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Identification of a Novel Mutation in *LRP6* Gene Responsible for Non-Syndromic Hypodontia in a Palestinian Family

By

Yasmeen Saleh Shokeh

In Partial Fulfillment of the Requirements for the Degree of

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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:

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"Identification of a Novel Mutation in *LRP6* Gene Responsible for Non-Syndromic Hypodontia in a Palestinian Family"

By: Yasmeen Saleh Shokeh

Abstract

Background: Hypodontia is considered one of the most common developmental anomalies in humans. It is defined as the missing of one or more primary or secondary teeth which results in disturbances during the early stages of tooth development. These disturbances are mostly due to genetic causes either syndromic or non-syndromic. Mainly, mutations in three genes have been identified in human: MSX1, PAX9 and AXIN2 through studying familial hypodontia pedigrees. Worldwide, permanent teeth missing prevalence ranges between 2.6%–11.3% of the population (missing primary teeth show very low prevalence). The aim of our study was to identify the genetic determinants of non-syndromic hypodontia in a Palestinian family.

Methods: Eight blood samples were collected from members of the study family and DNA was extracted. Whole exome sequencing then Sanger sequencing were used to investigate the causative mutation. The mutation detected in the study was screened by Sanger Sequencing in 200 normal controls.

Results: Whole exome sequencing revealed a novel heterozygous mutation (C>G) in Low-density lipoprotein receptor-related protein 6 (LRP6) gene which results in R675G alteration and hence alter the protein structure. The 200 controls were negative for the LRP6 variant.

Conclusion: Our results revealed a novel mutation in LRP6 gene that disrupts tooth development and results in non-syndromic hypodontia in human.

Keywords: hypodontia, LRP6, Wnt signaling pathway.

" تعريف طفرة جديدة في جين *LRP6* مسؤولة عن نقص عدد الأسنان الغير تلازمي في عائلة فلسطينية "

ياسمين صالح شوكه

مُلخّص

خلفية: يعتبر نقص عدد الأسنان من التشوهات الأكثر شيوعا في الإنسان. وتعرف بأنها نقص لواحد أو أكثر من الأسنان اللبنية أو الدائمة والتي تنتج بسبب اضطرابات خلال المراحل المبكرة من نمو الأسنان. و هذه الاضطرابات هي في الغالب نتيجة لأسباب وراثية إما المتلازمة أو غير المتلازمة. وقد تم تحديد طفرات في ثلاث جينات في الإنسان مسؤولة عن هذا النقص هي Msx1, Pax9, Axin2. يعد نقص الأسنان الدائمة هو الأكثر إنتشارا بنسبة تتراوح بين 2.6%-11.3% (في حين نسبة نقص الأسنان اللبنية منخفضة). تهدف دراستنا إلى تحديد الأسباب الجينية المسؤولة عن نفص الأسنان الوراثي في عائلة فلسطينية.

الطرق المستخدمة: تم جمع عينات دم من ثمانية أفراد من العائلة، ثم استخراج الحمض النووي DNA، ثم باستخدام whole exome sequencing ثم Sanger sequencing للبحث في الأسباب الجينية المتسببة في نقص الأسنان. ثم تم إختبار صحة الطفرة باستخدام 200 شخص ضابط.

النتائج: تم الكشف عن طفرة جديدة في الجين LRP6 و التي نتجت في تغيير البروتين الناتج عنه إذ تم إستبدال الحمض الأميني أرجينين في الموقع 675 إلى الحمض الأميني جلايسين. لكن هذه الطفرة كانت غائبة في ال 200 شخص ضابط.

الإستنتاج: كشفت نتائجنا عن طفرة جديدة في جين ال LRP6 والذي أدى إلى تعطيل نمو الأسنان فنتج عنه نقص عدد الأسنان الخلقي غير المتلازمة في الإنسان.

الكلمات الدالة: نقص عدد الأسنان,LRP6, WNT.

Declaration

I declare that the Master Thesis entitled "Identification of a Novel Mutation in *LRP6* Gene Responsible for Non-Syndromic Hypodontia in a Palestinian Family" 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: Yasmeen Saleh Shokeh

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Date: December 2014.

Dedication

I dedicate this thesis to my beloved parents who have been always there for me whenever I needed. You are my strength in this life and I will always feel of gratitude for all what have you done for me. I also dedicate this thesis to my sisters (Sarah, Sabreen, Hiba, and Haneen) and brothers (Hamzeh, Abedrahman, and Ezzdin) who are my closest persons in the world and have supported me and provided me with a strong love and never lets sadness enter inside. I dedicate it also to my best friend Nurah Ayesh.

I dedicate this thesis and give a special thanks to my ante Maha who have supported me throughout the entire master program.

Finally, I dedicate my thesis to my grandmother soul who was my best person in the world.

Acknowledgment

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Final thanks to the family that participated in this work and the dentist Yasmin Issa.

Abbreviation

APC	Adenomatous polyposis coli
Axin2	Axis inhibition 2
Barx1	BarH-Like homeobox 1
BMP	Bone morphogenetic protein
DKK1, 2	Dickkopf 1, 2
Dlx1, 2	Distal-less 1, 2
EDA	Ectodysplasin A
EDAR	Ectodysplasin A receptor
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast growth factor
Fz	Frizzled
Gli1, 2, 3	Glioma associated 1, 2, 3
GSK3	Glycogen synthase kinase 3
Kremen (Krm)	Kringle-coding marking the eye and the nose
LDLR	Low density lipoprotein receptor
Lef1	Lymphoid enhancer-binding factor 1
LTBP3	Latent Transforming Growth Factor Beta Binding Protein
Lhx6, 7	LIM homeobox 6, 7
LRP5, 6	Low density lipoprotein receptor-related protein
MSX1, 2	Muscle segment homeobox homolog 1, 2
NF-KB	Nuclear factor kappa B
Pax 9	Paired box 9
PCR	Polymerase chain reaction
Pitx1, 2	Paired-like homeodomain transcription factor 1
Ptc	Patched
SHH	Sonic hedgehog
TGFβ	Transforming growth factor beta
TNF	Tumor necrosis factor
Wnt	Wingless-related integration

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CHAPTER 1

Introduction

1.1 Background

Hypodontia is considered one of the most common developmental anomalies in humans (AlShahrani, 2013; Kotecha, 2012; Larmour et al, 2005). It is defined as the missing of one or more primary or secondary teeth that fail to erupt in the oral cavity and remained invisible in the radiograph. This happens due to perturbation during the early stages of tooth development (Kotecha, 2012; Pemberton et al, 2005). Hypodontia is a complex phenotype with variable penetrance and expressivity affecting various numbers of teeth in different regions (Matalova et al, 2008). Commonly, the missing teeth are the permanent teeth with prevalence ranging between 2.6%–11.3% in population worldwide. The missing of primary teeth shows very low prevalence between 0.1%-0.9% , but the third molars are not included since they occur in approximately 20% of the population (AlShahrani, 2013; Kotecha, 2012; Larmour et al, 2005). The majority of hypodontia patients (80%) fall under the classification of mild hypodontia in which one or two teeth are missing, while 10% are moderate with four or more missing teeth. Less than 1% have severe hypodontia with six or more missing teeth, a symptom referred to as oligodontia. The most common missing teeth in descending order are the mandibular second premolars, the maxillary lateral incisors, the maxillary second premolars and mandibular incisors (AlShahrani, 2013; Kotecha, 2012; Larmour et al, 2005). Hypodontia is frequently associated with other oral anomalies such as cleft lip and/or palate, microdontia, increased free-way space, tooth size reduction and malformations, short root anomaly, delayed eruption of other teeth and impaction (Chhabra et al, 2014; P. J. De Coster et al, 2009).

Environmental factors such as infections (e.g. rubella), drugs, trauma, radiations and chemical substances can occasionally cause hypodontia. However, the majority of cases arise due to genetic causes which can be syndromic, non-syndromic and inherited as autosomal dominant, autosomal recessive or x-linked patterns (Chhabra et al, 2014; Kotecha, 2012; P. J. De Coster et al, 2009).

Teeth development in embryo results from epithelial-mesenchymal interaction. This turns out to be shared among other embryonic organs such as lung, kidney, hair, mammary gland and limbs (Thesleff et al, 1989) (Weiss et al, 1998). Teeth development can be disrupted in transgenic knock out mouse (Zhang et al, 2005) or in cultured tooth premordia manipulation which allow investigation of the specific role of receptors and ligands among developmental pathways (Thesleff et al, 1995). These reasons have made tooth development an ideal model to study different mechanisms involved in tissue interactions (Thesleff et al, 1989).

1.2 Literature Review

1.2.1 Teeth development (odontogenesis)

Typically, the adult human mouth contains 32 teeth distributed equally between the upper and lower jaws. The upper jaw is referred to as maxilla and the lower as the mandible. Each half side of the jaw contains 1 central incisor, 1 lateral incisor, 1 canine, 2 bicuspids (premolars) and 2 molars as illustrated in figure 1.1(ortho-experience.com, 2014). Each tooth is composed of a crown and root. The crown is the protruding part in the mouth while the root is embedded in the gum and attached to the bone (Nieminen, 2007).

