components that form the philitrum and the bony palatal component that includes the four maxillary incisor teeth form from the intermaxillary segments of the maxilla (Bender, 2000). The primary palate extends posteriorly to the incisive foramen, located immediately behind the alveolar ridge.

By gestational day 36, the two medial nasal processes fuse to form the upper lip and by gestational day 38,the external lip development is complete (Chiquet, 2011). The lower lip, jaw and lower cheek region are produced by the mandibular prominences, which merge across the midline (Jugessur and Murray, 2005).

During the fifth week of gestation, after fusion of the upper lip, the palate developed begins and is complete at the end of the twelfth week. It also follows up the initial development of the oral region with further proliferation and migration of the maxillary prominences (Bender, 2000).
The palate is formed in two stages; from the merging of the two medial processes the primary median palatal triangle is formed, which originated from the median frontonasal process (Hussein-BDS and Abughazaleh, 2012). The secondary palate, formed by fusion of the two palatine shelves that derived from the two maxillary outgrowths (Dudas, Li et al., 2007).

The secondary palate includes the hard and soft palate, the tissue that extends posteriorly from the incisive foramen, which is develop in the sixth week from the two palatal shelves (Jugessur and Murray, 2005). "The bilateral palatal shelves subsequently grow down vertically along the two sides of the tongue Then, the mandible starts to grow in length and the tongue moves downward, allowing the palatal shelves to be elevated above the dorsum of the tongue" (Meng, Bian et al., 2009). Later, shelves rapidly elevate to a horizontal position above the tongue (Dudas, Li et al., 2007) (Figure 2.1). The bilateral palatal shelves grow toward each other and adhere by the glycoprotein coat and desmosomal junctions of the Medial Edge Epithelia (MEE) to form the Midline Edge Seam (MES) (Meng, Bian et al., 2009). The formation of the palatal shelves involves proliferation of the mesenchymal cells and elevation of them is due the intrinsic forces developed due to the Extracellular Matrix (ECM) (Chitturi, Reddy et al., 2014).

In the shelf mesenchyme the hydration of ECM components is thought to provide the necessary intrinsic force to cause shelf elevation (Jugessur and Murray, 2005). There is programmed cell death of the medial edges once the shelves are elevated to the correct position, that thins the epithelium and allows the tissue from each side to join in the midline (Bender, 2000).Finally, degradation of this epithelial connection completes the process of palatal fusion after a transitory MES is formed from the adhered epithelia (Dudas, Li et al., 2007). "The exact fate of the epithelia in the MES is controversial, and three major pathways have been proposed for their disappearance: programmed cell death (apoptosis), migration to the oral or nasal side

of the palate, and Epithelial- mesenchymal transformation (EMT)" (Meng, Bian et al., 2009).

By the tenth week, fusion of the hard palate is completed also by the twelfth week development of the soft palate and uvula is completed with successful merging of the secondary growth centers (Bender, 2000).

Several cell adhesion molecules play a role in palatal fusion including nectin 1, desmosomes, type IX collagen, growth factors, such as *TGFα / EGFR* and *TGF-β3* (Jugessur and Murray, 2005). Several ECM molecules and growth factors are required for signalling facial primordia identity, differentiation of epithelial cells and remodeling of the palatal shelves.

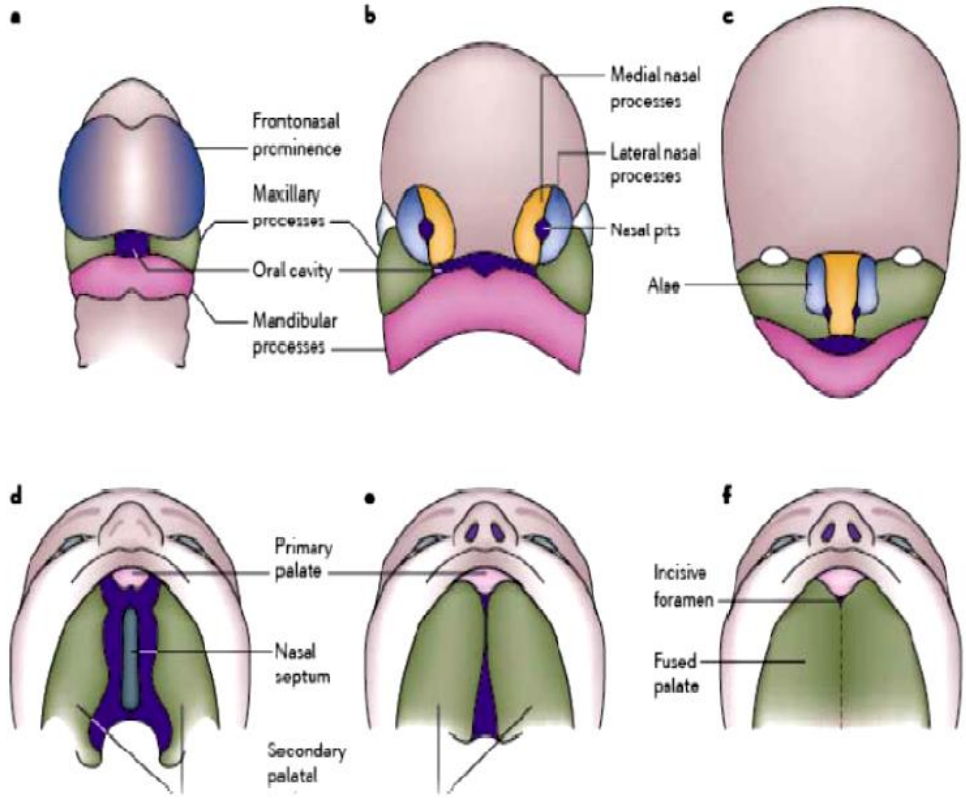


Figure 2.1:- Normal craniofacial development (Dixon, Marazita et al., 2011).

A. Developing of the five facial promordia (frontonasal prominence, paired maxillary processes and paired mandibular processes), which formed around the primitive mouth by the fourth week of gestation. **B.** By the fifth week, paired medial and lateral nasal processes form, by formation of the nasal pits. **C.** At the end of the sixth week, fusion between the medial nasal processes and the maxillary processes, this leads to the formation of the upper lip the primary palate. **D.** During the Sixth week, Secondary Palate formed by fusion of two maxillary outgrowths (palatal shelves), then the both shelves grow vertically around the tongue. **E.** Shelves rapidly elevate to a horizontal position above the tongue. **F.** Fusion between the lateral palatine shelves.

2.1.2 Development of cleft lip and palate

CL/ CP is the result of improper fusion of the processes that form the face. CL results from improper fusion between the frontonasal prominence and medial nasal process, and the cleft palate between the primary palate with the secondary palate or the lateral palatine shelves with each other.

CL/ CP caused by abnormal morphogenesis of the upper lip and primary palate either by misguided epithelial movement, disrupted EMT, or disrupted apoptosis (Chiquet, 2011). "Furthermore, Disruption in any of the processes of cell proliferation, migration, adhesion, differentiation and apoptosis involved in a highly coordinated growth and fusion of the facial processes and palatal shelves before the end of the sixth week of development can result in clefts of the lip and primary palate; between the sixth and tenth weeks, they cause clefts of the secondary palate" (Little and Nelson, 2013).

Two main mechanisms, were evidenced in this fusion defect: defective tissue development and/ or defective apoptosis in normal or defective tissues (François-Fiquet, Poli-Merol et al., 2014).

2.2 Classification and Phenotypes of Clefts

OC are commonly subdivided on the basis of anatomical, genetic and embryological findings, into those affecting the CL/ CP and those involving the CPO (Jugessur and Murray, 2005). OC have also been categorized into syndromic and non-syndromic clefts, according to the presence of other physical and developmental anomalies. Each category is further subdivided into complete or incomplete, unilateral (90%) or bilateral clefts (10%) and vermillion notch or microform (Table 2.1) (figure 2.2). Most studies suggest that about 70% of cases of CL/ CP and 50% of CPO are nonsyndromic. The syndromic cases can be subdivided into chromosomal syndromes, more than 350 Mendelian disorders, teratogens (e.g. phenytoin or alcohol) and uncategorized syndromes (Murray, 2002).

Table 2.1:- Comparison of different cleft classifications in the literature (Agrawal, 2014).

Authors and year	Number of groups/ types	Classification detail	Benefits	Drawbacks	Comments
Davis and Ritchie, 1922	3	Group I: Prealveolar (process) cleft Unilateral/bilateral/ median Group II: Postalveolar (process) cleft Soft palate/hard palate Group III: Alveolar (process) cleft Unilateral/bilateral/ median	Started the concept of cleft classification	Not an anatomical classification Lack of clarity Not a versatile one	First classification Has historical importance Not in use
Veau, 1931	4	Group 1: Cleft of soft palate Group 2: Cleft of hard palate Group 3: Complete unilateral CL, alveolus and palate Group 4: Bilateral CL, alveolus and palate	Still in use in modified forms	CL and CL with alveolus not included Sides of the cleft not mentioned No sound developmental basis	
Kernahan and Stark, 1958	2	Cleft of primary palate: Lip and premaxilla, anterior to incisive foramen Cleft of secondary palate: Hard and soft palate, posterior to incisive foramen	Most accepted principle for cleft classification	Not a versatile one Needs more descriptions for combinations	Basis for future classifications
Harkins et al., 1962	6	Cleft of primary palate: A — CL B — Cleft alveolus process Cleft of palate: A — Soft, B — Hard Mandibular process cleft Naso-ocular cleft Oro-ocular cleft Oro-aural cleft	Covers all types of clefts First descriptive classification	Elaborate classification Difficult to remember Did not gain popularity	Appointed by ACPA
Vilar-Sancho, 1962		Clefts were coded “Sk” (skisis) designates cleft “K” (keilos) for lip “G” (gnato) for alveolar process “U” (urano) for hard palate “S” (stafi los) for soft palate “2” for bilateral “d” for right, “l” for left, “I” for incomplete, “o” for operated, “+” for not affected	Objective Flexible Simple Adaptable Detail of cleft can be transcribed	Based on Greek nomenclature Difficult for non-Greek community	
Dahl, 1970	3/4	CL, CP, CLP-UCLP and BCLP	Easy to remember	Combination clefts cannot be classified	Basis for Indian Classification of Balakrishnan, 1975

Spina, 1973	4	Group I: Preincisive foramen cleft Group II: Transincisive foramen cleft Group III: Postincisive foramen clefts Group IV: Rare facial clefts	Simplified version of Harkin's cleft classification	Not popular	Modified ACPA classification, adopted by ISPRS
Berlin, 1979	3	Group 1: Cleft of anterior (primary) palate Group 2: Cleft of anterior and Posterior (secondary) palate Group 3: Cleft of posterior (secondary) palate Rare clefts			Like Spina's classification
Sandham, 1985	5	Types 1-4 are like Dahl's classification Type 5: Other types of clefts	Simple, embryologically sound		
Kernahan, 1971and modifications	Y	Schematic/logo /diagrammatic representation	Mental representation is perfect Ideal for recording the deformity for individual patients	Not fit for verbal communication Computer archiving and retrieval difficult	Not a true descriptive classification
Rossell-Perry, 2009		Clock diagram of 4 segments and 3 subdivisions-Lima clock diagram	Versatile	Diagrammatic representation	Not a true descriptive classification
Present, 2014	3	Tables 1-3(Ref: Agrawal, 2014) Combination of descriptive classification with brief notations		There is a learning curve, though short	Based on the Indian Classification of Balakrishnan, 1975

ACPA: American cleft palate association, CL: Cleft lip, CP: Cleft palate, CLP: Cleft lip and palate, UCLP: Unilateral cleft lip and palate, BCLP: Bilateral cleft lip and palate, ISPRS: International society for photogrammetry and remote sensing.

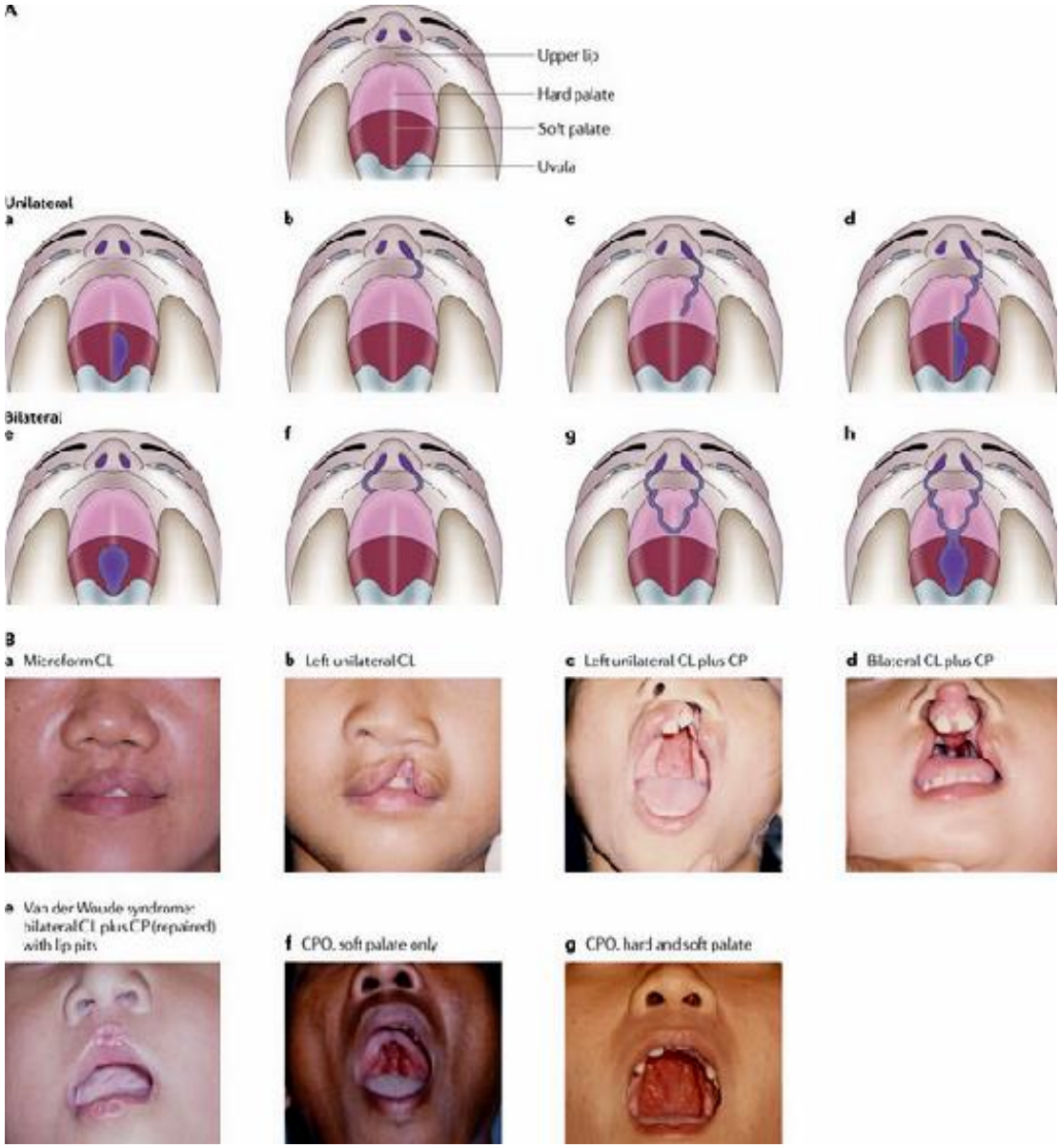


Figure 2.2:-Types of Cleft (Dixon, Marazita et al., 2011).

(A) Illustration of CL/ CP types. (**a** and **e)** unilateral and bilateral clefts of the soft palate; (**b**, **c** and **d)** degrees of unilateral cleft lip and palate; (**f**, **g** and **h)** degrees of bilateral cleft lip and palate. **(B** A collection of images of different types of clefts, some with associated anomalies such as lip pits. Descriptions are given above the images.

CL, cleft lip; CP, cleft palate; CPO, cleft palate only.

2.3 Consanguineous Marriages

Consanguineous marriages is an important factor contributing to increasing of congenital malformations and subsequent morbidity and mortality among the offspring (Bromiker, Glam-Baruch et al., 2004). "Consanguinity is known to be a risk factor for autosomal recessive disorder–related birth defects, increasing the chance of an infant being born with a homozygous genotype for a disease-associated allele" (Sabbagh, Hassan et al., 2013). Marriages between first cousins has 2.9 and 8.0% risk for congenital malformations in the offspring (Bromiker, Glam-Baruch et al., 2004). Estimates of the risk of recurrence for first degree relatives range from 24-fold to 82-fold (Sivertsen, Wilcox et al., 2008).

(Lidral, Moreno et al., 2008) reveled that among first degree relatives there is a 40 fold risk for CL/ CP of an affected individual and there is greater concordance in monozygotic compared to dizygotic twins. However, the concordance rate in monozygotic twins is only 40–60%, suggesting the influence of environmental factors is also important.