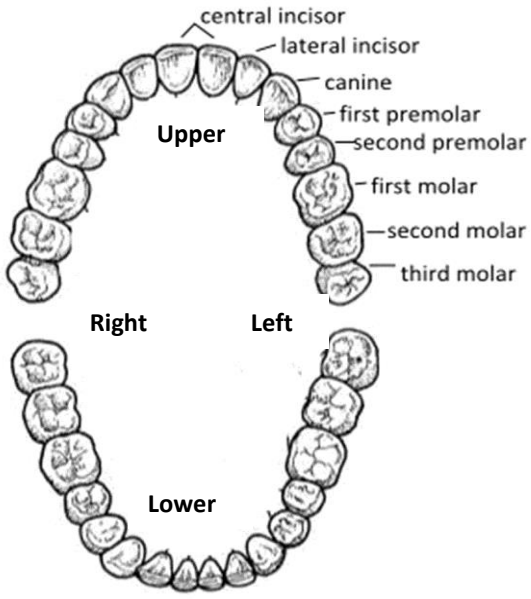


Figure 1.1 Teeth names and position in each jaw(ortho-experience.com, 2014).

Initiation of tooth development begins during the 6^{th}-7^{th} week of gestation when dental epithelium thickens to form dental lamina, which in turn proliferates into the underlying mesenchyme (neural crest derived layer). This leads to the formation of the tooth bud that grows and differentiates within the mesenchyme through cap and bell stages. Then, the crown and root of the tooth are formed, and finally eruption occurs (Bei, 2009; Cudney & Vieira, 2012). Figure 1.2 depicts these developmental stages.

During the initiation stage of the 6th week of embryo development, epithelial cuboidal cells elongate into columnar epithelial cells forming dental lamina. One week later dental lamina proliferates forming the 10 dental epithelial buds of the primary teeth. At week eight, another tooth bud forms to become a permanent tooth, but they pause at the bell stage until primary teeth replacement. Meanwhile, primary buds invaginate into the mesenchyme to form tooth germs. This process occurs during the cap stage, which ends during the tenth week (Zhang et al, 2005). At the tip of the bud, non-dividing cells called enamel knots are formed (Nieminen, 2007). During the cap stage, tooth budding to the mesenchyme induces mesenchymal cells to condensate around the bud resulting in epithelial-mesenchymal interaction. By the end of the bell stage, mesenchymal cells are well differentiated to form odontoblasts and establish the tooth crown (Thesleff et al, 1989). Both mesenchymal-odontoblasts and epithelial-ameloblasts cross talk to build the anatomical and functional parts of the tooth by secreting dentin and enamel matrices, respectively (Bei, 2009; Thesleff et al, 1989).

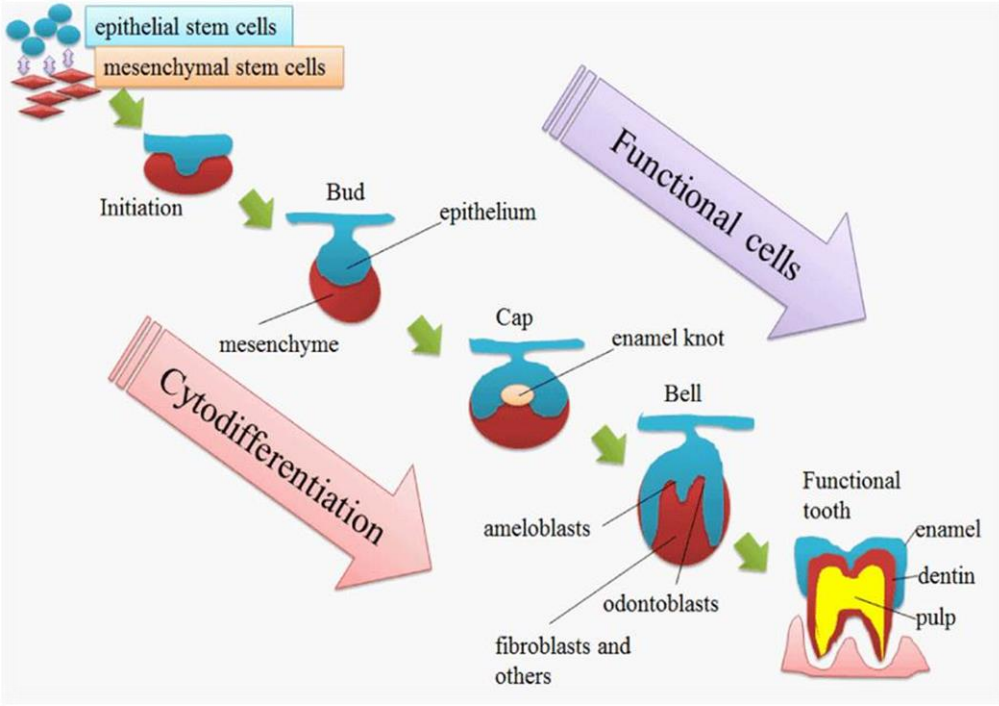


Figure 1.2: Teeth development stages from initiation to tooth eruption (Xiao, 2012).

1.2.2 Molecular pathways' interaction between epithelial-mesenchymal cells results in tooth development.

At the molecular level, there are four pathways: BMP, FGF, SHH and WNT. Combined, they interact in epithelial - mesenchymal tissue layers, regulating tooth development from tooth initiation to tooth patterning (determined by the location, identity, size and shape of the teeth) (Bei, 2009; Chhabra et al, 2014; P. J. De Coster et al, 2009; Thesleff et al, 1989).

Figure 1.3 summarizes signaling protein factors that are involved in tooth development. Epithelial-mesenchymal interaction was found to be sequential and reciprocal. Sequential refers to a chain of interactive events that carry on developmental process. Reciprocal alludes to molecular interactions that occur in both directions. This implies that regulation of morphogenesis and cell differentiation requires both epithelial and mesenchymal layers. It was also found that either the epithelium or the mesenchyme possess the information necessary for morphogenesis and differentiation of specific organs based on the organ type and its developmental stage (Thesleff et al, 1995). Traditionally, tooth development has been divided into phases of initiation, morphogenesis, and differentiation (Thesleff et al, 1995).

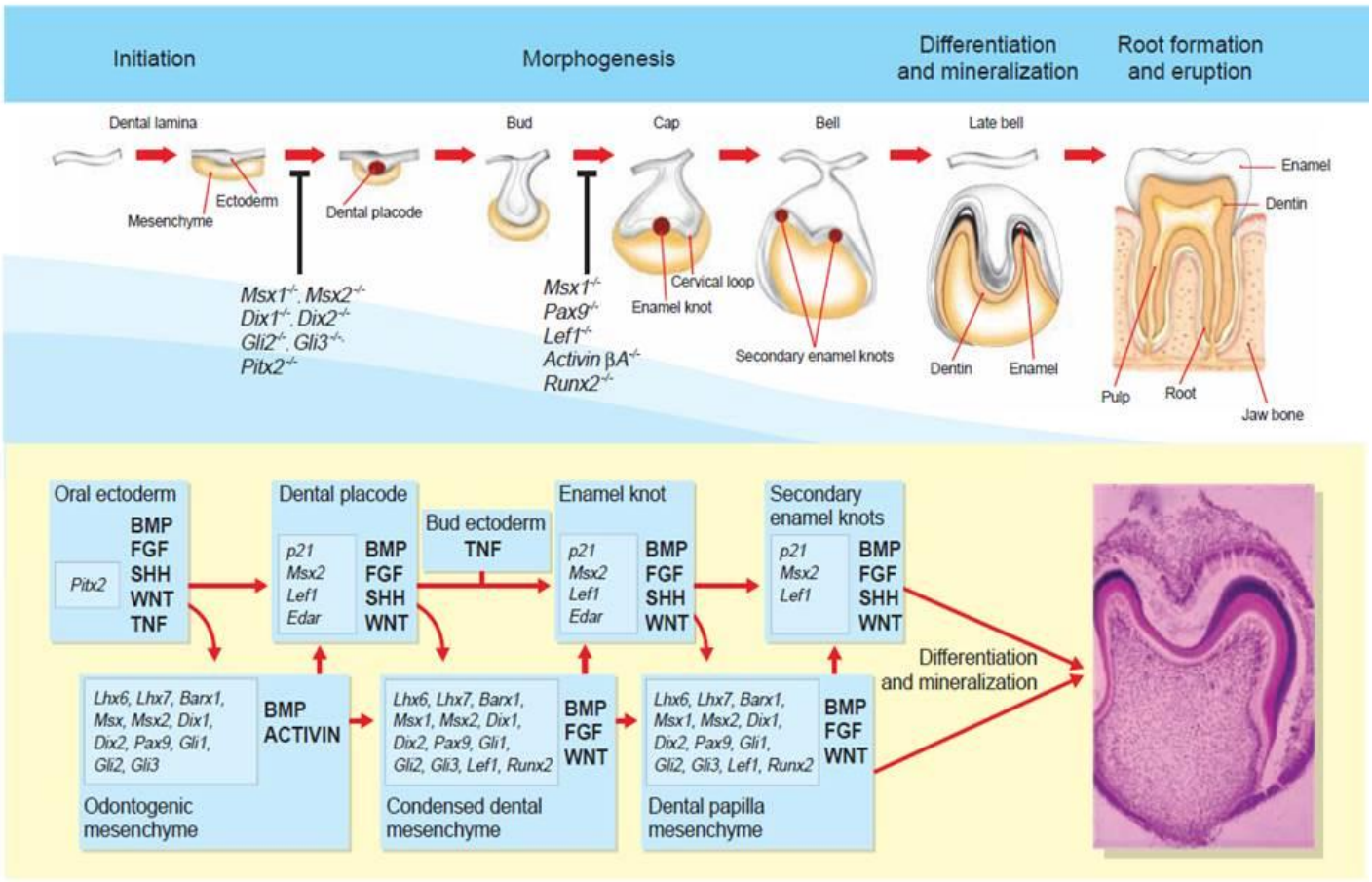


Figure 1.3 Several protein molecules act at specific stages of tooth development to regulate its patterning and differentiation process (Thesleff, 2003).

1.2.2.1 Initiation

At the initiation phase, early signals such as Bmp4 and FGF8 are expressed in the epithelium. These signals, in addition to Fgf9, Bmp2, Bmp7, Shh, Wnt10a, and Wnt10b, are believed to activate the expression of numerous transcription factors in the mesenchyme including: Msx1, Msx2, Lhx6, Lhx7, Dlx1, Dlx2, Lef1, Patched (Ptc), Gli1, and Syndecan-1 (Zhang et al, 2005). Bmp4 and Fgf8 restrict the expression of Pax9 and Pitx2 to the presumptive dental mesenchyme and to the presumptive dental epithelium, respectively. FGF signaling induces the expression of Pax9, Pitx1, and Pitx2. FGF8 is responsible for Lhx6 and Lhx7 expression in the mesenchyme prior to initiation and during tooth formation (Zhang et al, 2005). Additionally, the signaling genes Shh, Bmp2 and Wnt10b have been shown to be expressed early in the odontogenic epithelium, and thus up-regulated in the placodes. Wnt and Shh signaling is involved in the positioning of the tooth placodes where Wnt7b antagonizes the expression of Shh (Nieminen, 2007). Another recently identified pathway which plays a role in tooth development is the TNF pathway, particularly its member Eda (Zhang et al, 2005). The Eda pathway induces the expression of its main downstream target, the NF-κB transcription factor. This pathway acts to determine the size of the tooth during initiation, and contributes to tooth morphogenesis and cusp formation later (Matalova et al, 2008). The TGF-β signaling pathway consists of a large group of extracellular growth factors including BMPs and Activin-βA. Activin-βA is expressed only in the dental mesenchyme. It is expressed during tooth development from initiation to the late bell stage (Huang & Chai, 2010). Ultimately the initiation phase concludes with enamel knot formation which acts as a signaling center that controls cell proliferation and apoptosis, determining cusp number and position, and thus the tooth patterning (Zhang et al, 2005).

1.2.2.2 Morphogenesis and differentiation

Signals from the enamel-knot affect the reciprocal interactions between the mesenchyme and epithelium. Thus it is responsible for the maintenance of morphogenesis of the epithelium. The signal molecules expressed in the enamel knot include: Shh, Bmp-2, Bmp-4, Bmp-7, Fgf-3, Fgf-4, Fgf-9, Fgf-20, Wnt-3, Wnt-10a and Wnt-10b (Thesleff, 2003). Fgf8 is responsible for tooth patterning and positioning. Fgf4, on the other hand, is activated by LEF1 from the Wnt pathway and is responsible for enamel epithelium and dental papilla proliferation. Fgf10 stimulates cell proliferation in the epithelium. However, Fgf3 is expressed and stimulates cell proliferation in the mesenchyme (Cudney & Vieira, 2012). Shh is expressed at the epithelium and is responsible

for activating factors in the mesenchyme, such as the Ptc gene. Shh is also expressed as a downstream of Fgf8 and Fgf9. This is regulated by Bmp4 and is responsible for regulating Bmp2 (Cudney & Vieira, 2012). In addition, Bmp2, Bmp4, and Bmp7 are expressed in high amounts in the epithelium and play a role in cell proliferation, apoptosis, and tooth patterning (Cudney & Vieira, 2012; Zhang et al, 2005).

1.2.2.3 Transcription factors

Transcription factors are DNA-binding proteins that regulate target gene expression. Manipulation of their expression by knockout mice can alter a cell's commitment or change its capacity for differentiation (Nieminen, 2007). To this point, gene knockout mice have arrested tooth development, mostly during the bud stage. One of the main classes of transcription factors that regulate tooth development are the homeobox-containing genes, which include: Pax9, Msx1, -2, Dlx-3, 5, 6, 7, Barx1, Pitx2, Lhx-6, -7 (Zhang et al, 2005). The homeobox genes are expressed in an overlapping pattern. This results in jaw subdivision into different regions and thus specifies each tooth's position (Zhang et al, 2005). In other words, the expression of specific transcription factors during different stages of tooth development are responsible for competence, commitment and signaling (Nieminen, 2007).

Msx1 and Pax9 are two transcription factors that are known to cause hypodontia and play an important role in tooth development. Pax9 interacts with Msx1 to regulate Bmp4 expression in the mesenchyme (Cudney & Vieira, 2012; Zhang et al, 2005). Signals from the epithelium induce Msx1 in the mesenchyme. Msx1 is necessary for the expression of reciprocal signals Bmp4 and Fgf3, as well as for the expression of the Shh receptor Patched and the transcription factors Lef1 and Runx2 (Nieminen, 2007). Msx2 is co-expressed with Msx1 in the mesenchyme. However, part of its expression shifts to the epithelium and is restricted to the enamel knot (Zhang et al, 2005). Pax9 is also induced by epithelial signals and is an early molecular marker of the mesenchyme. It is the upstream regulator of Bmp4, Msx1, and Lef1 in the mesenchyme (Zhang et al, 2005). Pitx2 is another transcription factor involved in tooth development. It is positively regulated by FGF8 and negatively regulated by BMP4 (Nieminen, 2007; Zhang et al, 2005). Brax1 is induced by FGF8 and is expressed only in the molar region, thus it mediates tooth determination. Nevertheless, Brax1 activity could be expanded to the incisors region in case of BMP4 inhibition (Nieminen, 2007). A number of transcription factors were found to be expressed in the thickened presumptive dental epithelium, including Dlx-1 and Dlx-2. This

suggests that they are involved in the determination of the sites of initiation through their classification as downstream target genes for the morphogens and homeobox genes(Ambrish Kaushal 2009).

1.2.2.4 WNT signaling pathway

Wnts are secreted glycoproteins that trigger intracellular signaling pathways by binding to Fz and its co-receptor LRP5 or LRP6 to activate β-Catenin. In the absence of Wnt ligand, β-Catenin is kept at low levels by phosphorylation with the destruction complex— GSK-3, APC, CK1 and Axin complex. When Wnt ligands bind to an Fz receptor and co-receptor Lrp6, it initiates a cascade of events in the plasma membrane that lead to the disassembly of β-catenin-destruction complex. This behavior eventually causes the accumulation and translocation of β-catenin to the nucleus where it forms a complex with LEF/TCF transcription factor that is involved in regulating cell proliferation, stem cell maintenance, or differentiation. This entire process is called Wnt/β-catenin pathway, or canonical pathway. This pathway is inhibited by the DKK1 that binds to LRP/Kremen co-receptor (high-affinity receptors) resulting in the rapid internalization of Kremen-Dkk-LRP complexes and the removal of LRP from the plasma membrane (Cudney & Vieira, 2012; Zhang et al, 2005).

WNT/β-catenin signaling pathway is active during the whole tooth development process.

At the initiation stage the WNT signaling pathway plays a positive role in initiating laminar thickening in the epithelium. During the bud stage, WNT/β-catenin signaling pathway is necessary for proper epithelial-mesenchymal crosstalk. At cap stage, WNT/β-catenin is up-regulated in the knot (Cudney & Vieira, 2012). All the data available on WNT/β-catenin signaling pathway role in tooth development was obtained from several experiments on mice.

At the tooth initiation stage, several Wnt genes, including Wnt4, Wnt6, Wnt10a, and Wnt10b, as well as Lef1, are expressed in the presumptive dental epithelium (Chen et al, 2009). At bud stage, DDK1 is up-regulated by Msx1 and Msx2 (Cudney & Vieira, 2012). β-catenin is required for expression of Lef1 and Fgf3 in the mesenchyme and for the induction of primary enamel knot in the epithelium (Chen et al, 2009). To transfer from bud to cap stage, multiple interactions are required. First, Lef1 is important for reciprocal epithelial- mesenchymal interactions since it induces Fgf4 expression in the knot, which leads to the expression of Fgf3 and Shh in the mesenchyme and in the epithelium, respectively. Wnt1 and Wnt10b are expressed specifically in the epithelium at bud stage, inducing Lef1 in the mandibular mesenchyme (Chen et al, 2009).

Second, Brax1 is an antagonist to Wnt. It is up-regulated by Fgf8 and down-regulated by Bmp4. This antagonistic relationship between Wnt and Brax1 is important to differentiate molars from incisors as well as for the transition from bud to cap stage (Cudney & Vieira, 2012). Third, β-catenin function is required in the developing dental mesenchyme for the transition of tooth morphogenesis from the bud to cap stage. β-catenin is also required for the activation of odontogenic potential in the developing tooth mesenchyme for tooth development beyond the bud stage (Chen et al, 2009).