Table 2.2:- Frequencies of consanguineous marriages among Arabs (Teebi, 1994)

Population	Frequency (%)	Comments
Kuwaiti	54.3 37.8	Higher rates among Bedouin tribes
Egyptian	23.3 28.96	Sample from Kuwait Higher rates in rural areas
Iraqi Jordanian	57.87 36.2 50.0	Higher rates in rural areas Sample from Kuwait Lower rates among Christians
Lebanese Palestinian	26.0 39.0 38.7	Lower rates among Christians Arabs in Israel Arab village in Israel

The possibility of an association between facial clefting and consanguinity is (10/1000) among individuals who were products of consanguineous marriages (Jaber, Nahmani et al., 2002). NS OC was more frequently associated with than was found in the general population in first cousin consanguinity (Sabbagh, Hassan et al., 2013). The history of oral clefts either in the father's or in the mother's was strongly associated with both types of clefts, but parental consanguinity was associated only with CL/ CP (Leite and Koifman, 2009).

2.4 Epidemiology of Orofacial Cleft

CL/ CP is the most common orofacial congenital malformation found among live births, affecting 1 in every 500 to 1000 births worldwide (Cooper, Ratay et al., 2006), depending on ancestry, geographic residential location, maternal age and prenatal exposures, and SES (Wehby and Cassell, 2010). The prevalence varies according to race/ethnicity, sex, and cleft type (Al Omari and Al-Omari, 2004).

The prevalence rate shows a wide racial variation, low prevalence among blacks and a higher prevalence among Asians, whereas Caucasian lie in the middle (Al Omari and Al-Omari, 2004).

The incidence of clefts in Caucasian populations ranges from 1.0 to 2.21 per 1000 live births (Yazdee, Saedi et al., 2011), while, in American Indians have the highest incidence, 3.6:1000 births, (Muhamad, Azzaldeen et al., 2014) and African-derived populations have the lowest prevalence rates about 1/2500 births. (Dixon, Marazita et al., 2011; Murray, 2002). CL/ CP is more common 1-2/1000 births while, CPO is less common, with a prevalence of approximately 1/1500–2000 births in Caucasians, (Lidral, Moreno et al., 2008). European populations have intermediate prevalence rates are 1:1000 for NS CL/ CP and 1:2400 for NS CPO (Aldhorae, Böhmer et al., 2014).

The prevalence of CL\ CP varies in different racial backgrounds, Asian, American and Indians having the highest rate and Africans the lowest, while the prevalence of CPO does not vary. Also there are a gender ratio differences CPO more frequent in females and CL\ CP more in males (Lidral, Moreno et al., 2008).

"There is considerable international variation in the frequency of OC, but validity and comparability of data are adversely affected by numerous factors, among which are: source population of births considered (hospital versus population), time period, method of ascertainment, inclusion/ exclusion criteria, and sampling fluctuation" (Skuladottir, H et al., 2004).

2.4.1 Worldwide prevalence data on CL/ CP

In Canada for CL\ CP the mean birth prevalence was 0.82 per 1000 live births and for CPO is 0.58 per 1000 live births (Matthews, Oddone-Paolucci et al., 2014).

In China, The Prevalence rate of CL/ CP per 1000 live births was 1.30 and In Japan is 1.34. The prevalence of NS CL/ CP in Shanghai and China, was 1.12 per 1000 live births. The prevalence of NS CL/ CP was 1.54 in 1000 live in Filipino population (Cooper, Ratay et al., 2006).

There was approximately an eightfold variation in the prevalence at birth of CL/ CP with a range from 0.3 (USA) to 2.3 (India) per 1,000 births internationally (Little and Nelson, 2013).

In Norway, the birth prevalence is 2.2 per 1000 live births, which is among the highest rates of clefts in the Western world (Sivertsen, Wilcox et al., 2008).

In Finland, The incidence of CPO is 1.36 cases per 1000 live births, which is greater than the other European countries which have 0.53 cases per 1000 births (Lithovius, Ylikontiola et al., 2013).

In Finland For CPO, 15.2 per 10,000 was the only European center to report a higher prevalence, and both Australia and Finland have substantially higher prevalence than the average for Europe 6.2 per 10,000 births (Bell, Raynes-Greenow et al., 2013).

Prevalence rates for OC in Australia . range between 15 and 21 per 10,000 births which is a good example of differences in the reported prevalence of congenital anomalies. In Australia CL/ CP 12.05 per 10,000; CPO 10.12 per 10,000 compared with most other parts of the world. Odense in Denmark, Northern Netherlands, Saxony-Anhalt in Germany, and Styria in Austria have slightly higher prevalence than Australia (Bell, Raynes-Greenow et al., 2013).

2.4.2 Epidemiology of orofacial clefts in Palestine and Arab world

The reported incidence in the Middle East, was from 0.3 to 2.19 per year and is generally thought to be similar to rates reported in white populations (Borno, Hussein et al., 2012). The few available studies in Arab regions, suggest that the incidence of syndromic forms of clefting range between 0.5 and 2.19 in every 1000 live births (Aldhorae, Böhmer et al., 2014).

The incidence rate of CL/ CP in Sudan was 0.9 per 1000 live births, in Oman is 1.5 per 1000 live births. The prevalence of clefts in Iran is 1.03 per 1000 births (Aljohar, Ravichandran et al., 2008).

In Saudi Arabia, the incidence of facial clefts is 0.3 per 1000 live births. However, another study indicated the highest reported incidence of clefts 2.19 per 1000 live births (Aljohar, Ravichandran et al., 2008).

In Israel and Jordan, the incidence is recorded as 1.39 per 1000 live births per year. In Palestinians living in the occupied Palestinian territory the incidence of oral clefts has not been reported so far (Borno, Hussein et al., 2012). A study for Borno, 2012 in Palestine revealed that, "between Jan 1, 1986, and Dec 12, 1995, 33239 live births were recorded at the hospital. 35 infants had OC, yielding an incidence of 1.05 per 1000 live births per year: six infants (0.18 per 1000 live births) had isolated CL, 14 (0.421 per 1000 live births) CLP, five had other anomalies, and ten had other non-specified OC" (Borno, Hussein et al., 2012).

The reported prevalence rate for OC among Jordanians is similar to the previously reported prevalence rate in white Caucasians. which was 2.4 per 1000 live births (Sabbagh, Mossey et al. 2012).

In Israel, the incidence of facial clefts in 1967 was 0.76/1000 live births. Others have found variable rates, ranging from 0.54/1000 to 1.6/1000. Harlap et al. found differences in the rates between the Jewish and Arab populations in Jerusalem: 3.9/1000 and 1.5/1000 clefts/ live births, respectively (Silberstein, Silberstein et al., 2012). However, Jaber and colleagues found the prevalence of facial clefts in the Arab population of the city Taibe to be 1.56/1000, which is similar to rates in other western communities as well as the Jewish population in Israel (Silberstein, Silberstein et al., 2012).

2.5 Syndromic CL/ CP

Syndromic forms of CL/ CP often have simple Mendelian inheritance patterns and are thus more suitable for conventional genetic mapping strategies (Lidral, Moreno et al., 2008). The syndromic cases can be subdivided into chromosomal syndromes, more than 350 Mendelian disorders, teratogens (e.g. phenytoin or alcohol) and uncategorized syndromes (Murray, 2002).

Many genes implicated in Mendelian syndromic forms also play a role in the etiology of nonsyndromic clefts (Nikopensius, Jagomägi et al., 2010). "The candidate genes have been chosen based on expression patterns during facial development, cleft phenotype in transgenic or knockout mouse models, association with syndromic forms of clefting, previous positive findings in humans, role in nutritional or xenobiotic pathways, and cytogenetic location adjacent to chromosomal anomalies associated with OC phenotypes" (Lidral, Moreno et al., 2008).

Many studies from mouse and human have helped identify several genes known to underlie Mendelian syndromic forms of CL/ CP also have a role in the etiology of isolated clefts (Table 2.3), These include *IRF6*, *MSX1*, *PVRL1*, *TBX22* and *FGFR1* (Jugessur and Murray, 2005). Most likely that those mutations have a mild effect on genes which cause the syndromes could cause phenotypes not distinct from NS CL/ CP, means those genes could be involved in the etiology of NS CL/ CP (Scapoli, Palmieri et al., 2005).

Van der Woude syndrome (VDWS) is most common syndromic form of OC, which is an autosomal dominant disorder characterized by the presence of CL/ CP or CPO and/or lower lip pits (Nikopensius, Jagomägi et al., 2010), accounts for approximately 1% of all oral cleft cases it is consider as one of the largest contributors to the syndromic clefting population (Prescott, Winter et al., 2001). Most reported cases caused by heterozygous mutation in the gene encoding *IRF6* on chromosome 1q32 (MIM, #119300).

Table 2.3: CL/ CP syndromes with the gene and genomic locus (Setó-Salvia and Stanier, 2014, Sharaha, 2013).

Locus	Gene	Phenotype
1p21.1	*COL11A1*	Stickler syndrome type II
1p34	*WDR65*	Van der Woude syndrome
1p36.11	*GRHL3*	Van der Woude syndrome
1q32-q41	*IRF6*	Van der Woude syndrome
2p21	*SIX3*	Holoprosencephaly-2
2p25.3	*COLEC11*	3 MC syndrome
2q14.2	*GLI2*	Holoprosencephaly-9
2q31.1	*GAD1*	Cerebral palsy spastic quadriplegic
2q33.1	*SATB2*	Pierre Robin sequence with or without ankyloglossia
2q33.1	*SATB2*	Intellectual disability
3q27.3	*MASP1*	3 MC syndrome
3q28	*TP63*	Ectrodactyly, ectodermal dysplasia, and CL/ CP syndrome3
3q28	*TP63*	Ankyloblepharon-Ectodermal Defects-Clefting Syndrome
3q28	*TP63*	Limb-Mammary syndrome
3q28	*TP63*	Orofacial Cleft 8 (OFC8)
3q28	*TP63*	Rapp-Hodgkin syndrome
4p16.2	*MSX1*	Tooth agenesis
4p16.2	*MSX1*	CLP without dental problems
4p16.2	*MSX1*	Witkop syndrome
4p16.3		Wolf-Hirschhorn syndrome (WHS)
5q15-q21	*CDH1*	CL/ CP and gastric cancer
5q32	*TCOF1*	Treacher Collins syndrome with eye problems
6p21.32	*COL11A2*	Stickler syndrome type II
6q22.31	*GJA1*	Oculodentodigital dysplasia (ODDD) with CLP
7q21.11	*SEMA3E*	CHARGE syndrome
7q36.3	*SHH*	Holoprosencephaly-3
8p11.23-p11.22	*FGFR1*	Kallmann syndrome
8p11.23-p11.22	*FGFR1*	Non-syndromic CLP
8q12.1-q12.2	*CHD7*	CHARGE syndrome
8q13.3	*EYA1*	Branchiootorenal syndrome type I
8q24.21	*MYC*	Burkitt lymphoma
9q22.32	*PTCH1*	Holoprosencephaly-7
10q26.13	*FGFR2*	Cruzon/Apert/Pfeiffer syndromes
10q26.13	*FGFR2*	Non-syndromic CLP
11q23.3	*PVRL1*	CLP Ectodermal dysplasia 1 (CLPED1)
11q23.3	*PVRL1*	Non-syndromic CLP
11q24.2	*CDON*	Holoprosencephaly-11
12q12-q14	*MLL2*	Kabuki syndrome
12q13.11	*COL2A1*	Stickler syndrome types I with or without eye problems
13q32.3	*ZIC2*	Holoprosencephaly-5
14q13.3	*PAX9*	Tooth agenesis
14q23.1	*SIX1*	Branchiootorenal syndrome type III
15q12	*GABRB3*	Childhood Absence Epilepsy
16q22.2	*DHODH*	Miller syndrome
17q24.3	*SOX9*	Pierre Robin syndrome with or without campomelic dysplasia
18p11.31	*TGIF*	Holoprosencephaly-4
19q13.32	*SIX5*	Branchiootorenal syndrome type II
21q22.3	*RIPK4*	Bartsocas-Papas syndrome
22q11.2		22q11.2 deletion syndrome, DiGeorge syndrome, Velocardiofacial syndrome

22q11.21	*TBX1*	Velocardiofacial syndrome
Xp22.31	*KAL1*	Kallmann syndrome
Xq21.1	*TBX22*	Cleft palate with or without ankyloglossia
Xq21.1	*TBX22*	Abruzzo-Erickson syndrome
Xq21.1	*TBX22*	Hypodontia
Xq28	*FLNA*	Otopalatodigital syndrome
Xq28	*FLNA*	Facial features
7p22	*ACTB*	Autosomal - dominant developmental malformations, deafness and dystonia
Xq12	*EFNB1*	Craniofrontonasal
8p21.1	*ESCO2*	Roberts
7p13	*GLI3*	Oro-facial-digital
11q24.2	*HYLS1*	Hydrolethalus
Xp11.22	*PHF8*	X-linked mental retardation and CL/ CP
6p24	*TFAP2A*	Branchio-oculo-facial
17q21	*WNT3*	Tetra-amelia with CLP
1p32.3	*DHCR24*	Desmosterolosis
11q13.4	*DHCR7*	Smith–Lemli–Opitz
Xq12	*EFNB1*	Craniofrontonasal
Xp11.4	*BCOR*	Oculofaciocardiodental
3p14.3	*FLNB*	Larsen syndrome; atelosteogenesis
16q24.1	*FOXC2*	Hereditary lymphedema- distichiasis
9q22	*FOXE1*	Bamforth–Lazarus
7p13	*GLI3*	Oro-facial-digital'
17q24.3	*KCNJ2*	Andersen
5p13.2	*NIPBL*	Cornelia de Lange
Xp11.23	*PQBP1*	X-linked mental retardation
5q31-q34	*SLC26A2*	Diastrophic dysplasia
9q22	*TGFBR1*	Loeys – Dietz
3p22	*TGFBR2*	Loeys–Dietz
7p21.2	*TWIST1*	Saethre- Chotzen
Xp22	*MID1*	Opitz G/BBB
Xp22	*OFD1*	Oro-facial-digital type I

Table 2.4: Chromosomal abnormalities in CL/ CP syndromes (Chiquet, 2011).

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

2.6 Nonsyndromic Cleft Lip with or without Cleft Palate (NS CL/ CP)

NS CL/ CP is defined as no physical or development anomalies except the CL/ CP and no known teratogenic exposures that cause CL/ CP (Chitturi, Reddy et al., 2014). It is a genetically complex trait involving genetic heterogeneity, low penetrance and the influence of various environmental factors (Lidral, Moreno et al., 2008). There is currently little progress in identifying and understanding of the genetic etiology of NS CL/ CP cases (Allam and Stone, 2014).

Studies of NS CL/ CP suggest that the genetic heterogeneity have the highest incidence rates being observed in Asians, followed by Caucasians and Africans (Aldhorae, Böhmer et al., 2014).

"The majority of affected patients have no positive family history and the evaluation of inheritance patterns in the familial cases has not revealed a simple Mendelian mode of inheritance" (Lidral, Moreno et al., 2008). The nonsyndromic forms of orofacial clefts are likely due to gene – environment interactions, whereas the syndromic forms are mainly due to genetic alterations (Ghassibé, Bayet et al., 2005).

According to the studies, there are 3–14 genes interacting multiplicatively may be involved in CL\ CP phenotype, which indicate that is heterogeneous disorder. Currently there is not any method to identify different genetic subsets, also only a portion of affected individuals will have a mutation in the same gene, those making it more difficult to map these genes (Lidral, Moreno et al., 2008).

"Although extensively studied, due to factors such as the genetic heterogeneity, departure from Mendelian inheritance patterns, the limited availability and high cost of genomic tools, and the necessity for very large data sets, the exact genetic association, especially in non-syndromic OC cases, remains poorly characterized" (Allam and Stone, 2014), because there are many environmental and genetic factors involved and also, exists of a complex and diverse mechanism in embryogenesis at a molecular level (Chitturi, Reddy et al., 2014). Another limitation is that families with CL/ CP are rare, thus when using a linkage approach,it is necessary to combine the LOD scores across families, reducing power and increasing the likelihood for missing a gene (Lidral, Moreno et al., 2008).

2.6.1. Etiology of NS CL/ CP

2.6.1.1. Genetic causes of NS CL/ CP

"Evidence for a genetic etiology for NS CL/ CP comes from studies that show (1) a heritability for NS CL/ CP of 76%, (2) monozygotic twins are ten-fold more likely to be concordant for a cleft compared to dizygotic twins (40% vs. 4.2%), (3) siblings of affected individuals have an increased risk of having a cleft compared to the general population, and (4) clefting aggregates in families" (Chiquet, 2011).

Many of genetic approaches have been used to identify a candidate genes and loci that responsible for clefting such as linkage and association studies Genome-wide linkage scans have also provided some important clues. To date, for NS CL/ CP there are 13 genome-wide scans have been performed, also scans for CL\ CP individual revealed significant heterogeneity LOD score by meta-analysis studies on chromosomes 1p, 6p, 6q, 14q and 15q, and a particularly strong signal on 9q (Jugessur and Murray, 2005).

IRF6 gene on chromosome 1q32.2 consider as the first risk factor identified as functionally relevant in the development of NS CL/ CP. It is one of the most prominent candidate genes for NS CL/ CP (Table 2.5) and its functional mechanism is conclusively identified underlying orofacial defects (Aldhorae, Böhmer et al., 2014).