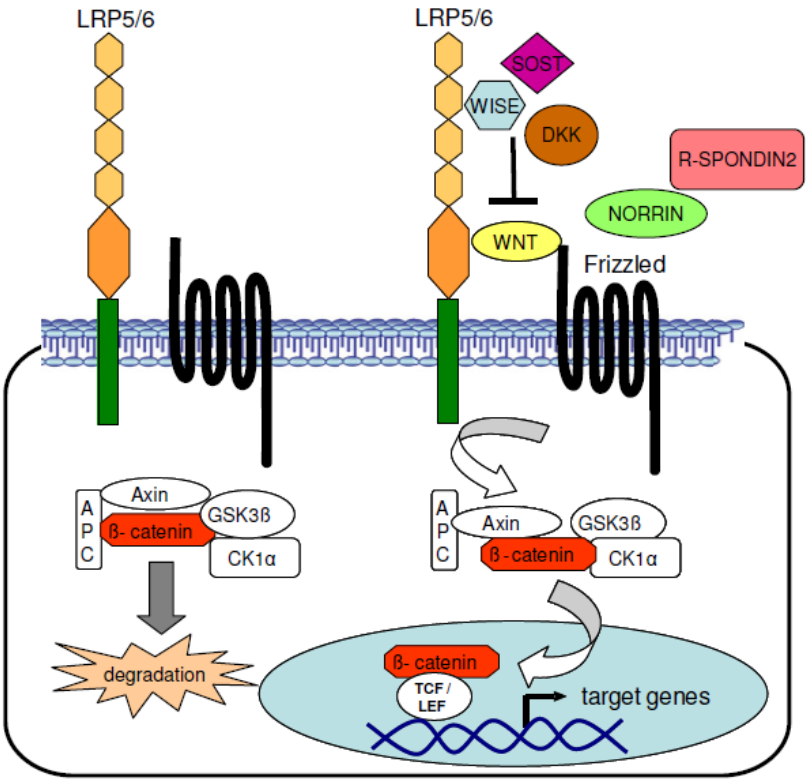


Figure 1.4 WNT/ β-catenin pathway (Med, 2010).

1.3 Genetic causes of hypodontia

Hypodontia occurs in two forms: syndromic and non syndromic:

1. Syndromic in association with other congenital phenotypes as a part of syndrome, or
2. Non syndromic as an isolated phenotype where it may be sporadic or familial which is inherited in an autosomal dominant, autosomal recessive or x-linked mode.

1.3.1 Syndromic hypodontia

More than 60 syndromes that involve hypodontia as part of their phenotypic spectrum are listed on Online Mendelian Inheritance in Man (OMIM) (Chhabra et al, 2014). On the list, ectodermal dysplasia (ED) is a congenital disorder that affects the development of two or more ectodermal structures. This includes the hair, nails, teeth, and sweat glands. It is caused by an X-linked dominant mutation in the ectodysplasin-A (EDA) coding gene (Xq12-q13.1). A mutation in EDAR (2q11-q13) coding for a TNF receptor can cause both autosomal dominant and recessive mode of ED (Chhabra et al, 2014; P. J. De Coster et al, 2009). Recently, a novel homozygous variant of Kremen1 gene was identified. This mutation T>C substitution is predicted to result in an amino acid change (Phe209Ser) (Issa, 2014).

Van Der Woude syndrome (VWS), the most common cleft syndrome, is another syndrome where 70% prevalence of hypodontia has been reported and most of cases are linked to interferon regulatory factor-6 (IRF6) mutation. Hypodontia is also associated with oral-facial-digital syndrome type I (OFD1) that is transmitted as an X-linked dominant disorder with mutations in CXORF5 (Chhabra et al, 2014; P. J. De Coster et al, 2009).

1.3.2 Non-syndromic hypodontia

To date, Msx1 and Pax9 are the only two genes known to cause non-syndromic hypodontia in humans. More recently, Axine2 was found to cause hypodontia in association with colorectal cancer. EDA and LTBP3 genes were also found to cause hypodontia but these cases are less common.

1.3.2.1 Msx1

Msx1 is a transcription factor that is located at chromosome 4p16.3-16.1. Msx1 contains a highly conserved 60 amino acids DNA-binding homeodomain. It plays a key role in tooth development and is expressed in the mesenchyme. Five mutations were identified in the Msx1gene, two of which (M61K, S105X) fall outside the homeodomain in the N-terminal region. The other three (Q187X, R196P and S202X) fall within the homeodomain itself. These mutations include missesnse and nonsense mutations. They are inherited in autosomal dominant and autosomal recessive pattern that affects the second premolars and third molars where they are arrested in the bud stage (Chhabra et al, 2014; Cudney & Vieira, 2012; Pemberton et al, 2005). Another two novel mutations in Msx1 were identified recently at the Hereditary Research Lab/Bethlehem

University. The first is a missense mutation that is predicted to result in an amino acid change p.Pro24Leu. The second mutation (4862095 A>T) is a heterozygous splicing mutation that leads to the loss of 166bp of the sense transcript (Issa, 2014).

1.3.2.2 Pax9

Pax9 is another transcription factor that has an important role at multiple stages of embryogenesis including odontogensis. Pax9 gene is located on chromosome 14q12-q13.It belongs to a nine transcription regulators family that shares 128 amino acid DNA-binding domains (paired box). In teeth development, Pax9 is expressed in the dental mesenchyme and interacts with Msx1 to maintain the Bmp4 expression. This is in order to drive morphogenesis of the dental organ, particularly the transition from bud to cap stage. This is why any loss of function mutations in Pax9 or Msx1 will arrest the development at the bud stage (Chhabra et al, 2014; Kapadia et al, 2007; P. J. De Coster et al, 2009).

Eleven mutations were identified in Pax9 gene and inherited in an autosomal dominant fashion. These mutations cause permanent molars absence and in severe cases the absence of the 2nd premolars and mandibular central incisors. These mutations include missense, nonsense and frameshift mutations (Cudney & Vieira, 2012; Pemberton et al, 2005).

1.3.2.3 Axin2

Two mutations have been found in the Axin2 gene (Lammi et al, 2004). One of the mutations was found in a four-generation Finnish family and the other in an unrelated patient (Lammi et al, 2004). Axin2 gene is localized on 17q23-q24 (Chhabra et al, 2014). Axin2 gene expresses the Axin2 protein that plays as negative regulator of the canonical Wnt pathway by β-catenin degradation (Chhabra et al, 2014; P. J. De Coster et al, 2009). The Arg656Stop nonsense mutation of Axin2 was found in eleven patients with oligodontia of the Finnish family eight of whom also had colorectal cancer. This suggests that there is an association between tooth agenesis and a predisposition for colorectal cancer in a tissue specific manner. The other mutation is Axin2 1994 insG which causes a frame-shift in the gene, and is associated with oligodontia (Lammi et al, 2004).

1.3.2.4 Other mutations

Other less common mutations have also been found. The LTBP3 mutation was identified in a Pakistani family with autosomal recessive oligodontia, and another X-linked mutation was

identified in EDA gene in Chinese families with non-syndromic hypodontia (Chhabra et al, 2014).

1.4 Problem statement and objectives

Teeth are the hardest constituent of the oral cavity and are essential for eating, pronunciation and esthetics (Honda et al, 2008). Missing teeth, particularly in hypodontia have an impact on overall health of patients comprising: functional limitations, impact on emotional well-being as well as social well-being. Additionally, hypodontia results in malocclusion (imperfect positioning of the teeth when the jaws are closed) and masticatory (chewing food) (Kotecha, 2012). However, hypodontia results in more serious changes on the facial appearance as a consequence of growth disturbances of the maxillofacial skeleton. These changes encompass: hypoplasia, maxillary and mandibular retrognathism, anterior rotation of mandible and low anterior face height (Worsaae et al, 2007). Hypodontia restorative treatment varies from no treatment to closing/ opening a space orthodontically with adhesive bridges. In severe cases, a combination of orthodontic, restorative and surgical approaches is needed to prepare for prostheses or implants (McNamara et al, 2006; Meaney et al, 2012).

The prevalence of hypodontia varies widely from 2.6% in Saudi Arabia (Salama & Abdel-Megid, 1994) to11.3% in Ireland (O'Dowling & McNamara, 1990). Hypodontia shows high prevalence in Denmark and Sweden with 7.8% (Rolling, 1980; Rolling & Poulsen, 2009) and 7.4% (Bergstrom, 1977) respectively, while it shows low prevalence in the United States and Malaysia with 3.5% (Muller et al, 1970) and 2.8% (Nik-Hussein, 1989) respectively.

There is no study in Palestine that discusses the prevalence nor the causes of hypodontia (mainly genetic causes) before that of Dr. Yasmin Issa in cooperation with the Hereditary Research Lab/Bethlehem University. Together, they initiated a research that aimed to discover the underlying genetic causes of inherited hypodontia in Palestinian families. In this study, I will continue the work that Dr. Yasmin Issa has initiated.

This study aims to resolve the causative mutation of non-syndromic hypodontia in a Palestinian family (HyF) from the Bethlehem area with an autosomal dominant inheritance.

This study aimed specifically to:

1. Profile for possible mutations in all known genes that cause non-syndromic hypodontia.

2. Perform Whole Exome Sequencing to identify novel mutations of non-syndromic hypodontia.
3. Validate mutation novelty and study its segregation in the affected family.
4. Genotype of 200 healthy controls to exclude any probability of the mutation being a normal SNP in the Palestinian population.