The genetic polymorphisms at the *IRF6* locus have stronger association in Asian and South American populations (Scapoli, Palmieri et al., 2005). Approximately 15% of isolated clefts is contributions from the single genes *IRF6*, *MSX1* and *FGFR1* are seem to explain (Jugessur and Murray, 2005). In addition, other gene associations have been identified but have been inconsistent and account for only a small percentage of the underlying genetic heritability, including *RARA, TGFα, TGFβ, p63, MYH9, BCL3,* and *MSX1*, but (Chiquet, 2011).

In addition, point mutations in *FOXE1, GLI2, MSX2, SKI, SATB2, TBX10*, and *SPRY2* may be rare causes of isolated CL/ CP which suggested by sequence analysis alone. The linkage disequilibrium data supported a larger, as yet unspecified, role for variants in or near *MSX2, JAG2*, and *SKI* (MIM, **%**119530)

Table 2.5: Summary of replicated NS CL/ CP GWAS loci including the implicated candidate gene with previously associated phenotypes (Setó-Salvia and Stanier, 2014).

Locus	Gene	Population	First GWAS study CL/ CP	Other anomalies associated
1p22.1	*ABCA4*	European and Asian	[Beaty et al., 2010]	Retinal dystrophies
1p22.1-p21.3	*ARHGAP29*	European and Asian	[Beaty et al., 2010; Ludwig et al., 2012]	e
1p36.13	*PAX7*	European and Asian	[Ludwig et al., 2012]	Neural crest cell development, muscle satellite cell marker
1q32.3-q41	*IRF6*	Norway, Denmark, EUROCRA, Philippines	[Rahimov et al., 2008]	Popliteal pterygium syndrome
2p21	*THADA*	European and Asian	[Ludwig et al., 2012]	Truncation mutations in thyroid tumours
3p11.1	*EPHA3*	Chinese	[Pan et al., 2013]	Role in lymphoid malignancies
3q12.1	*COL8A1/ FILIP1L*			Skin keratinocytes, eye and calvaria
8q21.3	*DCAF4L2*	European and Asian	[Ludwig et al., 2012]	e
8q24	*MYC*	European	[Birnbaum et al., 2009; Grant et al., 2009]	Prostate, colorectal, bladder and breast cancer,
9q22.2	*GADD45G*	European and Asian	[Beaty et al., 2013]	Pituitary adenomas growth suppressor controlling pituitary cell proliferation
9q22.33	*FOXE1*	European and Asian	[Beaty et al., 2013]	Thyroid gland organogenesis, hair follicle; Bamforth Lazarus syndrome (CP thyroid dysgenesis)
10q25	*VAX1*	European	[Mangold et al., 2010]	Brain anomalies and eye coloboma, syndromic micropthalmia
13q31.1	*SPRY2*	European and Asian	[Beaty et al., 2013]	FGF signalling, mouse null-abnormal gastrointestinal tract, ear problems
15q22.2	*TMP1*	European and Asian	[Beaty et al., 2013]	e
17p13.1	*NTN1*	European and Asian	[Ludwig et al., 2012]	Axon guidance; related with tumor cell survival in metastatic breast cancer
17q22	*NOG*	European	[Mangold et al., 2010]	BMP signalling, synostosis and symphalangism, tarsal, carpel, brachydactyly, etc
17q25.3	*RBFOX3*	European and Asian	[Beaty et al., 2013]	Rolandic epilepsy
20q12	*MAFB*	European and Asian	[Beaty et al., 2010]	Renal disease, malignant myeloid disorders, carpotarsal osteolysis syndrome.

Table 2.6: overview of other Currently Known NS CL/ CP Risk Loci (Sharaha, 2013, Aldhorae, Böhmer et al., 2014, MIM)

Locus	Gene	Gene description
14q22.2	*BMP4*	Bone morphgenetic protein 4
8p12	*FGFR1*	Fibroblast growth factor receptor 1
4p16.3-p16.1	*MSX1*	msh homeobox 1
22q12.3	*MYH9*	myosin, heavy chain 9, non muscle
10q24.32	*FGF8*	Fibroblast growth factor 8
22q11.23	*GSTT1*	glutathione S-transferase theta 1
1p36.22	*MTHFR*	Methylenetetrahydrofolate reductase (NAD(P)H)
4q32	*PDGFC*	Platelet derived growth factor c
11q23	*PVRLI*	Poliovirus receptor-related 1
2p13	*TGFA*	Transforming growth factor,alpha
2q33	*SUMOI*	SMT3 suppressor of mif two3 homolog 1
14q24	*TGFB3*	Transforming growth factor, beta 3
2p21	*ZFP36L2*	Zinc Finger Protein 36- Like 2
9q22	*C9orf156*	Chromosome 9 Open Reading Frame 156
9q22	*HEMGN*	Erythroid Differentiation- Associated Gene
10q25	*KIAA1598*	Shootin1
17p13	*PIK3R5*	Phosphatidylinositol 3- Kinase, Regulatory Subunit 5

2.6.1.2. Environmental causes of NS CL/ CP

Environmental factors that could increase the risk of CL/ CP are divided into four broad categories: womb environment, external environment, nutrition, and drugs (Bender, 2000). Many epidemiological studies make an evidence for the environmental causes of NS CL/ CP which show an increased birth prevalence of clefting after in utero exposure to putative teratogens, such as corticosteroids, high dietary intake of preformed vitamin A, insufficient amounts of folic acid and multivitamins, maternal hyperhomocystinemia, high quantities of alcohol consumption, and exposure to agricultural and industrial chemicals (Wyszynski and Wu, 2002, Chiquet, 2011).

However, some of maternal disease as chronic or infectious, during or before pregnancy are considered risk factors for CL/ CP. Influenza, common cold, orofacial herpes, gastroenteritis, sinusitis, bronchitis, epilepsy and angina pectoris, diabetes and obesity all of these are considered as risk factor. Additional reported factors are exposure to nicotine poisoning, and prescription drug use during pregnancy, such as amoxicillin, phenytoin, oxprenolol, thiethylperazine, oxytetracycline, and carbamazepine (Acuña - González, Medina-Solís et al., 2011).

The most studied environmental risk factor for oral clefting is maternal cigarette smoking is (Wyszynski and Wu, 2002). The relationship between maternal smoking and CL/ CP is not strong, but it is significant. Several studies have consistently yielded a relative risk of about 1.3-1.5. The effect is more significant, when maternal smoking was considered together with a positive genetic background (Muhamad, Azzaldeen et al., 2014). Tobacco smoking increase risk of CL/ CP during pregnancy by 34% and CPO by 22% (Bell, Raynes-Greenow et al., 2013).

In other hand, heavy maternal drinking, increases the risk of CL/ CP also causing fetal alcohol syndrome. Maternal alcohol drinking increases the risk for CL/ CP by 1.5-4.7 times in a dose dependent manner (Muhamad, Azzaldeen et al., 2014). Another study shows 3.4 times increased risk of delivering an infant with CL/ CP for the mothers who consumed more than five drinks per occasion (Muhamad, Azzaldeen et al., 2014).

Maternal nutrition during pregnancy also appears to play an important role. For example, low dietary intake of B-complex vitamins, and exposure to deficient or excessive amounts of vitamin A; have been linked to increased risks of cleft (Jugessur and Murray, 2005).

Based on both observational studies and interventional trials, folate deficiency, have been suggested to influence risk of CL/ CP, by using folate supplementation to prevent recurrences of CL/ CP in families (Stanier and Moore, 2004). If the folic acid were not taken during early pregnancy the risk for CL/ CP could be tripled. "The role of folic acid supplementation in the prevention of CL/ CP has been investigated in several studies, low dose folic acid supplementation cannot protect against CLP, Only a very high dose of supplementary folic acid (10 mg/day) could reduce the risk of CLP significantly (65% reduction was observed)" (Muhamad, Azzaldeen et al., 2014).

Other nutrient and micronutrient studies will need to be expanded to look for evidence of effects. For example, zinc deficiency there are some data to support its roles in the risk of oral clefts in populations in which zinc status is highly compromised, also for the role in cholesterol deficiency, as well for as multivitamins in general in cleft prevention (Dixon, Marazita et al., 2011).

2.6.1.3. Gene-environment interaction etiology of NS CL/ CP

As variations in numerous genes, together with environmental factors, the genetic basis of nonsyndromic clefting is complex, which play a role in its etiology (Thomason, Zhou et al., 2010). Clearly, environmental agents interact with maternal gene products, but it is not always clear if the same is true for fetal gene products, although it is likely in some situations. May the fetus have a low risk for CL/ CP due to its genes, but the risk increases due to maternal environmental exposures and her genetic susceptibility to these exposures (Lidral, Moreno et al., 2008).

A variety of genetic polymorphisms have been studied in population based association studies and candidate genes studies. "Results have suggested a role for genes responsible for growth factors (e.g. *TGFα, TGFβ3*), transcription factors (e.g. *MSX1, IRF6, TBX22*), factors which influence xenobiotic metabolism (e.g. *CYP1A1*, *GSTM1, NAT2*), nutrient metabolism (e.g. *MTHFR, RARA*), and immune response (e.g. *PVRL1, IRF6*)" (Allam and Stone, 2014).

TGFA and smoking have been most widely studied, with an interaction suggested but not confirmed. Some data also support interactions between alcohol, nutritional factors and the *MSX1* and *TGFB3* genes in addition to *TGFA* (Table 2.7) (Murray, 2002).

In addition, in the presence of maternal smoking *GSTT1* (glutathione S-transferase theta) or *NOS3* (nitric oxide synthase 3) genes appear to influence risk of CL/ CP. The *GSTT1* markers are gene deletion variants, which suggests deficiencies in detoxification pathways may underlie some of this susceptibility (Dixon, Marazita et al., 2011).

Table 2.7: Currently reported gene-environment interaction in cleft lip and palate (Allam and Stone, 2014).

Gene-environment interaction in cleft lip and palate
TGFA / Smoking
TGFA / Alcohol
TGFA / Vitamins
MSX1 / Smoking
MSX1 / Alcohol
TGFB3 / Smoking
TGFB3 / Alcohol
RARA / Smoking
MTHFR / Vitamins
P450 / Smoking
GST / Smoking
EPHX1 / Smoking

2.7 Interferon Regulatory Factor 6 (*IRF6*)

Interferon (IFN) regulatory factors are a family of ten transcription factors that act broadly in host defense, specifically in the innate immune response, immune cellular development, and tumor suppression (Savitsky et al., 2010).

2.7.1 Structure

All *IRF* proteins contain a highly conserved helix-turn-helix DNA - binding domain (DBD) also has a helix-turn-helix motif forms by a penta-tryptophan repeat (Figure 2.4) and recognizes a DNA consensus sequence: 5'-AANNGAAA- 3'(Biggs, 2012), and less conserved protein-binding domain in the carboxy- terminal end which is varies among family members, and giving them their distinct functions in human disease such as cancer (Figure 2.5). At the carboxyl terminal, *IRF* members possess the *IRF* associated domains (IAD) to mediate hetero- or homodimerization between the family members (JIN, 2007).

IRF6 is a 467 amino acid transcription factor. *IRF6* contains a winged-helix DBD from 13-113 amino acids, and an interferon associated domain (IAD) protein-binding domain from 226-394 amino acids (Su, 2011). The *IRF6* gene located in 1q32.3-q41,

it contains 10 exons. Exons 1, 2, and 10 are noncoding (Figure 2.3). There is strong structural conservation among human, mouse, zebrafish and Fugu *IRF6* orthologs, especially in the 7 coding exons (MIM: 607199).

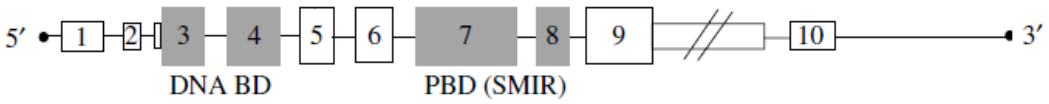


Figure 2.3: Structure of the *IRF6* gene. Exons (rectangles) are drawn to scale, except for exon 9, which is longer than shown. Untranslated regions are smaller rectangles. Exons 3 and 4 encode the DNA-binding domain and exons 7 and 8 code for the protein-binding domain (both in gray). Line connecting exons represents introns and 50-, 30-untranslated regions (Ghassibé, Bayet et al., 2005).

```
MALHPRRVRLKPWLVAQVDSGLYPGLIWLHRDSKRFQIPWKHATRHSPQQEEENTIFKAW 60
AVETGKYQEGVDDPDPAKWKAQLRCALNKSREFNLMYDGTKEVPMNPVKIYQVCDIPQPQ 120
GSIINPGSTGSAPWDEKDNDVDEEDEEDELDQSQHHVPIQDTFPFLNINGSPMAPASVGN 180
CSVGNCSPEAVWPKTEPLEMEVPQAPIQPFYSSPELWISSLPMTDLDIKFQYRGKEYGQT 240
MTVSNPQGCRLFYGDLGPMPDQEELFGPVSLEQVKFPGPEHITNEKQKLFTSKLLDVMDR 300
GLILEVSGHAIYAIRLCQCKVYWSGPCAPSLVAPNLIERQKKVKLFCLETFLSDLIAHQK 360
GQIEKQPPFEIYLCFGEEWPDGKPLERKLILVQVIPVVARMIYEMFSGDFTRSFDSGSVR 420
LQISTPDIKDNIVAQLKQLYRILQTQESWQPMQPTPSMQLPPALPPQ               467
```

Figure 2.4: Amino acid sequence of *IRF6* and its functional domains. *IRF6* contains two functional domains. The amino acid 13-113 constitute the conserved DNA-binding domain (green) and amino acid 226-394 represent the less conserved interferon association domain (pink). The signature penta-tryptophan residues are highlighted in red and the serine rich region is underlined (Su, 2011).

2.7.2 Members of the *IRF* family

The *IRF* transcription factors are ubiquitously expressed, except for *IRF4* and *IRF8* which expressed in the hematopoietic cells. In addition, regarding to the viral

infection or exposure to IFNs, their expression is either constitutive or induced. The *IRFs* have diverse transcriptional activity, can function as transcriptional activators (*IRF1, IRF3, IRF5* and *IRF9*), repressors (*IRF8),* or both (*IRF2*, *IRF4* and *IRF7*) (Su, 2011).

IRF 2 and 4 have oncogenic characteristics ,while *IRF* 1, 3, 5, 6, 8 and 9 act as tumor suppressors. Furthermore, *IRF* 3, 7, and 9 are active in the regulation of type I interferon while *IRF* 1, 2, 4, 6, and 8 regulate the growth and differentiation of a variety of cell types (Biggs, 2012).

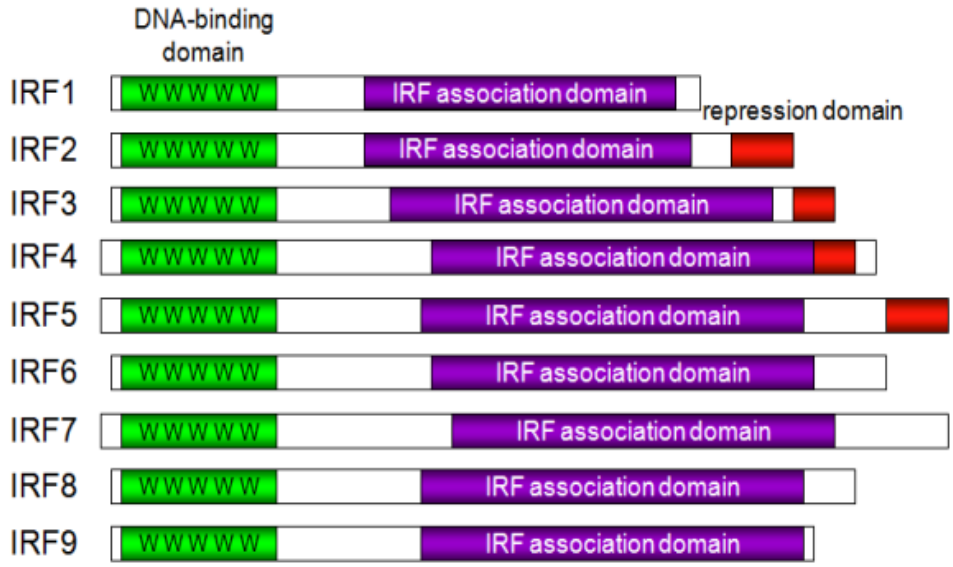


Figure 2.5: Members of the *IRF* family. The Schematic diagram shows the *IRF* family and their functional domains. All *IRF* members have a DNA-binding domain (green) located at the N-terminal. *IRF3, IRF4, IRF5, IRF8* and *IRF9* have an interferon association domain (purple) and *IRF2* has a repression domain (Su, 2011).

2.7.3 Function

In the MEE of the secondary palatal shelves, *IRF6* is expressed to regulate the expression of other genes during palatogenesis (Lidral, Moreno et al., 2008).

When the two palatal shelves begin to associate one with another, the expression of *IRF6* in the MEE increases immediately before fusion, then rapid increase in *IRF6* expression. The elimination of the MEE linked to three different processes: programmed cell death (apoptosis), cellular migration, and EMT. Most likely that the

IRF6 is involved in the elimination of the MEE, because mutations in it lead to a CL/ CP phenotype associated with VWS and PPS, (Bailey, 2006).