CHAPTER 2

Materials and Methods

2.1. Materials

2.1.1. Buffers, Gels and Solutions Preparation Protocols

A. DNA Extraction by Salting-Out Technique

- **Red Blood Cell Lysis Buffer**
    1. Add 8.28gr of NH4Cl (155mM) plus 0.79gr NH4HCO3 (1mM) to 1L flask.
    2. Then mix with 0.2 ml EDTA (0.1mM, pH 7.4).
    3. Complete the volume to 1L with double distilled water (dd H_2O).
    4. Store at 4^oC.
- **1X Lysis Buffer**
    1. Mix 50ml Tris-HCL (50mM, 7.5pH) with 33.4ml NaCl (100mM).
    2. Then add 2ml EDTA (1mM).
    3. Complete the volume to 1L with dd H_2O.
    4. Store at room temperature.
- **Proteinase K**

  Dissolve Proteinase K in double distilled water to a 5mg/ml final concentration. Then store at 20^oC freezer.

  Agarose Gel Dissolve Ethidium Bromide in ddH2O to final concentration of 1mg/ml.

B. Electrophoresis

- **Ethidium Bromide**
- **50X TAE Buffer**
    1. Mix 242gr Tris base (8.0pH) with 57.1ml Acetic Acid (1M).

2. Then add 100ml EDTA (0.5M, 8.0pH)
3. Complete to 1L with ddH_2O
4. Store at room temperature

C. PCR products running gel

- **5X loading buffer**
  0.25% bromophenol blue with 0.25% Xylene cyanol FF in 30% Glycerol in water.

D. DNA Storage

- 0.02 % Sodium Azide.

E. Ethanol/EDTA Precipitation in the cleaning step
125mM EDTA, 100% Ethyl Alcohol (EtOH), and 70% EtOH are needed for this step.

2.1.2. Reagents, Instruments and Kits

Reagent	Supplier	Product Specifications
Ready Mix PCR	ABgene	Cat# AB-0575-DC-LD
Agarose	Amresco	Cat# : 9012-36-6
Sodium Azide	Sigma-Aldrich	Cat# : S2002
Hi-Di-Formamide	Applied Biosystem	P/N#:4311320
GeneRuler100bp DNA Ladder	Thermo Scientific	Cat # :SM0241

Instrument	Supplier	Product Specifications
Thermal Cycler	Applied Biosystems	Gene Amp PCR Sysytem 9700
Spectrophotometer-DNA Concentration Measurement	NanoDrop Technologies	NanoDrop ND-1000
Automated Sequencing-Electrophoresis	Applied-Biosystems /HITACHI	ABI Prism 3130 DNA sequencing (S/N:20355-023)

Illumina Analyzer	Genome	Illumina	Illumina HiSeq 2000

Kit	Supplier	Product Specifications
PCR Purification Kit	New England BioLabs	Antarctic Phosphates cat# M0289L
Sequencing Kit	Applied Biosystem	The BigDye® Terminator v1.1 Cycle Sequencing Kit Cat# 4337450

2.2. Methods

2.2.1 Family Asserting and Blood Collection

The family HyF spans three generations and shows the hypodontia phenotype inherited in all generations in autosomal dominant mode as displayed in the pedigree in Figure 5. The hypodontia phenotype was first determined by two dentists, and then 5-10ml blood samples were collected from nine members of the family who have participated in this study after obtaining informed consent (in accordance with the guidelines of the Bethlehem University Helsinki Committee) for the DNA extraction.

2.2.2 DNA Isolation by Salting-Out technique

After nine blood samples were collected in a sterile EDTA vacutainer tubes (5-10ml) DNA was extracted from each as follow: Red Blood Cell lysis buffer was added 4 times the volume of the blood and mixed gently, then tubes were kept on ice for approximately 30 minutes till the blood becomes transparent. Tubes then were centrifuged at 2000 rounds per minute for 10 minutes at 4°C and the resulted supernatant was carefully removed and the pellet was re-suspended in 3 ml Red Blood Cell lysis buffer and re-centrifuged at same conditions. The resulted pellet after that was broken and suspended in a mix of 3ml of 1X Lysis buffer, 100ul of 20% *SDS* and 100ul of 5mg/ml Proteinase K , followed by incubation at 37°C overnight. After the incubation period, 1ml of 6M NaCl was added to the lysate and vigorously mixed until the solution get a foamy appearance, then centrifugation was done at 3000 rpm for 20 minutes at room temperature. The clear supernatant (upper phase) then was transferred carefully to a 15ml tube, avoiding the salt protein deposit. Cold 100% ethanol (EtOH) was added twice the volume to the upper phase and

mixed gently by tube inversion till fine threads appear which are the DNA that will be fished using glass Pasteur pipette and washed in 70% EtOH and let it for air dry for few minutes on the Pasteur pipette, DNA then was dissolved in 200-1000ml 0.02% Sodium Azide (depending on the amount of the DNA) and left at room temperature overnight.

2.2.3 Excluding known mutations

Mutations in Msx1, Pax9 and Axin2 are known to be the major cause for autosomal dominant hypodontia, so these two genes were scanned by direct sequencing. Msx1and Pax9 were sequenced using primers that were designed at the Hereditary Research Lab/Bethlehem University. Axin2 primers were described earlier (Larmour et al, 2005) Primers are presented in table 4.2.

Gene	Template	Primer sequences	Size of amplicon
Msx1			
Exon1part1	DNA	F1: GTGCTCCCGGGAACTCTG R1: TGAAGGGCAGGAGCGAAG	401 bp
Exon1part2	DNA	F2: AAGTGTCCCCTTCGCTCCT R2: AGGTCTGGAACCTTCTTCCTG	497 bp
Exon1(complete)	DNA	F1: GTGCTCCCGGGAACTCTG R2: AGGTCTGGAACCTTCTTCCTG	871 bp
Exon2part1	DNA	F1: ACTTGGCGGCACTCAATATC R1: CAGCTCTGCCTCTTGTAGTCTC	350 bp
Exon2part2	DNA	F2: CGCCAAGGCAAAGAGACTAC R2:TGTGAGGGTTAAAGGGAAGG	353 bp
Exon2(complete)	DNA	F1: ACTTGGCGGCACTCAATATC R2:TGTGAGGGTTAAAGGGAAGG	669 bp
Exon1part2+Exon2part1	cDNA	F: AAGTGTCCCCTTCGCTCCT R: CAGCTCTGCCTCTTGTAGTCTC	548 bp
Pax9			
Exon2	DNA	F: GGTGCGGAAAGTTTCTGTCT	283 bp

		R: ATCAACAGCCACCCAGTAGC	
Exon3part1	DNA	F: GGGGACAGCCCCAGTAGTTA R: GCTTGTAGGTCCGGATGTGT	402 bp
Exon3part2	DNA	F: ACACATCCGGACCTACAAGC R: TCCCTGAGGCTGCAGATACT	462 bp
Exon4	DNA	F: GGTCTAAGCCCTCCAGCTCT R: GAAGGATCTGGCTCGTAGCA	385 bp
Exon5	DNA	F: TCAGAGCATTGCTGGCTTAC R: ATGTGAGACCTGGGAATTGG	443 bp
Axin2			
Exon1part1	DNA	F: TGGGTTTTTGGAAGGTTGTG R: GAACAGGTAAGCACCGTCTTG	727 bp
Exon1part2	DNA	F: CATCTCCGGATTCCCCTCT R: TCCACCCATCCACCATACTT	684 bp
Exon2	DNA	F: GCTGCCTCTGGAATACTCTCTG R: TAAGTGCTCAGGTGGCATCC	465 bp
Exon3	DNA	F: AGCACCGATGGTATCTGGAG R: CCACCACCCATTTCTTTTCTT	327 bp
Exon4	DNA	F:GATGGTTGACAACAGTCTTTGAAG R: CTAACGCACCCCATGCAC	549 bp
Exon5	DNA	F: CTTCTGCTTCCTGGGTCACT R: CTGCCGCCCTCTTAGAAACT	673 bp

Exon6	DNA	F: AGGAGTCCCGGAGATTTAACC R: AACAGCCATTCCCACAATACC	330 bp
Exon7	DNA	F: TTCCAGTTCTTCTAACCCAGTTTC R: TTGAGACCCAGGCAGAAAGAG	398 bp
Exon8	DNA	F: AATTGCTCTGGGGACAACAG R: GGACATGGATGGCAACATCT	320 bp
Exon9	DNA	F: GCACGTGTGTGTTTGCTTTAG R: TCTGGCTCTTGGTTCTGAGC	300 bp
Exon10	DNA	F: TCAACAATGTGGAAAATGCAG R: AGAAACCATGAACGCACTCC	684 bp

Table 4.2: Primers used for Msx1, Pasx9 and Axin2 amplification.

2.2.4 Exome Sequencing

Whole exome sequencing was conducted at the Department of Medicine, Division of Medical Genetics, University of Washington, Seattle, WA 98195, USA.

DNA libraries were prepared using the Illumina TruSeq DNA sample preparation protocol (low throughput) and kit (A cat#FC-121-1001, B cat#FC-121-1002). Extracted genomic DNA was broken up into small fragments using the Covaris S2 Ultrasonicator. Fragment's ends were then repaired by adding end Repair Control to each fragmented DNA sample in the IMP plate. Then AMPure XP paramagnetic beads were added to each well of the IMP plate containing End Repaired Mix to clean up IMP. 3'ends were then adenylated by adding A-Tailing Control preparing them for adaptors ligation.