Along the medial edge of the fusing palate, tooth buds, hair follicles, genitalia, and skin, studies have been showed a high levels of *IRF6* mRNA. Haploinsufficiency of *IRF6* disrupts orofacial development and were consistent with dominant-negative mutations disturbing development of the skin and genitalia (MIM: 607199).

Recent studies has shown that the hyper-proliferative epidermis in *IRF6* mutant mice fails to undergo terminal differentiation, which cause multiple epithelial adhesions that result finally in cleft palate (Dixon, Marazita et al., 2011). Many research indicated that *IRF6* plays a key role in the formation of oral periderm, spatio-temporal regulation of which is essential in ensuring appropriate palatal adhesion
"It has been speculated that mutations in *IRF6* might repress the *TGF- β* signaling pathway in a manner analogous to *IRF1*-mediated repression, leading to increased epithelial apoptosis before the bilateral processes have managed to fuse" (Jugessur and Murray, 2005). During palatal fusion, there are no apoptosis of MEE cells in the mutant mouse which indicates that *TGF-β*-mediated *IRF6* expression is critical for the apoptosis of MEE (Meng, Bian et al., 2009).
In addition, *IRF6* play a role in probable DNA-binding transcriptional activator also in regulating mammary epithelial cell proliferation (By similarity). It is key determinant of the keratinocyte proliferation and differentiation switch involved in appropriate epidermal development (By similarity), also plays a role in (GeneCards, GC01M209959).

2.7.4 Diseases for *IRF6*

Mutations in the *IRF6* gene associated with non-syndromic orofacial cleft type 6 also can cause van der Woude syndrome and popliteal pterygium syndrome (NCBI: 3664)
Van der Woude syndrome 1 (VWS1) is an autosomal dominant developmental disorder characterized by lower lip pits, CL/ CP, most cases of VWS are due to mutation in the *IRF6* linked to chromosome 1q32-q41 (MIM: 119300). Popliteal pterygium syndrome (PPS) is an autosomal dominant disorder characterized by oro-facial, skin and genital anomalies. Clinical features include CL/ CP, lower lip cysts, syngnathia, congenital ankyloblepharon filiforme in some cases, bifid scrotum,

hypoplastic scrotum, hypoplastic uterus, talipes equinovarus (MIM: 119500). This syndrome is caused by heterozygous mutation in the gene.

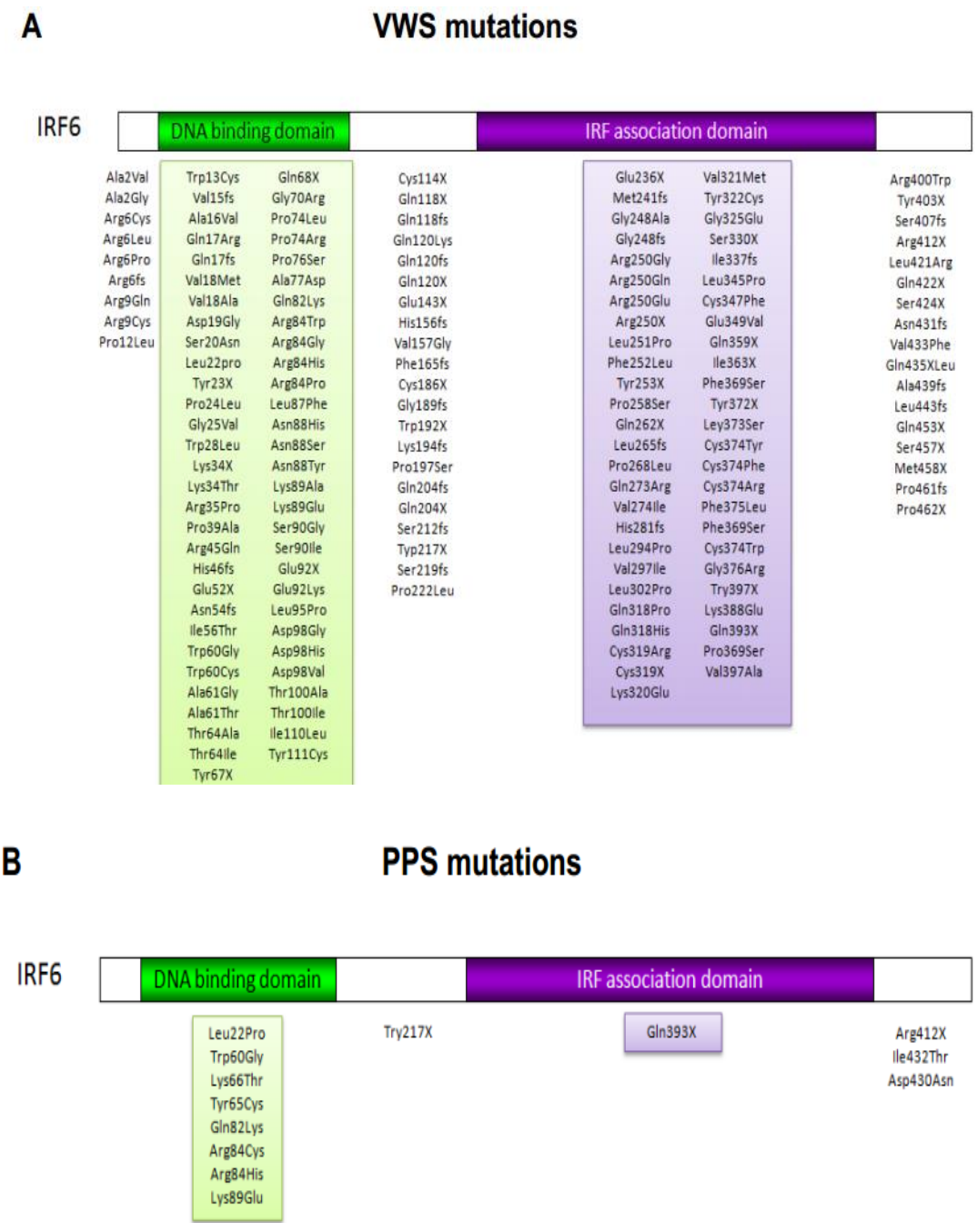


Figure 2.6: Lists of mutations in the *IRF6* protein that cause VWS and PPS. (A) List of amino acid missense mutation, truncation (X) and frameshift (fs) within the *IRF6* DNA-binding domain (green) and interferon association domain (purple) and other regions, that have been identified in individuals with VWS and (B) PPS patients (Su, 2011).

2.8 New Genes that might be Associated with Nonsyndromic Cleft Lip and Palate

2.8.1 *CCDC141*

Coiled-coil domain containing 141 (*CCDC141*) also known as (CAMDI) is a protein, type of tertiary structure composed of multiple coiled-coil domains and or more alpha helices which entwine to form a cable like structure. The helical cables serve a mechanical role in forming stiff bundles of fibers in proteins. It has also Immunoglobulin-like domains, one of the most common protein modules found in animals, also found in several diverse protein families, it are related in both sequence and structure. Ig- like domain consists of a beta-sandwich of seven or more strands in two sheets with a Greek-key topology, has a role in a variety of functions, including cell-cell recognition, cell-surface receptors, muscle structure and the immune system, also often involved in interactions, commonly with other Ig-like domains via their beta-sheets. *CCDC141* maps to chromosome 2q31.2, has 24 exons.
(GeneCards, GC02M179694; Uniprot, Q6ZP82; NCBI, 545428; MIM: 616031).

CCDC141 has Spectrin repeats forms a three-helix bundle, the second helix is interrupted by proline in some sequences, it involved in cytoskeletal structure which found in several protein, include spectrin alpha and beta subunits, alpha-actinin and dystrophin. At position 17 in helix A, the repeats are defined by a characteristic tryptophan (W) residue and a leucine (L) at 2 residues from the carboxyl end of helix C (EMBL, IPR002017).

During radial neuronal migration in developing nervous system *CCDC141* is predicted to play a role in centrosome positioning and movement based on experiments in mice. Based on Fukuda et al. (2010) experiments the deduced 1,451-amino acid protein has N-terminal spectrin-like repeats, a central coiled-coil domain, and I-set domains near the C terminus. In the embryonic day-16 of mouse brain western blot analysis detected a strong Camdi expression, but in the adult brain tissues a little to no expression. In situ hybridization detected Camdi expression in discrete cell layers of adult mouse cerebrum, hippocampus, and cerebellum, and in embryonic mouse eye. Database analysis detected orthologs of Camdi in humans and several vertebrates, but not in lower organisms (MIM: 616031).

Knockdown of *CAMDI* revealed severely impaired radial migration with disoriented centrosomes knowing that the centrosomes play a central role in the directed migration of developming neurons. A yeast two-hybrid screen identified myosin II as a binding protein of *CAMDI*. *CAMDI* interacts with phosphomyosin II and induces an accumulation of phosphomyosin II at the centrosome in a DISC1-dependent manner. Interestingly, one single nucleotide polymorphism of the *CAMDI* gene (R828W) is identified which its gene product reduce the binding ability to phosphomyosin II, also overexpression of R828W in neurons exhibit an impaired radial migration. "*CAMDI* is required for radial migration probably through *DISC1* and myosin II-mediated centrosome positioning during neuronal development In addition, knockdown of *CAMDI* or *DISC1* showed a similar phenotype, an impaired migration of cortical neurons with disorientated centrosomes".(Fukuda, Sugita et al., 2010).

Drerup et al. (2009) revealed that Disc1 knockdown has a role in the abnormal craniofacial development, which analyzed by investigating the CNC contribution to the developing pharyngeal arches. "Zebrafish CNC migrate a substantial distance from the dorsolateral neural rod into the pharyngeal arches before forming the ventral craniofacial cartilage Thus, Disc1 loss reduced the ectomesenchymal CNC cell in the pharyngeal arches and altered its arrangement, which probably led to the abnormal craniofacial development" (Drerup et al., 2009).

2.8.2 *BOD1*

BOD1 (biorientation of chromosomes in cell division 1) also known as *FAM44B* is a protein-coding gene (185 aa), highly conserved throughout metazoans. An important paralog of this gene is *BOD1*L1. Required for proper chromosome biorientation through the detection or correction of syntelic attachments in mitotic spindles. *BOD1* maps to chromosome 5q35.2, has 4 exons. It localizes at the centrosomes throughout the cell cycle, only dissociating during cytokinesis, and localizes at the kinetochore from prometaphase until anaphase. BOD1 has BOD1- Like domain, Bod1-like1 (BOD1L1) and Bod1-like2 (BOD1L2), the function of both is not clear.
(NCBI, 91272; UCSC, uc003mcq.2; GeneCards, GC05M172968; Uniprot, Q96IK1).

In human cells, depletion of *Bod1* causes severe biorientation defects, although kinetochores appear to generate force and oscillate, also causes a range of defects all of which are consistent with increased levels of PP2A activity (Porter et al.,2007, Porter, Schleicher et al., 2013). *Bod1* appears to be required for sister chromatid cohesion, and required for the efficient detection or removal of syntelic attachments but it is not required for the spindle assembly checkpoint. *BOD1* plays a critical role in defining and monitoring the proper attachment of microtubules to the kinetochore.

Furthermore, *Bod1* short interfering RNA (siRNA) depletion causes a loss of phosphorylation of MCAK, which is a microtubule depolymerase that is required for correction of improper kinetochore-microtubule attachment and to modulate it also.

Porter, Schleicher et al. (2013) revealed that *"Bod1*, a protein required for proper chromosome alignment at mitosis, shares with Ensa the sequence similarity also with Arpp-19 and specifically inhibits the kinetochore - associated *PP2A - B56* holoenzyme". By dephosphorylating several kinetochore proteins, *PP2A -B56* regulates the stability of kinetochore- microtubule attachments. Loss of *Bod1* causing defects in kinetochore function which change the balance of phosphorylation at the kinetochores. Therefore, *Bod1* is required to fine tune PP2A phosphatase activity at the kinetochore which play a role in chromosome congression and maintenance of chromatid cohesion.

CHAPTER THREE

Materials and Methods

3.1. Materials

3.1.1. Buffers, Gels and Solutions

- **Ethidium Bromide**

  Ethidium bromide was dissolved in the sterile double distilled water to a final concentration of 1 mg/ml.

- **Agarose gel**

  1.5% agarose

  1X TBE buffer

  Final concentration of 0.01% ethidium bromide

- **Red blood cell lysis buffer (One Liter)**

  155 mM NH_4Cl

  NH_4HCO_3

  0.1 mM EDTA with (PH=7.4)

- **1X lysis buffer (0.5 Liter)**

  50 M Tris HCL with (PH=7.5)

  100 mM NaCl

  0.5mM EDTA with (PH=8)

- **5X loading buffer**

  0.25% bromophenol blue

  0.25% Xylene cyanol FF

  30% Glyserol in water

- **50X TAE Buffer**

  2M Tris ph 8.0

  1M Acetic acid

  0.05M EDTA

  Adjust to PH=8.0

- **Proteinase K**

  Proteinase K was dissolved in sterile double distilled water to a 5mg/ml final concentration.

3.1.2. Reagents, Instruments and Kits

Reagents

Reagent	Supplier	Product specifications
Agarose	ORNAT	SeaKem® LE Agarose
Oligonucleotide primers	Hylabs	
Super therm polymerase	Eisenberg Bros	CAT# JMR-80
10x polymerase Buffer	Eisenberg Bros	CAT# JMR-420
dNTPs 100 mM	TAMAR	CAT# R0181,4X0.25mM
Exonuclease I	BioLabs	CAT# M0293L,15000 units
Antaractic Phosphatase	BioLabs	CAT# M0289L, 5000units
Q solution	Qiagen	
Proteinase K	aMReSCO®	LOT# 1311C384
Hi Di Formamide	Applied Biosystems	CAT# 4311320,
20% SDS	aMReSCO®	CAT # 083754-500ml
100bp plus DNA ladder	TAMAR	Thermo Scientific, GeneRuler™

Kits

Kit	Supplier	Product specification
BigDyeTM Terminators V1.1 Cycle Sequencing Reaction Kit	Applied Biosystems	CAT# 4337451-100

Instruments

Instruments	Supplier	Instrument Specification
NanoDrop®		
Agarose gel electrophoresis apparatus	BioRad	SUB-CELL® GT
Agarose gel electrophoresis power supplier	BioRad	Power PAC 300
Gel DOC	BioRad	Molecular Imager, Gel DOC™ XR+ Imaging System
PCR machine	Applied Biosystems	GeneAmp® PCR System 9700
Sanger Sequencing Machine	Applied Biosystems	ABI 3130XL Genetic Analyzer

3.2. Methods

3.2.1. Samples Collection

The families samples, were collected by Dr Hashem Shahin and his team at the Human Genetics Research lab (HGRL). Also sampling of those family members, pedigree construction and family history of the disease were done through family interviews either at their homes or in the HGRL.

Consent forms were signed by each family member who accepts to participate in the study. For those individuals who are under 18 years old, the consent form was signed by one of their parents.

3.2.2 Families and their Phenotypes

The study shows that the affected individuals of the CP-E and CL-BM families have NS CL/ CP phenotype and CP-AL family has mental retardation and\or CL\ CP, those families are from different regions in the West Bank. All the information regarding the family pedigrees, the medical reports and the personal details are kept in locked cabinets at the HGRL and only those who work under the supervision of Dr. Shahin have access to such data.

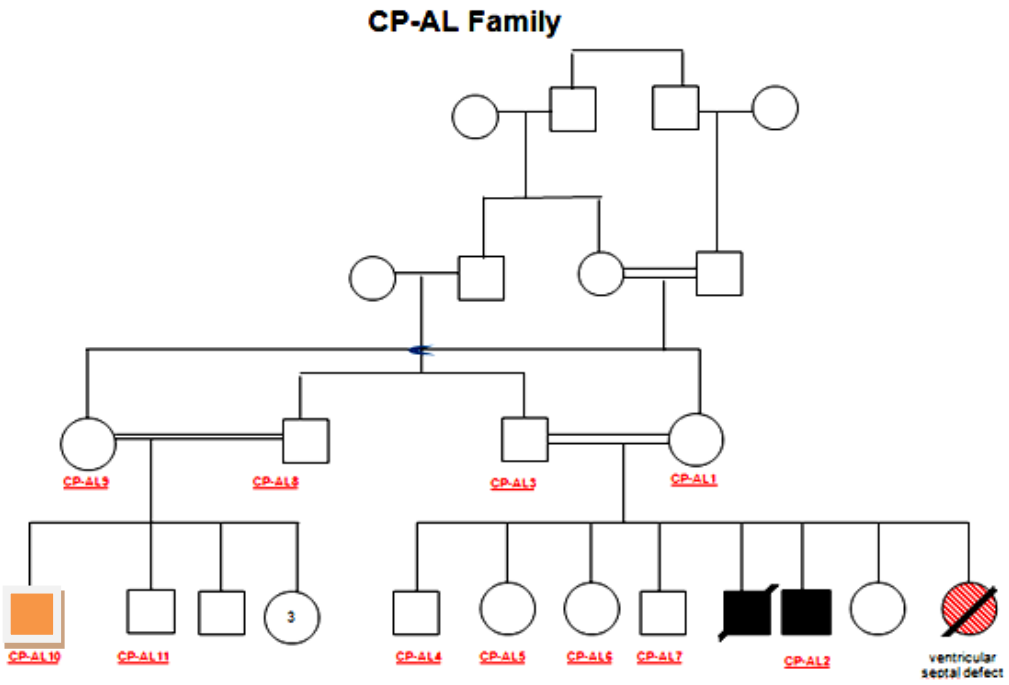


Figure 3.1: CP-AL Family

CP-AL2: 17 years old, has Left complete CLP and simple mental retardation.

CP-AL10: 16 years old, has the same phenotype of CP-AL2 with MR and without CL\ P.

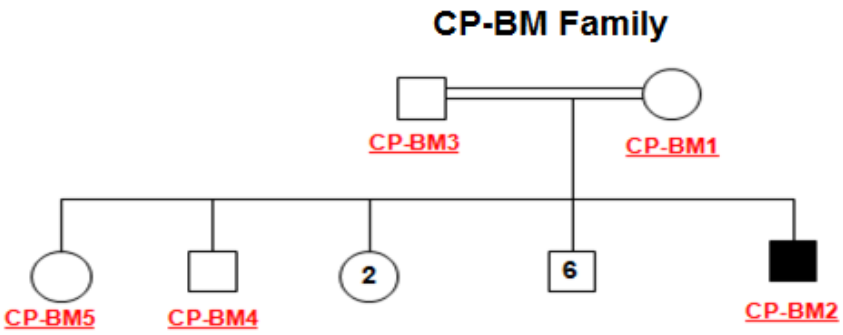


Figure 3.2: CP-BM Family

CP-BM2: 19 years old, has bilateral complete CLP.

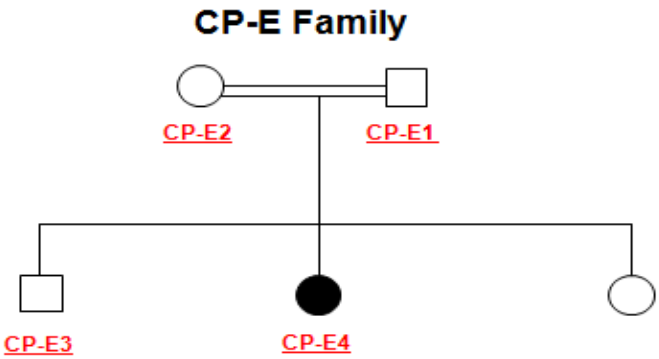


Figure 3.3: CP-E Family

CP-E4: 9 years old, has unilateral cleft lips.

3.2.3. Blood collection

Blood samples were collected from affected and non- affected individuals from each family member for DNA extraction. Five to seven ml blood was mixed with EDTA via EDTA tube to prevent clotting.

3.2.4. Isolation of the DNA by Salting-Out technique

Five to seven ml of blood was collected in a sterile EDTA tube for DNA extraction. Almost 20 ml of the red blood cell lysis buffer (section 3.1.1) was added to each tube, and then it was mixed briefly and kept on ice for 10-20 minutes (min). From time to another the blood was shaken by hand, until it becomes transparent, after that centrifugation was done for 10 min at 2000 round per minute (rpm) at 4C. The supernatant was carefully removed and the pellet was resuspended in 3 ml red blood

cell lysis buffer, then the centrifugation was repeated for the pellet at the same manner. The supernatant was carefully removed and the pellet was suspended in a mix of 3 ml of 1x lysis buffer, 100µl of 20% SDS and proteinase K (section 3.1.1), overnight at 37C or 55C for 3 hours.

One ml of 6M NaCl was added and vigorously mixed until the solution appeared foamy, and then centrifugation was done at 3000 rpm for 20 min. at room temperature. The supernatant (upper phase) was gently transferring into a 15 ml tube, avoiding the salt protein deposit and the precipitated protein pellet was left behind at the bottom of the tube.

Two volumes of absolute ethanol (100% EtOH) were added to the upper phase followed by its gentle inversion several times until the DNA precipitates. DNA was removed with a glass Pasteur pipette, followed by washing in 70% EtOH (in eppendorf tube) and then it was left to air dry on Pasteur pipette for a few minutes. DNA was dissolved in DDW (200- 600µl depending on the amount of DNA) and left at room temperature overnight. Quantity and quality of DNA was checked by using a Nanodrop.

3.2.5. Next generation sequencing

Next generation sequencing (NGS) is often referred to as massively parallel sequencing, which means that millions of small fragments of DNA can be sequenced at the same time.

Exome sequencing was used in this research to detect the mutations that is responsible for the CL/ CP phenotypes in each of the affected individuals of CP-E, CP-AL and CP-BM families.

Exome sequencing was done by Dr. Hashem Shahin, on the 3 affected individuals (AL2, BM2 and E4) of the CP-AL, CP-BM, and CP- E families in Prof. Mary-Claire King's Laboratory at the University of Washington in – Seattle, USA.

3.2.6. Mutation analysis

3.2.6.1 Detection of the *BOD1*_R112X, *IRF6*_R250X and *CCDC141*_I295L by direct Sequencing

3.2.6.1.1. Polymerase Chain Reaction (PCR)

The standard PCR reaction mix is a solution containing Taq DNA Polymerase, PCR Buffer, dNTPs and Q- solution. PCR Buffer has been developed to save time and effort by reducing the need for PCR optimization. Furthermore, Q-Solution facilitates amplification of difficult templates by modifying the melting behavior of DNA and it will often improve suboptimal PCR. Taq DNA Polymerase is a high-quality recombinant enzyme that is suitable for general and specialized PCR applications.
Oligonucleotide primers are generally 20–40 nucleotides in length and ideally have a GC content of 40–60%. Primer3 and UCSC programs were used to design the primers.

CP-E Family primers

Primer	Primer sequence
Forward Primer	5'-TTCTGGAAGCCCTGTCAATG- 3'
Reverse Primer	5'- AAGTTTTGAAAGGTTGAGGGACT- 3'

CP-AL Family primers

Primer	Primer sequence
Forward Primer	5'- GCAGACTGCTGCAACTCCTA - 3'
Reverse Primer	5'- TCAGGTGGGCACTTGTGTTA - 3'

CP-BM Family primers

Primer	Primer sequence
Forward Primer	5'- ATCAGGTTGGGAGCAACAAG - 3'
Reverse Primer	5'- CTTGCAGTGACTGACCTGGA - 3'

Table 3. 1: Standard PCR reaction mix per 25µl of total volume

Reagent	Volume in µl
10X Buffer	2.50
Polymerase (super therm polymerase)	0.25
Q-Solution	5.00
dNTPs (2.5Mm)	2.00
Forward primer	0.50
Reverse primer	0.50
Nuclease free H2O	13.25
100ng/ µl DNA Template	1.00

Program, (T.D 50 and 55 Programs):

By using the PCR machine GeneAmp-PCR system 9700 from Applied Biosystem.

CP-E family	**CP-AL/ BM families**
94 °C 5 min[1]	94 °C 5 min[1]
94 °C 30sec[2] 58 °C 30sec[3] X2 72 °C 30sec[4]	94 °C 30sec[2] 63 °C 30sec[3] X2 72 °C 30sec[4]
94 °C 30sec 56 °C 30sec X2 72 °C 30sec	94 °C 30sec 61 °C 30sec X2 72 °C 30sec
94 °C 30sec 54 °C 30sec X2 72 °C 30sec	94 °C 30sec 59 °C 30sec X2 72 °C 30sec
94 °C 30sec 52 °C 30sec X2 72 °C 30sec	94 °C 30sec 57 °C 30sec X2 72 °C 30sec
94 °C 30sec 50 °C 30sec X35 72 °C 30sec	94 °C 30sec 55 °C 30sec X35 72 °C 30sec
72 °C 7 min[5]	72 °C 7 min[5]
4 °C ∞	4 °C ∞

1) Initial denaturation
2) An initial denaturation of 30 seconds at 94°C is sufficient for most amplicons from pure DNA templates.
3) The annealing step is typically 15–60 seconds. Annealing temperature is based on the T_m of the primer pair and is typically 45–68°C.
4) Extension, the recommended extension temperature is 68°C. Extension times are generally 1 minute per kb.
5) Final Extension

3.2.6.1.2. Electrophoresis of PCR product using Agarose gels.

The amount of Agarose (grams) required to make the desired Agarose gel concentration and volume was prepared by adding 1.5 gram of agarose to a 100 ml TAE buffer. The agarose was heated in a microwave until the solution became clear. After a short period of cooling to about 50-55°C, the ethidium bromide (section 3.1.1) was added to a final concentration of 0.5µg/ml. The percentage of agarose dissolved in the TAE was determined by the size of the PCR product.

Three µls of the loading dye was mix with three µls of the PCR product then the mixture was loaded onto the gel and run in 1X TAE running buffer at 120V for 20-30 minutes, depending on the fragment size. Once all samples have been loaded into the gel, 6µl of molecular weight marker (DNA ladder) (ThermoScientific, GeneRuler™) was loaded into well number 1 of each row of wells for size estimation. DNA fragments were observed using ultraviolet light and Photographed using the (Molecular Imager®, Gel DOC TM Imaging System, BioRAD).

3.2.6.1.3. Cleaning of PCR Product

This procedure was used to chew up excess primers and remove excess dNTPs from the PCR product. Many classical methods used to clean up PCR products prior to sequencing include gel electrophoresis, ethanol precipitation, and column chromatography.

In this protocol two hydrolytic enzymes were used Exonuclease I and Antarctic Phosphatase which can be used to remove unwanted materials.

Exonuclease I and Antarctic Phosphatase were added directly to the PCR product to degrade primers and dephosphorylate dNTPs that were not consumed in the reaction so that they cannot interfere with downstream sequencing reactions.

Exonuclease I digests single stranded DNA, thus removes leftover primers on the other hand, Antarctic Phosphatase removes 3' phosphate groups from single nucleotides.

Enzyme purification of PCR Program

Treatment is carried out for 30 minutes at 37°C, followed by 20 minute incubation at 80°C to completely inactivate both enzymes. Then cooled to 4°C to be ready for downstream sequencing reactions without any additional manipulation.

Table 3. 2: PCR clean reaction mix per 7 µl of total volume

Reagent	Volume in µl
Exonuclease I	0.25
Antaractic Phosphatase	0.25
Nuclease free H2O	1.5
PCR Product	5

3.2.6.1.4. DNA Sequencing

DNA sequencing was done using 10ng of PCR product per 100 bp of the length of PCR fragment. In the sequencing reaction 10pms of a forward or reverse primer were added to 1µl of BigDyeTM Terminators V1.1

The sequencing reaction was carried out with the 0.30 µl BigDye^{TM} Terminators V1.1 Cycle Sequencing Reaction Kit along with 1.5 5x buffer and 0.75 64x buffer, the reaction was completed to 16µl total volume using nucleas free water. Sequence amplification of these samples was performed using GeneAmp-PCR system 9700 from Applied Biosystem.

Sequencing PCR Reaction Program:

Sequencing was carried out for 1 minute at 96°C, followed by 25 cycle of (96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min) then ramp to 4°C for 10 min.

Table 3. 3: Sanger sequencing reaction mix per 16µl total volume

Reagent	Volume in µl
5x buffer	1.50
64x buffer	0.75
BigDye Terminator V1.1	0.30
Forward primer	0.50
DNA Template	2.00
Nucleas Free H2O	10.95

3.2.6.1.5. EDTA/ Ethanol Precipitation of Cycle-Sequenced Products

This is a critical stage, In order to properly clean the PCR product from primers, excess dNTPs, and unincorporated dyes. Incorrect concentration of ethanol can lead to loss of short products and incomplete precipitation of DNA fragments. EDTA helps to stabilize extension products during precipitation, and also helps to wash out unincorporated dyes from the completed reaction.

For each 20 µl of sequencing reactions 5 µl of 125mM EDTA and 100 µl of absolute ethanol were added to each sample and mixed by vortexing briefly. The samples were left in the dark at -20°C for 30 min or -80°C for 10 min to precipitate products, and then it was spin in centrifuge at 3800 RPM for 30 min at 4°C. After that, the supernatant was discarded completely; 60µl of 70% ethanol was added to each sample and centrifuged again directly as before at 3800 RPM for 20 min at 4°C. After spinning, the supernatant was also discarded and spin upside down at 500 rpm for 1 min. The samples were placed on 95°C for 5min in order to dryness and remove any residual ethanol.

Finally, the samples were resuspended in 10µl Hi Di Formamide (CAT#4311320, Applied Biosystem) and placed on 95°C for 5 min for denaturation, then it was placed on ice for 5 min. Samples were loaded on the 96 –well Optical Reaction Plate from Applied Biosystems and run on sequencing machine (3130XL Genetic Analyzer from Applied Biosystems). The results were analyzed with FINICH TV program.

CHAPTER FOUR

Results

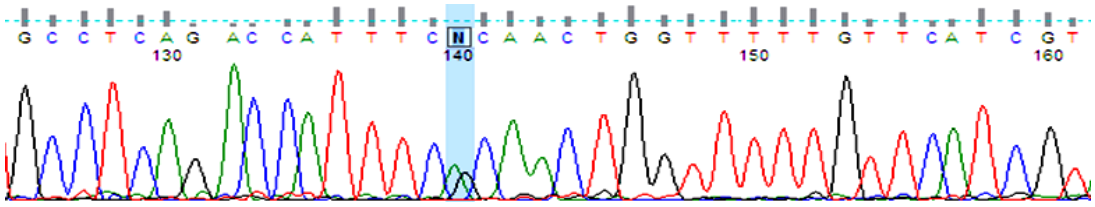
4.1. Next Generation Sequencing Results

The exome sequencing revealed in AL family a nonsense substitution mutation in *BOD1* gene, a single base pair change in the second exon converts the Arginine codon (CGA) at amino acid 112 to a stop codon (TGA), (*BOD1*_R112X, chr5: 173040162 G>A). BM family has also nonsense substitution mutation in *IRF6* gene, a single base pair change in the exon 7 converts the Arginine codon (CGA) at amino acid 250 to a stop codon, (*IRF6*_R250X, chr1:209964152 G>A). Insertion mutation in *CCDC141* gene have been found in family E in exon 6, one base pair inserts (A) converts the isoleucine codon (ATA) at amino acid 295 to leucine codon (TTA), due to this, the second codon leucine (CTG) at amino acid 294 converts to a stop codon (*CCDC141*_I295L, chr2:179809274 ins A). Those mutations cause premature termination of transcription and release of incomplete almost nonfunctional polypeptides.

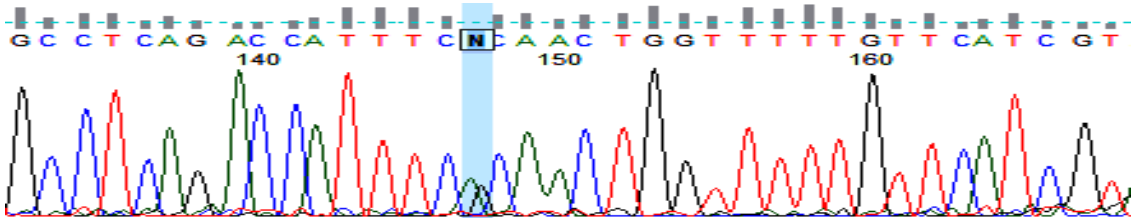
4.2. Sanger Sequencing Results

4.2.1 Segregation of the R112X mutation with the cleft lip and palate phenotype and\ or MR in CP-AL family.

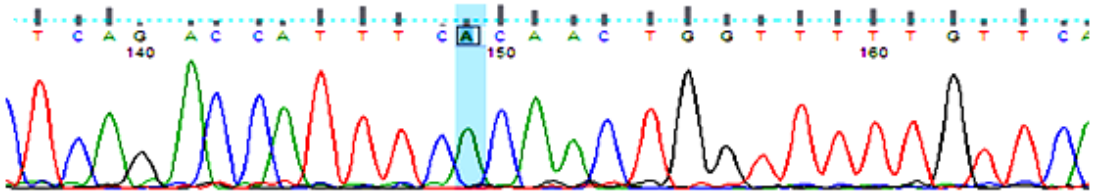
Sanger sequencing revealed that the nonsense mutation in *BOD1* gene segregate perfectly in CP-AL family under an autosomal recessive mode of inheritance, it also revealed that the identified mutation is not responsible for CL\ CP phenotype because CP-AL10 has carry the same mutation; which means that BOD1 mutation is most likely responsible for an known body phenotype and the mental retardation syndrome (Figure 4.2). Sanger sequencing revealed also that CP-AL10 and CP-AL2 were homozygous for the mutation moreover; the parents and the non-affected siblings were heterozygous carriers for the mutation except CP-AL11 was wild type (Figure 4.1).