DNA libraries were hybridized to biotinylated DNA oligonucleotide baits from the SeqCap EZ Human Exome Library v.2.0 (Roche NimbleGen). Hybridized DNA was pulled down using capture beads. Unbound fragments were removed by washing. The enriched fragment pool was amplified by PCR and sequenced on a HiSeq 2000 instrument (Illumina) (see Appendix 1).

2.2.5 Mutation Validation by direct Sanger sequencing

2.2.5.1 Polymerase Chain Reaction (PCR)

A 537 bp fragment from LRP6 gene was amplified by PCR using the following primer pairs:

Primer	Sequence 5' ⟶ 3'	Start	Length	Temperature	GC%
Forward	TTGAGATTGGGAGCAAGACA	206	20	59.37	45.00
Reverse	TGAACTCTGCCTGTCAAACAA	736	21	59.47	42.86

These two primers were mixed with the other components of the PCR reaction as follows:

Reagent	Volume/µl
2X PCR ReadyMix (ABgene)	12.5 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Sample DNA	1.0 µl
DDH_2O	10.5 µl

In which, the primer concentration is 10pM and the optimal DNA concentration is 50-100/ reaction, and each fraction is multiplied by the number of samples that we are doing +1 (for a control).

Then touch down 60 program was used for the PCR reaction:

95°	5 min	95^o	30 sec	
95°	30 sec			
60°	30 sec	56^o	30 sec	
72°	1.0 min			
95°	30 sec	72^o	1.0 min	
59°	30 sec	95^o	30 sec	
72°	1.0 min			
95°	30 sec	55^o	30 sec	30 cycles
58°	30 sec			
72°	1.0 min	72^o	1.0 min	
95°	30 sec			
57°	30 sec	72^o	10 min	
72°	1.0 min			
		4^o	10 min	

2.2.5.2 Electrophoresis of PCR products using agarose gels

The concentration of the agarose gel used was 1.5% and this was determined depending on the DNA fragment size that should be visualized on the gel. 2.25g of agarose was dissolved in 150ml of 1X TAE buffer and boiled together then two drops of Ethidium Bromide was added. The agarose gel then was poured into the electrophoresis tray till it becomes solid where 3µl of PCR product were loaded onto the wells and run in 1X TAE running buffer at 100V for 30 minutes. Finally, DNA fragments were visualized and documented using BioRad ultraviolet imaging system.

2.2.5.3 PCR product cleaning

After PCR and before sequencing step we clean the PCR mix from the remaining primers and the free nucleotides using the two enzymes: Exonuclease I and Antaractic Phosphatase, in which, for each 1µl of volume we add 0.25µl Exonuclease I, 0.25µl Antaractic Phosphatase, 1.5µl nuclease free H2O, and 5µl PCR product to get final volume of 7µl, and then put in PCR machine at the following program: 37oC for 30min, 80oC for 20min, and 4^{o}C until using.

2.2.5.4 DNA Sequencing

In the sanger sequencing step, we add for each 1X reaction 0.2µl of BigDye, 1.5µl of 5X buffer, 0.75µl of 64X buffer, 0.2µl of forward or reverse primer, 2.0µl of cleaned PCR product, and completed with 11.05µl of dd H_2O to reach final volume of 16µl, in which the concentration of the PCR product is10ng per 100 bp of the length of PCR fragment. Then the mix was run on ABI 3130 DNA sequencer (Applied Biosystem) at 64X program:

96^{o}C for 1min
96^{o}C for 10sec
50^{o}C for 5sec } 30 cycles
60^{o}C for 4min
4^{o}C for 10min

3.2.5.5 EDTA/Ethanol Precipitation of sequenced PCR

The final step before running the PCR product on the sequencing machine is the cleaning up the PCR products from primers, excess dNTPs, unincorporated dyes and that is done by adding for each 16 µl of sequencing reactions a 5 µl of 125mM EDTA and 100 µl of absolute ethanol and mix them gently, then incubate the reactions at -20° for 30 minutes followed by centrifugation at 3800 RPM for 30 minutes at 4°C. The supernatant was discarded and 60 ul of 70% Ethanol were

added to each reaction, and centrifuged again for 20 minutes. Then the supernatant was discarded again and the reaction wells were inverted on tissue and centrifuged for 1 minute at 500 RPM. Then the samples were placed on 95^{o}C hot plate to dry them for 5 minutes and finally 10 ul of Hi-Di were added, and the samples were dried again at 95°C for 2 minutes and they were placed on ice for 5 minutes so they become ready to be loaded on the 96 –well Optical Reaction Plate and run on sequencing machine.

CHAPTER 3

Results

3.1 Clinical Diagnosis and Description

Family HyF from Bethlehem was orally examined and confirmed to have hypodontia by the dentist Yasmin Issa. Family pedigree was constructed through the information that was obtained from the family interview. The pedigree shown in Figure 4.1, demonstrates the inheritance of hypodontia phenotype through three generations in autosomal dominant pattern. Clinically, the family displays non-syndromic hypodontia with generally missing several permanent incisors and premolars with spacing and large diastema too. Missing teeth are ranging from 1-13teeth per individual, as shown in table 4.1. Moreover, intraoral photographs with panoramic X-rays for II-5, III-1, III-2 and III-3 members in figure 4.2 illustrated detailed description for each member phenotype.

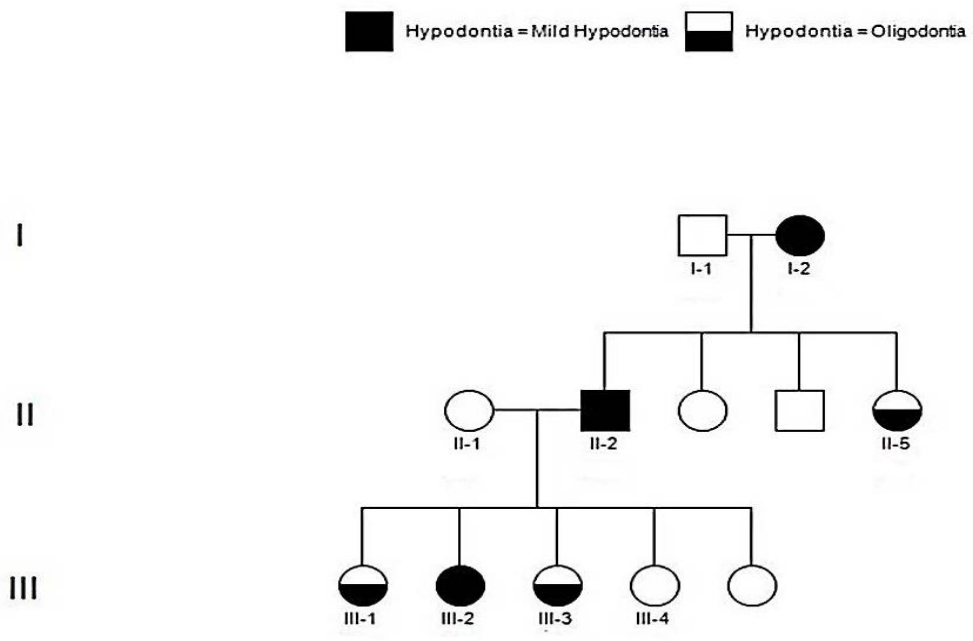


Figure 4.1: Hypodontia phenotype appears in the three generation of HyF family in autosomal dominant pattern with variable expression among individuals.

Individual	Gender	Age	Number of Missing Teeth in each jaw	Total Number of Missing Teeth	Jaw	Right Side 8 7 6 5 4 3 2 1	Left Side 1 2 3 4 5 6 7 8
I-2	F	56y	0 1	1	Max Man	* 	
II-2	M	36y	Unkown 2	At least 2	Max Man	 *	 *
II-5	F	15y	8 At least 1	9	Max Man	* * * * 	* * * * *
III-1	F	12y	2 4	6	Max Man	* * *	* * *
III-2	F	11y	2 2 with some premolars	at least 4	Max Man	* * *	* * *

III-3	F	6y	7 6	13	Max * * * * Man * * *	* * * * * *

Table 4.1: Phenotypes of the affected members of HyF family. Missing teeth are indicated with an asterisk. In which: (1)central incisor; (2)lateral incisor; (3)canine; (4)first premolar; (5)second premolar; (6)first molar; (7)second molar; (8)third molar (wisdom tooth). Max: maxilla: Man: mandible.