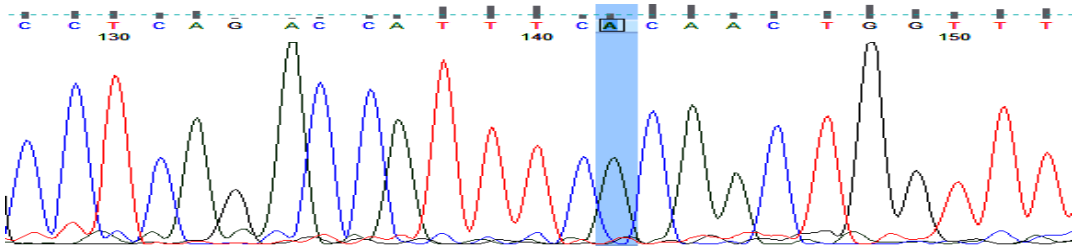
CP_AL1: Unaffected mother



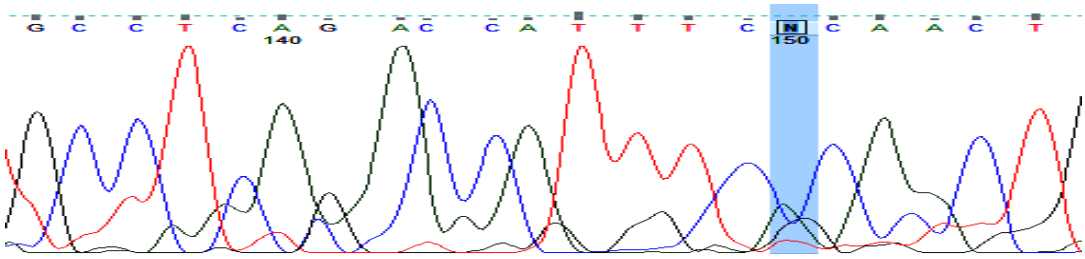
CP_AL3: Unaffected father



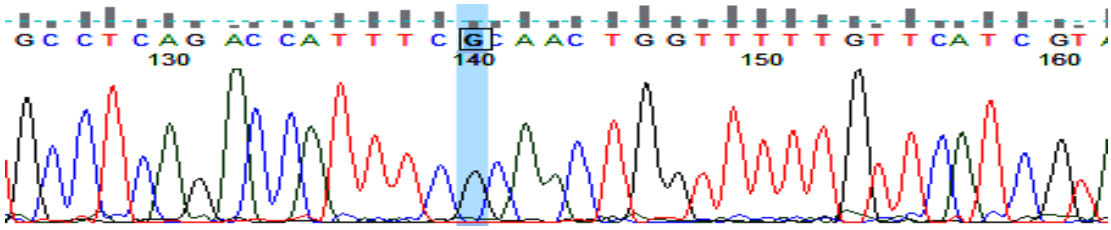
CP_AL2: Affected child (chr5:173040162 G>A)



CP_AL10: Affected child (chr5:173040162 G>A)



CP_AL9: Unaffected mother



Healthy control

Figure 4.1: Sanger sequencing results for the both parents and the affected child of the CP-AL family compared with healthy control.

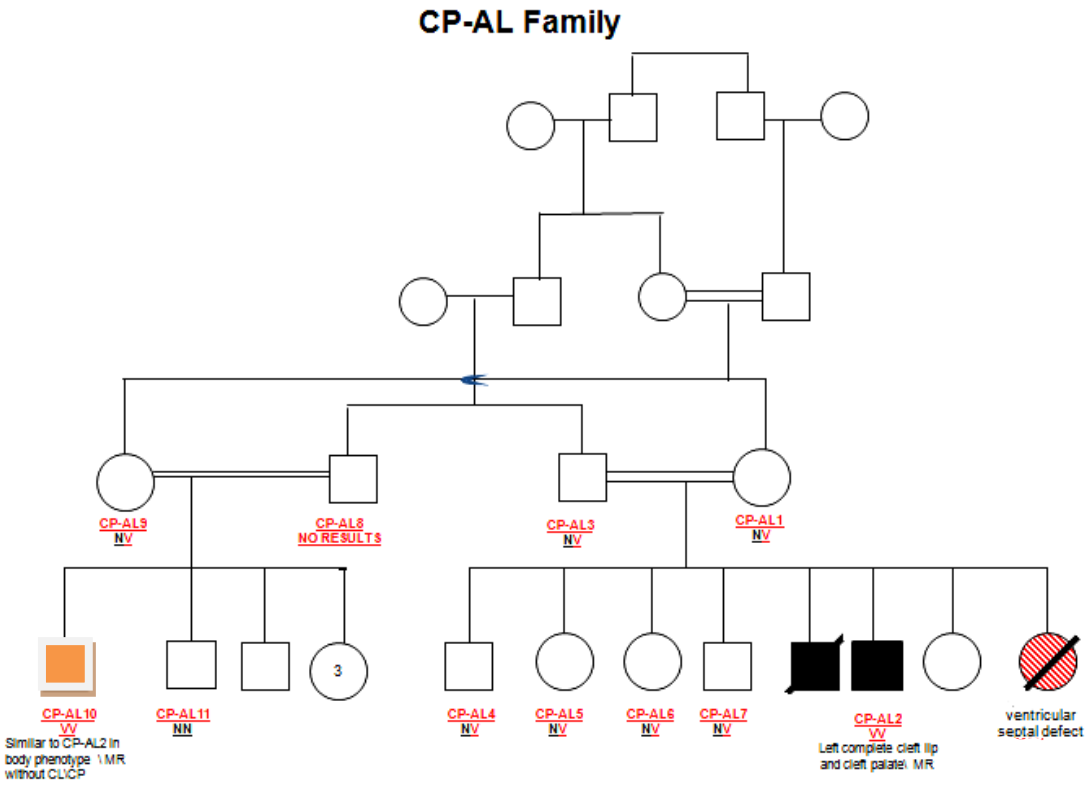
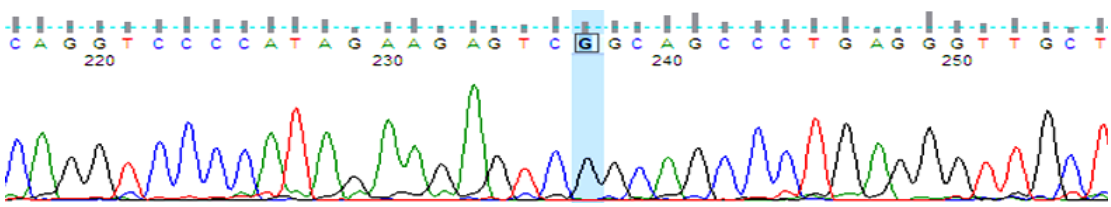


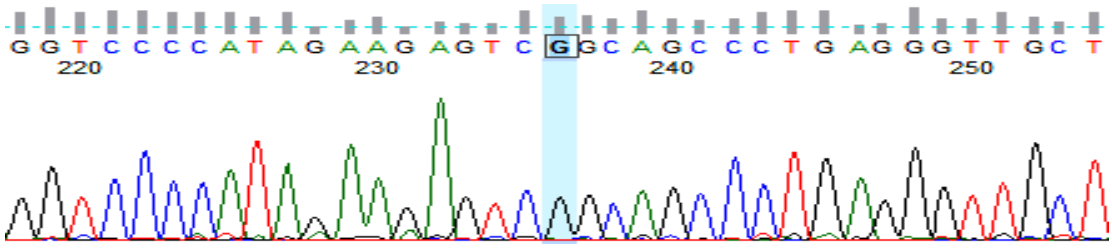
Figure 4.2: Segregation of the *BOD1* mutation in family AL which most likely responsible for mental retardation (unknown syndrome), with two affected individuals (CP-AL2\10) who had homozygous nonsense substitution.

4.2.2 Segregation of the R250X mutation with the cleft lip and palate phenotype in CP-BM family.

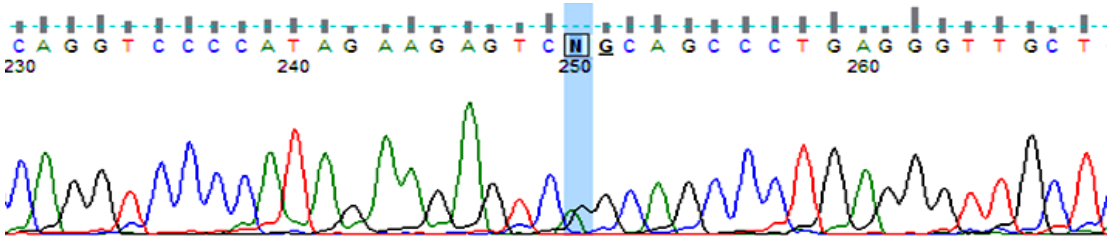
Sanger sequencing for all affected and unaffected individuals of the CP-BM family revealed that the nonsense mutation in *IRF6* gene was de novo, and the mutation did not segregate within the family (Figure 4.4). Sanger sequencing showed that the affected one was heterozygous for the mutation, while both parents and siblings were wild type for the mutation (Figure 4.3).



CP_BM1: Unaffected mother



CP-BM3: Unaffected father



CP_BM2: Affected child (chr1:209964152 G>A)

Figure 4.3:- Sanger Sequencing results for the both parents and the affected child of the CP-BM family.

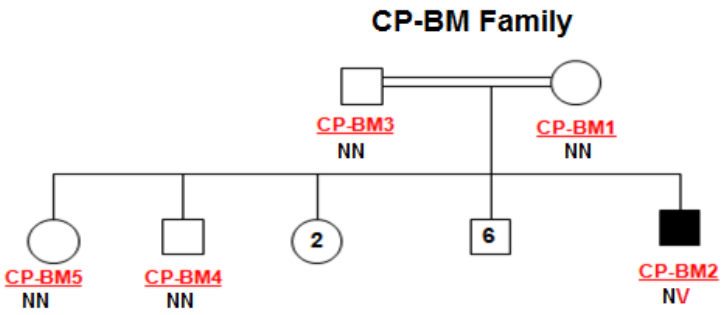
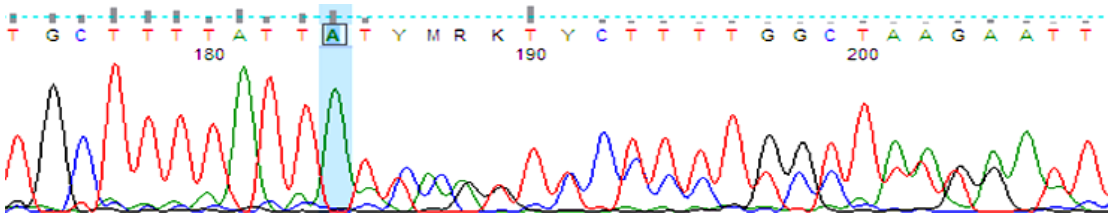


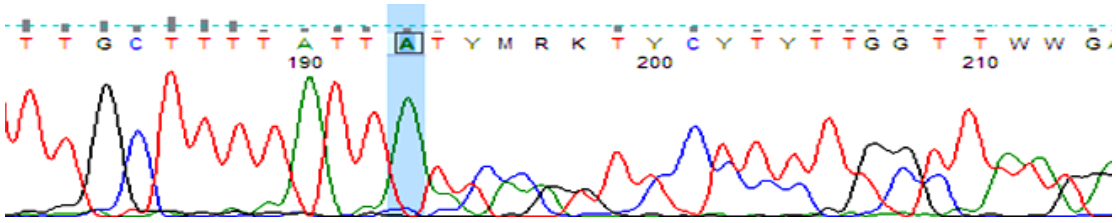
Figure 4.4: CP-BM2 had heterozygous nonsense de-novo mutation, with no Segregation within the family.

4.2.3 Segregation of the I295L mutation with the cleft lip phenotype in CP-E family.

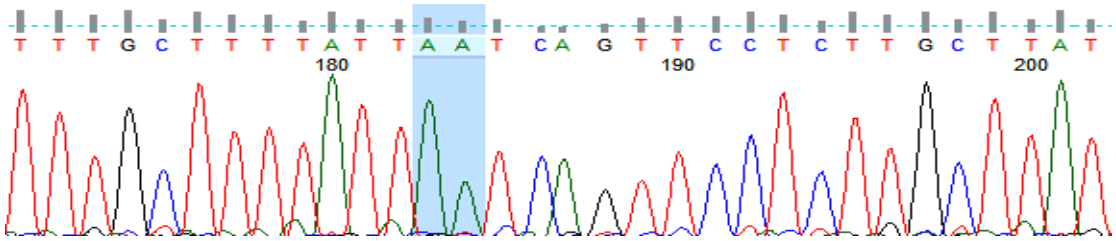
Sanger sequencing revealed that the insertion mutation in *CCDC141* gene segregate perfectly in CP-E family under an autosomal recessive mode of inheritance (Figure 4.6). It revealed also that the affected one was homozygous for the mutation, the parents were heterozygous carrier of the mutation and the non-affected brother was wild type (Figure 4.5).



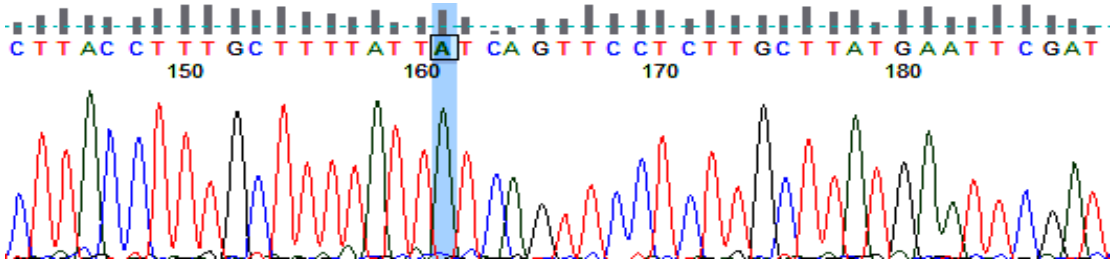
CP_E1: Unaffected father



CP_E2: Unaffected mother



CP_E4: Affected child (chr2:179809274 ins A)



Healthy control

Figure 4.5:- Sanger Sequencing results for the both parents and the affected child of the CP-E family compared with healthy control.

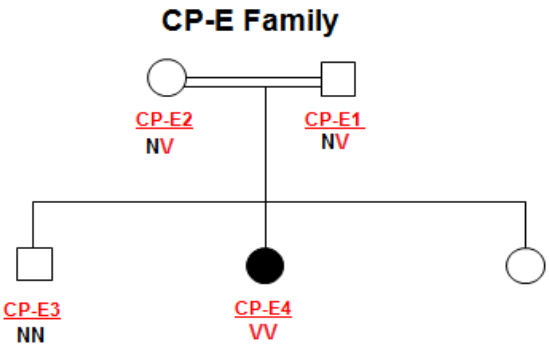


Figure 4.6: Segregation of the *CCDC141* mutation in family E, with one affected individual (CP-E4) who had homozygous insertion substitution.

4.3. Sanger sequencing for Healthy Controls Results

The identified mutations were subsequently verified and screened in at least 200 healthy, unaffected Palestinian controls from the West Bank for each CP-AL and CP-E families by using Sanger sequencing. Results showed no one of these individuals carrying those two mutations either in homozygous or heterozygous forms.

4.4. Bioinformatics Tools Results

By using the UCSC program and the NCBI data bases, we show that the *CCDC141* gene consists of 24 exons and has 220,303 base pairs (bp). Within the sixth exon, we identified a nonsense insertion mutation (*CCDC141*_I295L, chr2:179809274 ins A) which truncates the normal gene product, eliminating more than one half of the peptide chain, including the 4 major domains, which are SMC_prok_B from 221→846, SPEC; Spectrin repeats from 132→356, I-set; Immunoglobulin I-set domain from 1409→1498 and Ig; Immunoglobulin domain from 1425→1498 (Figure 4.7).

This nonsense mutation, which comes directly after the insertion mutation in the previous codon resulting in the truncation of the encoded polypeptides at position 294 which leads to loss of 1157a.a from the whole protein length 1450 a.a.

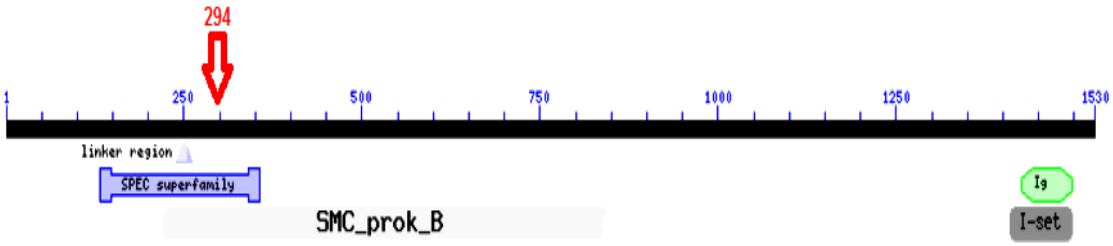


Figure 4.7: CCDC141 domains and mutation position. (Screenshot from NCBI, Dec 2015)

ExAC browser beta "The Exome Aggregation Consortium (ExAC, 2015)" showed that the frequency of the dominant allele in worldwide Population (Frequency of heterozygote) for the *CCDC141* mutation is 0.00005181, which distributed in south Asian and European (non Finnish) populations (Figure 4.8).

Variant	Chrom	Position	Protein Consequence	Filter	Annotation	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
2:179809254 T / A	2	179809254		PASS	splice region	1	13456	0	0.00007432
2:179809274 T / TA	2	179809274	p.Ile296AsnfsTer11	PASS	frameshift	8	15442	0	0.0005181
2:179809277 C / T	2	179809277	p.Leu294Leu	PASS	synonymous	1	15580	0	0.00006418

Population	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
South Asian	7	6872	0	0.001019
Other	0	196	0	0
Latino	0	290	0	0
European (Non-Finnish)	1	5090	0	0.0001965
Total	**8**	**15442**	**0**	**0.0005181**

Figure 4.8: Population frequencies for the dominant allele (screenshot from ExAC browser, 2015)

Protein interaction network, which was predicted by using STRING 9.1 program, indicates that the *CCDC141* interact with 4 different proteins (Figure 4.9) particularly, has strong interaction with DISC1. For this reason, *CCDC141* has an alternative name coiled -coil protein associated with myosin II and DISC1.

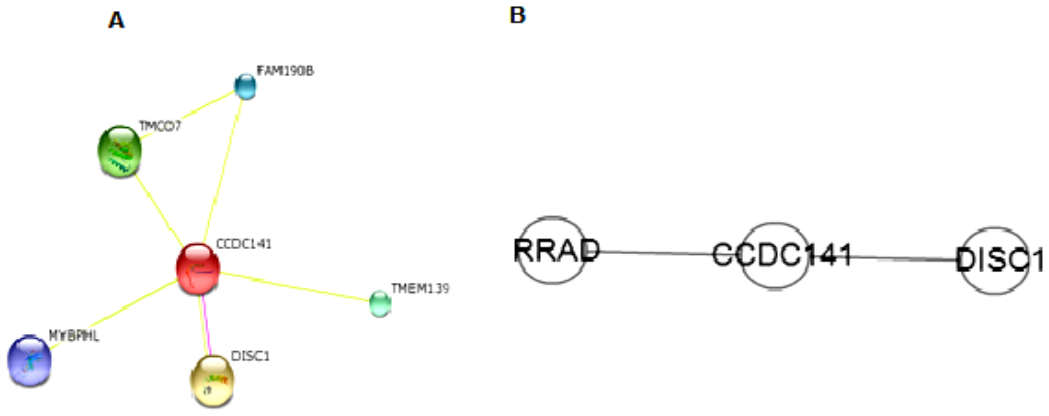


Figure 4.9: A- Action view of CCDC141 protein interaction network. Different line colors represent the types of evidence for the association. Evidence for the interaction between the genes above and the CCDC141 is textmining, but the evidence for the interaction between DISC1 and CCDC141 is experimental (screenshot from String program, 2015). **B-** Screenshot from IntAct Molecular Interaction Database, 2015.

BOD1 gene consists of 4 exons and has a 9,519 bp. Within the second exon, we identified a nonsense substitution mutation *BOD1*_R112X, chr5: 173040162 G>A that truncates the normal gene product, eliminating more than one third of the peptide chain, including the major domain, which is the COMPASS-Shg1 domain from 53→160 a.a. The nonsense mutation, resulting in truncation of the encoded polypeptides at position 112, leads to loss of 73 a.a from the whole protein length 185 a.a (Figure 4.10).

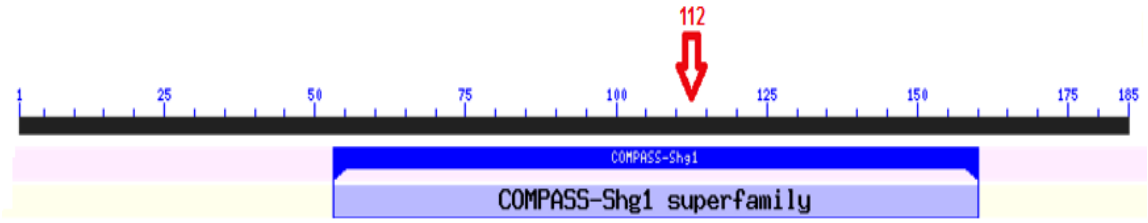


Figure 4.10 : BOD1 domains and mutation position. (Screenshot from NCBI, Dec 2015)

ExAC browser beta showed that the frequency of the dominant allele in worldwide Population for the *BOD1* mutation is zero so far. STRING 9.1 predicted that the *BOD1* interact with 4 different proteins (Figure 4.11).

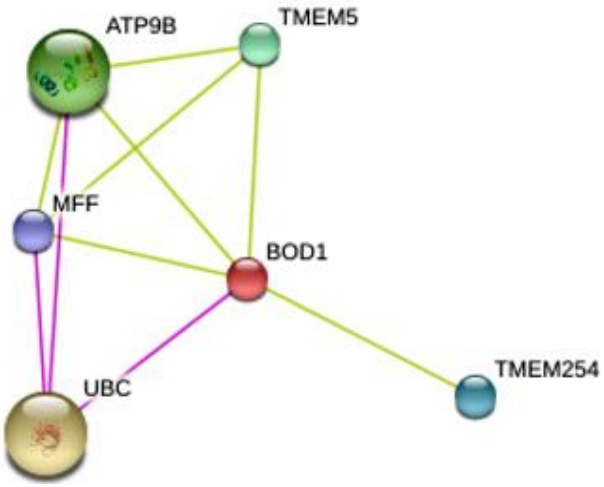


Figure 4.11: action view of BOD1- protein interaction network. Green line represent that the type of evidence is textmining but the purple line is experimental one. (screenshot from String program, Dec 2015)

IRF6 gene consists of 9 exons and has a 20,553 bp. Within the seventh exon, we identified a nonsense substitution mutation *IRF6*_R250X, chr1:209964152 G>A that truncates the normal gene product, eliminating one of its major domains, which is

IRF3; Interferon-regulatory factor 3 from 223→407 a.a. The second domain is far from the truncated region located from 7→115 called *IRF*; Interferon Regulatory Factor (*IRF*); also known as tryptophan pentad repeat.

The nonsense mutation resulting in truncation of the encoded polypeptides at position 250, leads to loss of 217 a.a from the whole protein length 467 a.a (Figure 4.12). ExAC browser beta showed that the frequency of the dominant allele in worldwide Population frequency for the *IRF6* mutation is zero so far.

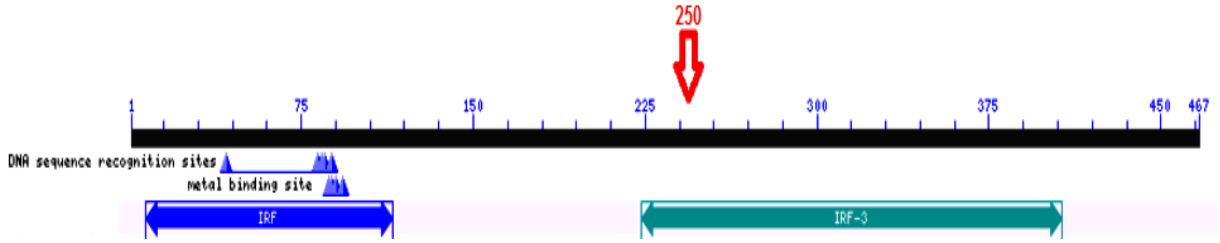


Figure 4.12: IRF6 domains and mutation position. (Screenshot from NCBI, Dec 2015).

CHAPTER FIVE

Discussion and conclusion

5.1. Discussion

CL/ CP is the most common congenital malformation that affects the upper lip and the roof of the mouth, it also possesses significant medical, psychological, social, and financial implications on the affected individuals and families (Allam and Stone, 2014). Currently, treatment includes multiple surgeries over the first 18 years of life, in addition to speech therapy, dental, and orthodontic treatment. CL/ CP is one of the most frequent congenital anomalies, affecting 1 in every 500 to 1000 births worldwide (Cooper, Ratay et al., 2006).

The majority of NS CL\ CP patients have no positive family history and the evaluation of inheritance patterns in the familial cases has not revealed a simple Mendelian mode of inheritance (Lidral, Moreno et al., 2008). It is a genetically complex trait involving genetic heterogeneity, low penetrance and the influence of various environmental factors (Lidral, Moreno et al., 2008). Most studies suggest that about 70% of cases of CL/ CP and 50% of CPO are nonsyndromic (Murray, 2002).

"Evidence for a genetic etiology for NSCLP comes from studies that show (1) a heritability for NSCLP of 76%, (2) monozygotic twins are ten-fold more likely to be concordant for a cleft compared to dizygotic twins (40% vs. 4.2%), (3) siblings of affected individuals have an increased risk of having a cleft compared to the general population, and (4) clefting aggregates in families" (Chiquet, 2011).

As it has been reported previously, the mutations in *IRF6* gene cause NS CL/ CP. We report a novel de novo mutation of the *IRF6* gene in one Palestinian family, resulting in the truncation of the encoded polypeptides at position 250, which leads to truncate the normal gene product and loss of 217 a.a. The mutation produced a stop codon within exon 7 of the *IRF6* gene. A base substitution changes the arginine codon at amino acid position 250 into a stop codon (*IRF6*_ R250X, chr1: 209964152 G>A). The premature stop codon was responsible for a truncated protein lacking of the Smad-interferon regulatory factor - binding domain (SMIR) probably essential for

interactions with the Smad transcription factors. The mutation is presumably expected to disturb the transcription regulatory function of *IRF6*.

The SMIR domain mediate an interaction between IRFs and Smads, a family of transcription factors known to transduce TGF-β signals. *IRF6* is expressed in the MEE of the secondary palatal shelves, it plays a role in palatal development, and regulate the expression of other genes during palatogenesis (Lidral, Moreno et al., 2008).
This IRF6 mutation can be caused by environmental factors such as ultraviolet radiation, vitamins deficiency , or can occur if a mistake is made as DNA copies itself during cell division. The mother was not exposed to any environmental factor as she said, in another hand consanguinity could play a role. In addition the family are from small village has low SES. All these points could be the causative for CL\ P phenotype.

Also we report a novel mutation of the *BOD1* gene in another one Palestinian family, resulting in the truncation of the encoded polypeptides at position 112, which lead to truncate the normal gene product and loss of 73 a.a. The mutation produced a stop codon within exon 2 of the *BOD1* gene. A base substitution changes the arginine codon at amino acid position 112 into a stop codon (BOD1_R112X, chr5:173040162 G>A). The premature stop codon is responsible for a truncated protein lacking of the COMPASS-Shg1 domain (Complex proteins associated with Set1p) component shg1.

The Shg1 subunit is one of the eight subunits of the COMPASS complex, complex associated with SET1, conserved in yeasts and in other eukaryotes up to humans. The function of Shg1 seems to be to slightly inhibit histone 3 lysine 4 (H3K4) di- and tri-methylation. The function of the COMPASS complex, silencing of genes close to the telomeres of chromosomes and to methylate the fourth lysine of Histone 3 and for (NCBI, pfam05205).

Porter, Schleicher et al. (2013) recently identified *Bod1* as a small kinetochore-associated protein required for mitotic chromosome congression. Loss of *Bod1* changes the balance of phosphorylation at kinetochores, causing defects in kinetochore function also causes severe biorientation defects

It plays a critical role in defining and monitoring the proper attachment of microtubules to the kinetochore.
Further ascertainment of unaffected individuals from family CP-AL revealed that this mutation can't be the causative one for the clefting phenotype in this family rather it is causing another phenotype in the family.

Despite the numerous attempts to study AL-family patients to obtain the need information for our research, the family has refused to respond with us also refused to presented their patients to a geneticists in order to know their exact phenotype and the syndrome that the patients have. We don't have sufficient information to complete the research, the information which is available to us indicates that the mutation is not responsible for cleft lip and palate and we don't have any info about this syndrome.
By exon sequencing this is the only deleterious mutation that we found. CL\ CP phenotype most likely coming from another mutation in regions that do not covered by exome sequencing like intronic regions, mutations in micro RNA also epigenetic changes.

Also we report a novel mutation of the *CCDC141* gene in another one Palestinian family, resulting in the truncation of the encoded polypeptides at position 294, which leads to truncate the normal gene product and loss of 1157 a.a. The mutation produced a stop codon within exon 6 of the *CCDC141* gene. The premature stop codon was responsible for a truncated protein lacking of the *CCDC141* major domains. *CCDC141* is required for radial migration during neuronal development probably through DISC1 and myosin II-mediated centrosome positioning (Fukuda, Sugita et al., 2010).

Through the second coiled-coil domain, *CCDC141* directly binds with DISC1, CCDC141 or DISC1 Knockdown showed an impaired migration of cortical neurons with disorientated centrosomes (Fukuda et al., 2010).

Drerup et al. (2009) revealed that Disc1 knockdown results in abnormal craniofacial development. Loss of Disc1 reduced the ectomesenchymal CNC cell population in the pharyngeal arches and altered its arrangement, which probably led to the abnormal craniofacial development. May the missing part of the *CCDC141* polypeptide be necessary for Disc1 interaction through the second domain which leads to orofacial

cleft. CP-E family is almost small family we can't took samples from other individuals to make the picture more clear.

By looking at the family pedigrees we note that the three families CP-AL, CP-E, CP-BM have first cousin marriage. We expected that a recessive mode of inheritance responsible of both cleft lip and cleft palate phenotypes in the affected individuals of CP-E and CP-BM families and responsible of mental retardation phenotype in CP-AL family. It is recognized that the consanguineous unions lead to increased incidences of autosomal recessive disorders. The closer biological relationship between parents, the greater is the probability that their offspring will inherit identical copies of one or more detrimental recessive genes.

I recommended to knocking out the activity of a those genes (CCDC141, BOD1, IRF6) to provides information about what that genes normally does. Consequently, observing the characteristics of knockout mice or any other animal model like Zebra fish gives researchers information that can be used to better understand how a similar gene may cause or contribute to disease in humans. The loss of gene activity often causes changes in the animal phenotype, which includes appearance, behavior and other observable physical and biochemical characteristics.

5.2. Concluding Remarks

- Mental retardation and NS CL/ CP phenotype in the CP-AL and CP-BM families respectively, are as a result of a single base pair change that converts the arginine codon (CGA) to a stop codon (TGA). In addition, CP-E family which suffers from NS CL phenotype is as a result of an insertion mutation that converts the isoleucine codon (ATA) to leucine codon (TTA) due to this, the second codon leucine (CTG) converts to a stop codon.
- The mutation in *BOD1* and *CCDC141* segregates perfectly in a recessive pattern of inheritance with the cleft palate phenotype in the CP-E family and with mental retardation in the CP-AL family. On the other hand, the mutation in *IRF6* is de novo.
- No healthy control individuals (200) were either in homozygous or heterozygous forms.
- CP-AL and CP-E families are the first examples of a recessive mutation in *BOD1* and *CCDC141* respectively that cause MR and NS CL/ CP respectively. also CP-BM family is the first example of a de novo mutation in *IRF6*.

CHAPTER SIX

References

- Abdul Wahab, N., et al. (2004). "Nasalance scores in Malay children with repaired cleft palate (±lip)."
- Acuña-González, G., et al. (2011). "Family history and socioeconomic risk factors for non-syndromic cleft lip and palate: A matched case-control study in a less developed country." Biomédica **31**(3): 381-391.
- Agrawal, K. (2014). "Classification of cleft lip and palate: An Indian perspective." Journal of Cleft Lip Palate and Craniofacial Anomalies **1**(2): 78.
- Al Omari, F. and I. Al-Omari (2004). "Cleft lip and palate in Jordan: birth prevalence rate." The Cleft palate-craniofacial journal **41**(6): 609-612.
- Aldhorae, K. A., et al. (2014). "Nonsyndromic cleft lip with or without cleft palate in arab populations: Genetic analysis of 15 risk loci in a novel case–control sample recruited in Yemen." Birth Defects Research Part A: Clinical and Molecular Teratology **100**(4): 307-313.
- Aljohar, A., et al. (2008). "Pattern of cleft lip and palate in hospital-based population in Saudi Arabia: retrospective study." The Cleft Palate-Craniofacial Journal **45**(6): 592-596.
- Allam, E. and C. Stone (2014). "Cleft Lip and Palate: Etiology, Epidemiology, Preventive and Intervention Strategies." Anat Physiol **4**(150): 2161-0940.1000150.
- Amos, C., et al. (1996). "Nonsyndromic cleft lip with or without cleft palate: erratum." American journal of human genetics **59**(3): 744.
- Aquino, S. N., et al. (2014). "Analysis of susceptibility polymorphisms for nonsyndromic cleft lip with or without cleft palate in the Brazilian population." Birth Defects Research Part A: Clinical and Molecular Teratology **100**(1): 36-42.
- Bailey, C. M. (2006). "Expounding Maspin and IRF6: identification and characterization of a novel serpin partnership." Theses and Dissertations: 52. University of Lowa.
- Bell, J. C., et al. (2013). "Descriptive epidemiology of cleft lip and cleft palate in Western Australia." Birth Defects Research Part A: Clinical and Molecular Teratology **97**(2): 101-108.
- Bender, P. L. (2000). "Genetics of cleft lip and palate." Journal of pediatric nursing **15**(4): 242-249.
- Bianchi, F., et al. (1999). "[Environment and genetics in the etiology of cleft lip and cleft palate with reference to the role of folic acid]." Epidemiologia e prevenzione **24**(1): 21-27.
- Biggs, L. C. (2012). "Interferon regulatory factor 6: a novel regulator of keratinocyte differentiation and migration." Doctoral dissertation. University of Lowa.
- Blanco, R., et al. (2013). "Genetic risk score for nonsyndromic cleft lip with or without cleft palate for a Chilean population." Genetic counseling (Geneva, Switzerland) **25**(2): 143-149.
- Blanton, S. H., et al. (2005). "Variation in IRF6 contributes to nonsyndromic cleft lip and palate." American Journal of Medical Genetics Part A **137**(3): 259-262.