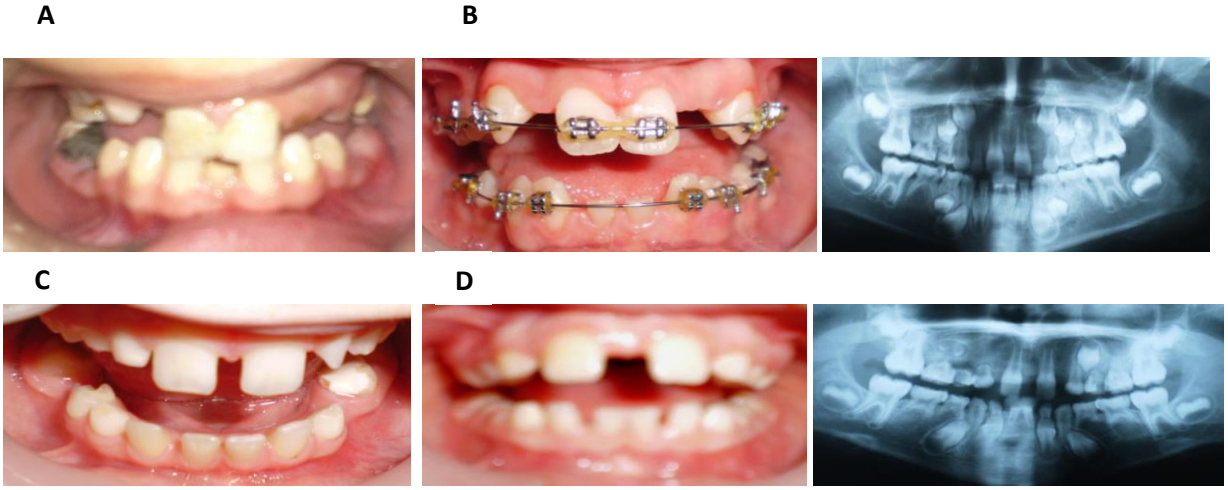


Figure 4.2: An intraoral photographs with panoramic X-rays. A)II-5 member with permanent teeth missing including: upper premolars, canines, lateral incisors, at least one lower central incisor with underdeveloped skeleton. B) III-1 member missing at least 6 permanent teeth: upper lateral incisors, lower central incisors and second premolars plus missing primary left upper lateral incisor.C) III-2 member missing permanent teeth include: upper lateral incisors, lower central incisors and some premolars (exact number not known because of X-ray absence) also missing primary upper left lateral incisor with large spacings, square-shaped upper permanent central incisors. D) III-3 member missing at least 11 permanent teeth: upper lateral incisors, lower central incisors, lower first and second premolars, upper left first and second premolars, upper right canine plus missing primary upper lateral incisors.

3.3 Exome sequencing results

Targeted exome sequencing was applied for 6 affected and one unaffected individuals in HyF family. Data revealed one shared variant among all the affected. This variant turned to be G>C substitution mutation in exon 9 of the Low-density lipoprotein receptor-related protein 6 (LRP6) gene, which was read in almost half of the reads (half of the alleles were mutated and the other half was wild type) in the exome sequencing data. This substitution mutation in one base pair resulted in conversion of the Arginine codon at amino acid 675 of third YWTD domain to a codon of the amino acid Glycine (LRP6_R675G, chr12: 12,317,236 G>C).

LRP6 gene maps to human chromosome 12p11-p13. This gene encodes the transmembrane protein LRP6 protein which is a member of the LDLR family. It is composed of four epidermal growth factors (EGF) and three LDLR repeats in the extracellular domain, in addition to proline-

rich motifs (PPPSPxS) at the cytoplasmic domain (Brown et al, 1998). Figure 4.3 shows the LRP6 protein structure and where the mutation is.

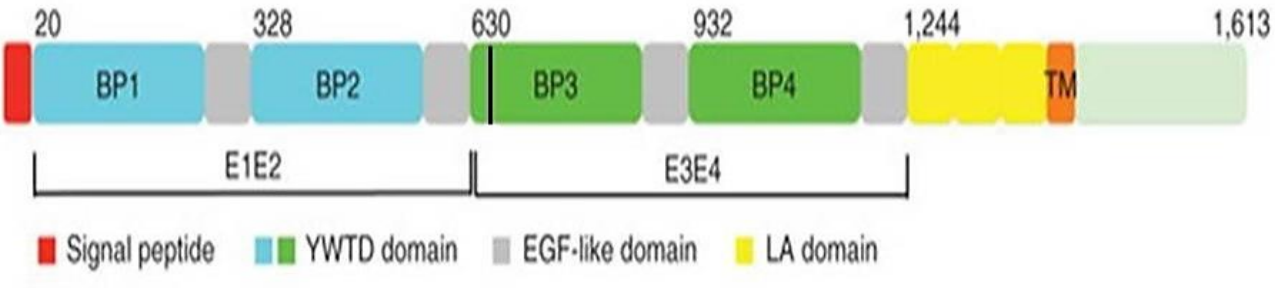
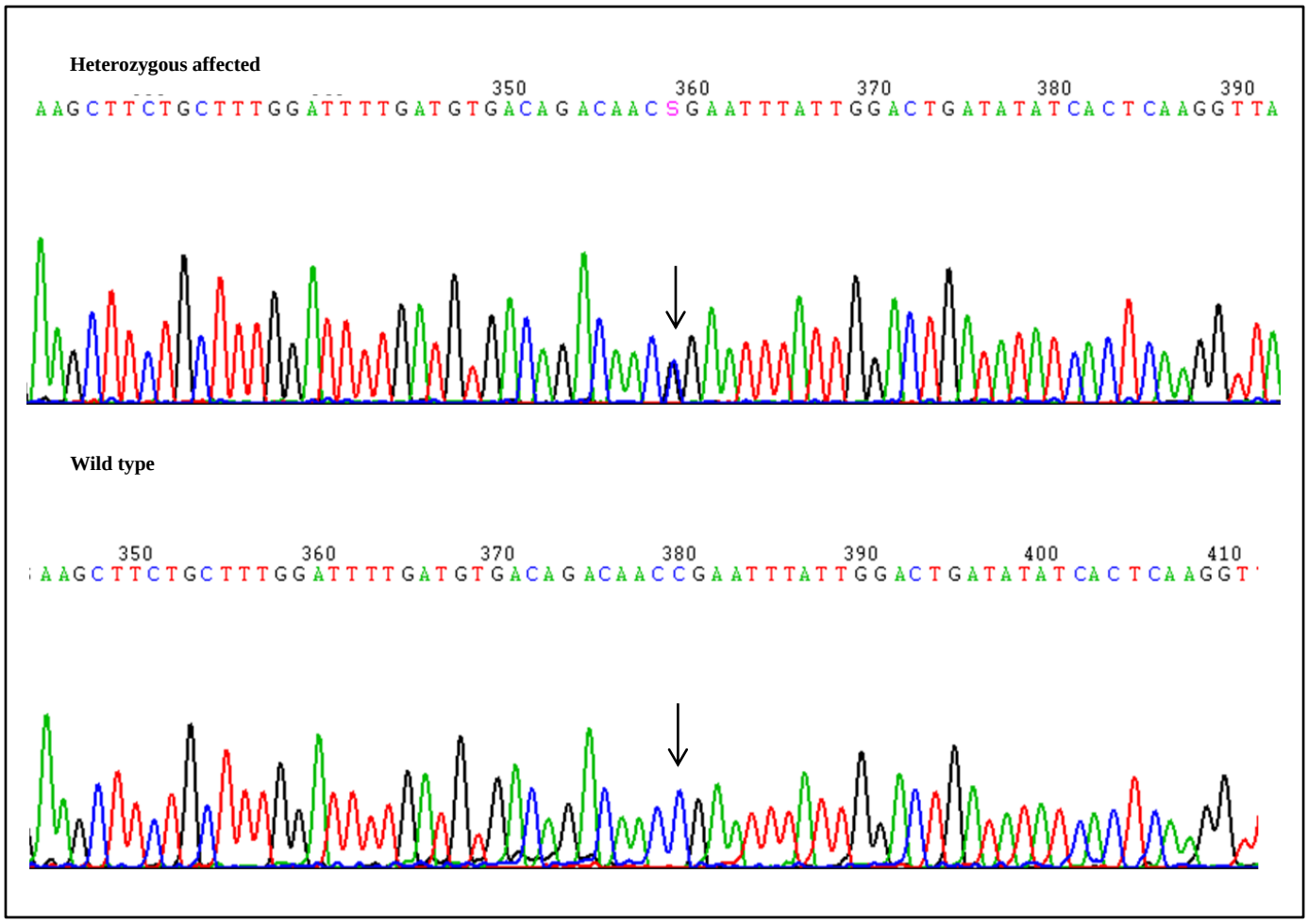


Figure 4.3 LRP6 protein structure where it composed of 4 EGF-like domains and 3 LDLR type A repeats in addition to single transmembrane region and cytoplasmic domain (Cheng et al, 2011).. The black bar shows the site (675) of the mutation at the third YWTD domain.

3.4 Validation of mutation by Sanger sequencing

Sanger sequencing for both affected and unaffected individuals of the family in addition to 200 controls was performed to firstly see mutation segregation, secondly to validate the mutation and thirdly to ascertain mutation absence in the controls. Sanger sequencing has revealed that LRP6 (G>C) mutation segregate perfectly in an autosomal dominant manner in hypodontia HyF family, Figure 4.4B. Additionally, all affected individuals were heterozygous for the mutation while the unaffected individuals were wild type for the mutation. Moreover, all the 200 controls were wild type or in another word no mutation was present. Figure 4.3A shows Sanger sequencing results for affected and unaffected sequences.