- Böhmer, A. C., et al. (2013). "Analysis of susceptibility loci for nonsyndromic orofacial clefting in a European trio sample." American Journal of Medical Genetics Part A **161**(10): 2545-2549.
- Borno, H. T., et al. (2012). "Incidence of cleft lip and palate in the occupied Palestinian territory: a retrospective study." The Lancet **380**: S42.
- Borno, H. T., et al. (2014). "Incidence of Cleft Lip and Palate in the Palestinian Territories: A Retrospective Study From the Makassed Hospital Neonatal Unit." The Cleft Palate-Craniofacial Journal **51**(4): 472-475.
- Bromiker, R., et al. (2004). "Association of parental consanguinity with congenital malformations among Arab newborns in Jerusalem." Clinical genetics **66**(1): 63-66.
- Brugmann, S. A., et al. (2010). "A primary cilia-dependent etiology for midline facial disorders." Human molecular genetics **19**(8): 1577-1592.
- Carinci, F., et al. (2007). "Human genetic factors in nonsyndromic cleft lip and palate: an update." International journal of pediatric otorhinolaryngology **71**(10): 1509-1519.
- Carroll, K. and P. Mossey (2012). "Anatomical Variations in Clefts of the Lip with or without Cleft Palate." Plastic surgery international **2012**.
- Chai, Y. and R. E. Maxson (2006). "Recent advances in craniofacial morphogenesis." Developmental Dynamics **235**(9): 2353-2375.
- Chiquet, B. T. (2011). "Gene Discovery in Nonsyndromic Cleft Lip with or without Cleft Palate." Doctoral dissertation. University of Texas.
- Chitturi, R., et al. (2014). "Genetic Aspects of Cleft Lip and Palate–A Review." J Clin Diagn Res **2**(111): 2376-0311.1000111.
- Compton, D. A. (2007). "Chromosome orientation." The Journal of cell biology **179**(2): 179-181.
- Cooper, M. E., et al. (2006). "Asian oral-facial cleft birth prevalence." The Cleft Palate-Craniofacial Journal **43**(5): 580-589.
- DeRoo, L. A., et al. (2008). "First-trimester maternal alcohol consumption and the risk of infant oral clefts in Norway: a population-based case-control study." American journal of epidemiology **168**(6): 638-646.
- Dixon, M. J., et al. (2011). "Cleft lip and palate: understanding genetic and environmental influences." Nature Reviews Genetics **12**(3): 167-178.
- Donoghue, L. J., et al. (2014). "The impact of participation in genetic research for families with cleft lip with and without cleft palate: a qualitative study." Journal of community genetics: 1-8.
- Dudas, M., et al. (2007). "Palatal fusion–Where do the midline cells go?: A review on cleft palate, a major human birth defect." Acta histochemica **109**(1): 1-14.
- Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org) (3, 2015) accessed.
- Farronato, G., et al. (2014). "Cleft lip and/or palate: review." Minerva stomatologica **63**(4): 111-126.
- Flynn, T., et al. (2013). "A longitudinal study of hearing and middle ear status in adolescents with cleft lip and palate." The Laryngoscope **123**(6): 1374-1380.
- Francis-West, P., et al. (1998). "Signalling interactions during facial development." Mechanisms of development **75**(1): 3-28.
- François-Fiquet, C., et al. (2014). "Role of angiogenesis-related genes in cleft lip/palate: Review of the literature." International journal of pediatric otorhinolaryngology **78**(10): 1579-1585.

- Fukuda, T., et al. (2010). "CAMDI, a novel disrupted in schizophrenia 1 (DISC1)-binding protein, is required for radial migration." Journal of Biological Chemistry **285**(52): 40554-40561.
- Ghassibé, M., et al. (2005). "Interferon regulatory factor-6: a gene predisposing to isolated cleft lip with or without cleft palate in the Belgian population." European journal of human genetics **13**(11): 1239-1242.
- Group, I. W. (2011). "Prevalence at birth of cleft lip with or without cleft palate: data from the International Perinatal Database of Typical Oral Clefts (IPDTOC)."
- Harville, E. W., et al. (2005). "Cleft lip and palate versus cleft lip only: are they distinct defects?" American journal of epidemiology **162**(5): 448-453.
- Hoffmeister, M., et al. (2014). "The Ubiquitin E3 Ligase NOSIP Modulates Protein Phosphatase 2A Activity in Craniofacial Development." PloS one **9**(12): e116150.
- Hopper, R. A., et al. (2007). "Cleft lip and palate." Thorne CH, Beasley RW, Aston SJ, et al. Grabb and Smith's Plastic Surgery **6**: 201-225.
- Hussein-BDS, E. and K. Abughazaleh (2012). "Cleft Lip and Palate: The Multidisciplinary Management." Smile dental journal.
- Hviid, A. and D. Mølgaard-Nielsen (2011). "Corticosteroid use during pregnancy and risk of orofacial clefts." Canadian Medical Association Journal **183**(7): 796-804.
- Jaber, L., et al. (2002). "Facial clefting in an Arab town in Israel." Clinical genetics **61**(6): 448-453.
- Jacobs, H., et al. (2011). "Retinoic acid drives aryl hydrocarbon receptor expression and is instrumental to dioxin-induced toxicity during palate development." Environmental health perspectives **119**(11): 1590.
- Jahanbin, A., et al. (2014). "Analysis of MTHFR Gene C. 677C> T and C. 1298A> C Polymor-phisms in Iranian Patients with Non-Syndromic Cleft Lip and Palate." Iranian Journal of Public Health **43**(6): 821-827.
- JIN, B. (2006). Molecular characterization and Developmental analysis of interferon regulatory factor 6 (IRF6) gene in Zebrafish. Department of paediatrics. Master dissertation. National university of Singapore.
- Joo, K., et al. (2013). "CCDC41 is required for ciliary vesicle docking to the mother centriole." Proceedings of the National Academy of Sciences **110**(15): 5987-5992.
- Jugessur, A. and J. C. Murray (2005). "Orofacial clefting: recent insights into a complex trait." Current opinion in genetics & development **15**(3): 270-278.
- Kling, R. R., et al. (2014). "Oral Clefting in China Over the Last Decade: 205,679 Patients." Plastic and Reconstructive Surgery Global Open **2**(10).
- Kondo, S., et al. (2002). "Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes." Nature genetics **32**(2): 285-289.
- Kuijpers, M. A., et al. (2014). "Three-dimensional Imaging Methods for Quantitative Analysis of Facial Soft Tissues and Skeletal Morphology in Patients with Orofacial Clefts: A Systematic Review." PloS one **9**(4): e93442.
- Langlois, P. H., et al. (2013). "Maternal occupational exposure to polycyclic aromatic hydrocarbons and risk of oral cleft-affected pregnancies." The Cleft Palate-Craniofacial Journal **50**(3): 337-346.
- Leite, I. C. G. and S. Koifman (2009). "Oral clefts, consanguinity, parental tobacco and alcohol use: a case-control study in Rio de Janeiro, Brazil." Brazilian oral research **23**(1): 31-37.

- Letra, A., et al. (2010). "Follow-up association studies of chromosome region 9q and nonsyndromic cleft lip/palate." American Journal of Medical Genetics Part A **152**(7): 1701-1710.
- Lidral, A. C., et al. (2008). Genetic factors and orofacial clefting. Seminars in orthodontics, Elsevier.
- Lithovius, R. H., et al. (2013). "A descriptive epidemiology study of cleft lip and palate in Northern Finland." Acta odontologica Scandinavica(0): 1-4.
- Little, J. and C. Nelson (2013). "OROFACIAL CLEFTS." CONGENITAL ANOMALIES IN CANADA 2013: 42.
- Lohmann, K. and C. Klein (2014). "Next generation sequencing and the future of genetic diagnosis." Neurotherapeutics **11**(4): 699-707.
- Ludwig, K. U., et al. (2012). "Genome-wide meta-analyses of nonsyndromic cleft lip with or without cleft palate identify six new risk loci." Nature genetics **44**(9): 968-971.
- Maarse, W., et al. (2012). "A systematic review of associated structural and chromosomal defects in oral clefts: when is prenatal genetic analysis indicated?" Journal of medical genetics **49**(8): 490-498.
- Martinelli, M., et al. (2014). "Evidence of the involvement of the< i> DHFR</i> gene in nonsyndromic cleft lip with or without cleft palate." European journal of medical genetics **57**(1): 1-4.
- Matthews, J. L., et al. (2014). "The Epidemiology of Cleft Lip and Palate in Canada, 1998 to 2007." The Cleft Palate-Craniofacial Journal.
- Meng, L., et al. (2009). "Biological mechanisms in palatogenesis and cleft palate." Journal of dental research **88**(1): 22-33.
- Muhamad, A.-H., et al. (2014) "The multifactorial factors influencing cleft Lip-literature." International journal of clinical medicin research.
- Muhamad, A.-H., et al. (2014). "CLEFT LIP AND PALATE; A COMPREHENSIVE REVIEW." International Journal of Basic and Applied Medical Sciences **4**(1):338-355.
- Murray, J. (2002). "Gene/environment causes of cleft lip and/or palate." Clinical genetics **61**(4): 248-256.
- Murthy, J. and L. Bhaskar (2009). "Current concepts in genetics of nonsyndromic clefts." Indian journal of plastic surgery: official publication of the Association of Plastic Surgeons of India **42**(1): 68.
- Naim, A., et al. (2012). "Birth defects in Gaza: Prevalence, types, familiarity and correlation with environmental factors." International journal of environmental research and public health **9**(5): 1732-1747.
- Nikopensius, T., et al. (2010). "Genetic variants in COL2A1, COL11A2, and IRF6 contribute risk to nonsyndromic cleft palate." Birth Defects Research Part A: Clinical and Molecular Teratology **88**(9): 748-756.
- Owens, J., et al. (1985). "Epidemiology of facial clefting." Archives of disease in childhood **60**(6): 521-524.
- Paradowska-Stolarz, A., et al. (2013). "Dental anomalies in the incisor-canine region in patients with cleft lip and palate-literature review." Medycyna wieku rozwojowego **18**(1): 66-69.
- Park, J. W., et al. (2007). "Association between IRF6 and nonsyndromic cleft lip with or without cleft palate in four populations." Genetics in Medicine **9**(4): 219-227.

- Porter, I. M., et al. (2007). "Bod1, a novel kinetochore protein required for chromosome biorientation." The Journal of cell biology **179**(2): 187-197.
- Porter, I. M., et al. (2013). "Bod1 regulates protein phosphatase 2A at mitotic kinetochores." Nature communications **4**.
- Prescott, N., et al. (2001). "Nonsyndromic cleft lip and palate: complex genetics and environmental effects." Annals of human genetics **65**(06): 505-515.
- Sabbagh, H. J., et al. (2013). "Parental consanguinity and nonsyndromic orofacial clefts in children: a systematic review and meta-analyses." The Cleft Palate-Craniofacial Journal.
- Sabbagh, H. J., et al. (2012). "Prevalence of orofacial clefts in Saudi Arabia and neighboring countries: A systematic review." The Saudi Dental Journal **24**(1): 3-10.
- Savitsky D, Tamura T, Yanai H, Taniguchi T (2010). "Regulation of immunity and oncogenesis by the IRF transcription factor family." Cancer Immunol Immunother **59**:489- 510.
- Scapoli, L., et al. (2005). "Strong Evidence of Linkage Disequilibrium between Polymorphisms at the< i> IRF6</i> Locus and Nonsyndromic Cleft Lip With or Without Cleft Palate, in an Italian Population." The American Journal of Human Genetics **76**(1): 180-183.
- Schutte, B. C. and J. C. Murray (1999). "The many faces and factors of orofacial clefts." Human molecular genetics **8**(10): 1853-1859.
- Semb, G. (2014). "International Confederation for Cleft Lip and Palate and Related Craniofacial Anomalies Task Force Report: Beyond Eurocleft." The Cleft Palate-Craniofacial Journal **51**(6): e146-e155.
- Setó-Salvia, N. and P. Stanier (2014). "Genetics of cleft lip and/or cleft palate: Association with other common anomalies." European journal of medical genetics.
- Sharaha, U (2013). " Novel Mutation of Syndromic Cleft Palate in Four Consanguineous Palestinian Families." Master dissertation. Bethlahem University.
- Silberstein, E., et al. (2012). "Epidemiology of cleft lip and palate among Jews and Bedouins in the Negev." IMAJ-Israel Medical Association Journal **14**(6): 378.
- Sivertsen, Å., et al. (2008). "Prevalence of major anatomic variations in oral clefts." Plastic and reconstructive surgery **121**(2): 587-595.
- Sivertsen, Å., et al. (2008). "Familial risk of oral clefts by morphological type and severity: population based cohort study of first degree relatives." Bmj **336**(7641): 432-434.
- Skuladottir, H., et al. (2014). "Corticosteroid use and risk of orofacial clefts." WHO. Birth Defects Research Part A: Clinical and Molecular Teratology.
- Souza, J. and S. Raskin (2013). "Clinical and epidemiological study of orofacial clefts." Jornal de Pediatria (Versao en Portugues) **89**(2): 137-144.
- Srichomthong, C., et al. (2005). "Significant association between IRF6 820G→ A and non-syndromic cleft lip with or without cleft palate in the Thai population." Journal of medical genetics **42**(7): e46-e46.
- Stanier, P. and G. E. Moore (2004). "Genetics of cleft lip and palate: syndromic genes contribute to the incidence of non-syndromic clefts." Human molecular genetics **13**(suppl 1): R73-R81.
- Strauss, R. P. and C. H. Cassell (2009). "Critical issues in craniofacial care: quality of life, costs of care, and implications of prenatal diagnosis." Academic pediatrics **9**(6): 427-432.

- Su, L.-I. (2011). "The effects of disease mutations on the transcription factor Interferon Regulatory Factor 6." Department of life science. Doctoral dissertation. University of Manchester.
- Teebi, A. S. (1994). "Autosomal recessive disorders among Arabs: an overview from Kuwait." Journal of medical genetics **31**(3): 224-233.
- Thomason, H. A., et al. (2010). "Cooperation between the transcription factors p63 and IRF6 is essential to prevent cleft palate in mice." The Journal of clinical investigation **120**(120 (5)): 1561-1569.
- Tolarová, M. M. and J. Cervenka (1998). "Classification and birth prevalence of orofacial clefts." American journal of medical genetics **75**(2): 126-137.
- Van Aalst, J. A., et al. (2008). "MOC-PS (SM) CME Article: Nonsyndromic Cleft Palate." Plastic and reconstructive surgery **121**(1S): 1-14.
- van Aalst, J. A., et al. (2011). "Natural disaster and crisis: lessons learned about cleft and craniofacial care from Hurricane Katrina and the West Bank." The Cleft Palate-Craniofacial Journal **48**(6): 741-749.
- Vanderas, A. P. (1987). "Incidence of cleft lip, cleft palate, and cleft lip and palate among races: a review." Cleft palate J **24**(3): 216-225.
- Wehby, G. and C. H. Cassell (2010). "The impact of orofacial clefts on quality of life and healthcare use and costs." Oral diseases **16**(1): 3-10.
- William (2004). "Global strategies to reduce the health care burden of craniofacial anomalies: report of WHO meetings on international collaborative research on craniofacial anomalies." The Cleft Palate-Craniofacial Journal **41**(3): 238-243.
- Womersley, J. and D. Stone (1987). "Epidemiology of facial clefts." Archives of disease in childhood **62**(7): 717-720.
- Wu, T., et al. (2010). "Evidence of gene–environment interaction for the IRF6 gene and maternal multivitamin supplementation in controlling the risk of cleft lip with/without cleft palate." Human genetics **128**(4): 401-410.
- Wu, T., et al. (2014). "Evidence of Gene− Environment Interaction for Two Genes on Chromosome 4 and Environmental Tobacco Smoke in Controlling the Risk of Nonsyndromic Cleft Palate." PloS one **9**(2): e88088.
- Wyszynski, D. F. and T. Wu (2002). "Use of US birth certificate data to estimate the risk of maternal cigarette smoking for oral clefting." The Cleft Palate-Craniofacial Journal **39**(2): 188-192.
- Yazdee, A. K., et al. (2011). "Epidemiological aspects of cleft lip and palate in Iran." Acta Medica Iranica **49**(1): 54-58.
- Zaghloul, N. A. and S. A. Brugmann (2011). "The emerging face of primary cilia." genesis **49**(4): 231-246.
- Zucchero, T. M., et al. (2004). "Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate." New England Journal of Medicine **351**(8): 769-780.