A


Heterozygous affected
350
360
370
380
390
AAGCTTCTGCTTTGGATTTTGATGTGACAGACAACSGAATTTATTGGACTGATATATCACTCAAGGTTA
Wild type
350
360
370
380
390
400
410
AAGCTTCTGCTTTGGATTTTGATGTGACAGACAACCGAATTTATTGGACTGATATATCACTCAAGGT

B

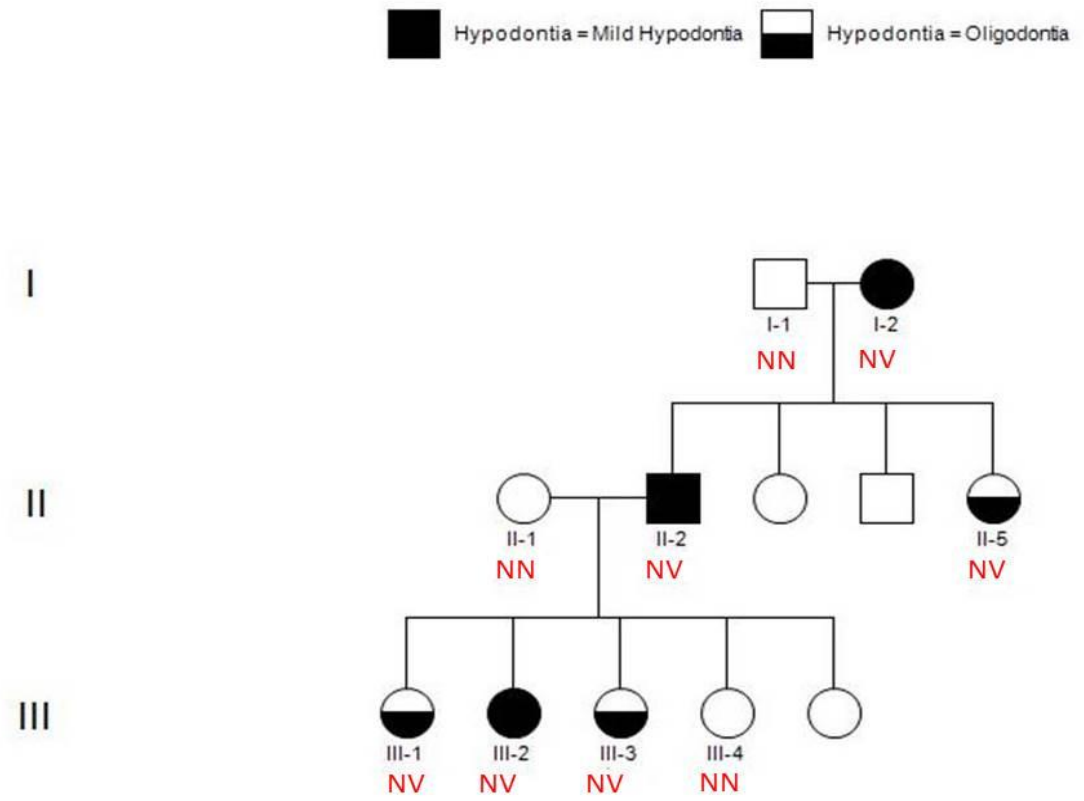


Figure 4.4: A) Sanger sequences for heterozygous affedted individual and wild type for unaffected along with health control. The upper arrow indicates the site of the mutated nucleotide and the lower indicates the wild type. B) Perfect segregation of the genotype with the phenotype. Where NN is the unaffected wild type and NV is heterozygous affected.

3.5 Bioinformatic analysis of results

The results have revealed that the hypodontia phenotype resulted from a single base pair substitution mutation that converts the codon of Arginine to the codon of Glycine. When we investigated this amino acid codon by using the UCSC genome browser, we found that it is completely conserved among the species from human to zebra fish as illustrated in Figure 4.4. Consequently, any changes in this amino acid will probably lead to damage in the structure and function of the protein. To predict the possible impact of R675G substitutions on the stability and function of LRP6 we used polyphen-2 tool and this mutation is predicted to be probably damaging with a score of 0.986 (sensitivity: 0.74; specificity: 0.96). This damage could be due to the differences between the two amino acids. In which, arginine is a polar, positively charge and prefers to be on the protein surface while glycine is small in size and it is polar but not charged amino acid (Betts & Russell, 2003).

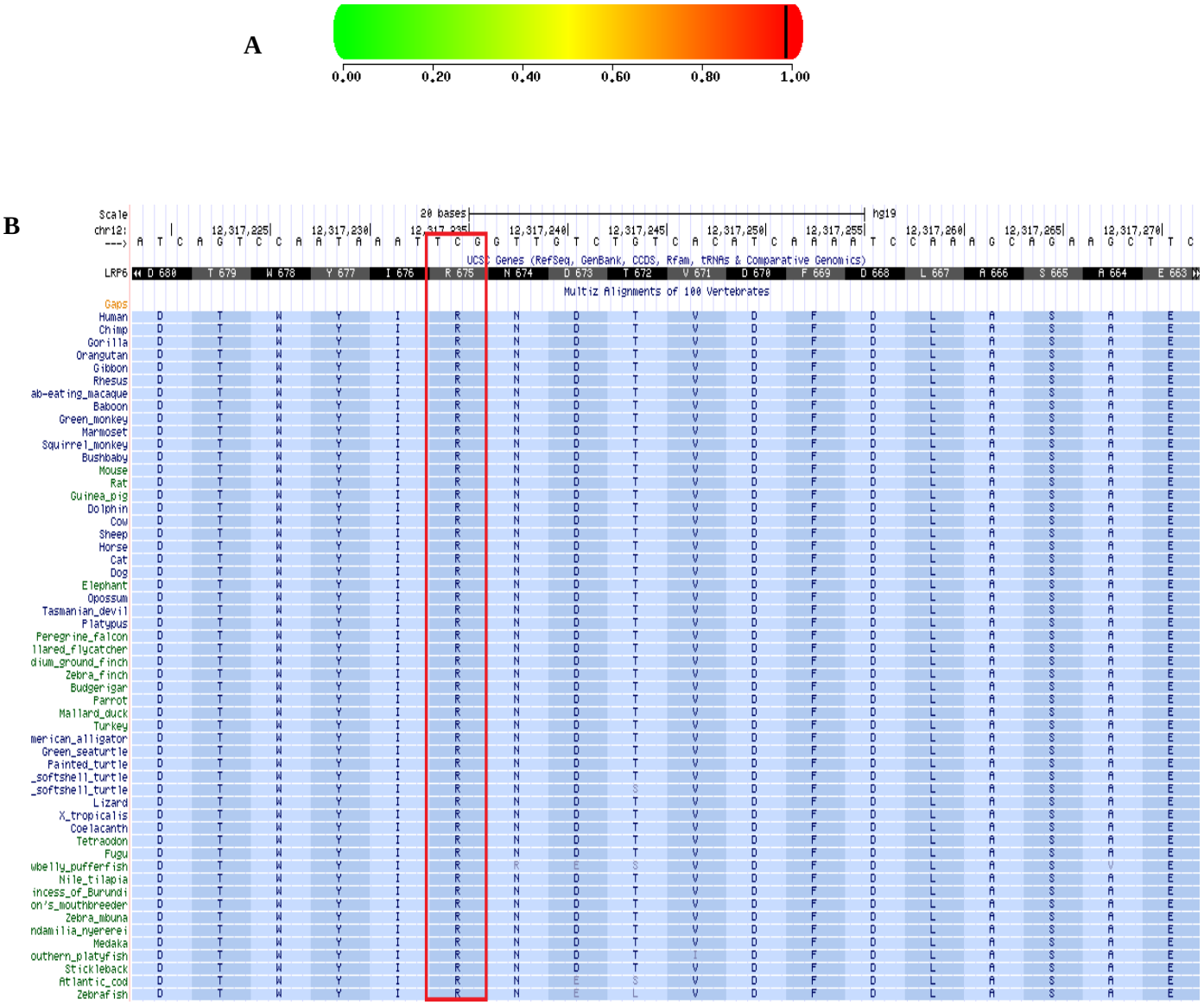


Figure 4.5 Result analyses by bioinformatic tools. A) The black bar at the red region indicates the mutation is damaging B) multiple alignments of LRP6 protein for variable organisms from human to zebra fish showing the conservation of the mutant site (R675G).

Chapter 4

Discussion

In this study, we have identified a novel mutation in LRP6 gene using exome sequencing. The mutation was found in a Palestinian family which inherited non-syndromic hypodontia in an autosomal dominant pattern. Then, by using Sanger sequencing the mutation was confirmed and

found to be perfectly segregated with the non-syndromic hypodontia phenotype. This substitution mutation G>C in exon 9 of LRP6 gene is predicted to cause conversion from Arginine amino acid to Glycine amino acid at the site of 675 of third YWTD domain of LRP6 protein and it was also predicted to be a deleterious mutation.

LRP6 plays a critical role in Wnt/β-catenin pathway. LRP6 is a co-receptor that binds to Fz receptor at the plasma membrane to mediate Wnt signaling. This results in β-catenin activation and translocation to the nucleus where it complexes with LEF/TCT family members to mediate transcriptional activation of target genes (Med, 2010). LRP6 plays a dual role in Wnt/β-catenin pathway. On one hand, Wnt binds to LRP6 which leads to pathway activation by LRP6 phosphorylation. LRP6 phosphorylation begins primarily with PPPSP motif phosphorylation by Gsk3. This induces the subsequent phosphorylation of xS in the PPPSPxS motif by CK1. Both phosphorylation events provide a docking site for axin to complex with the phosphorylated Lrp6. This finally leads to β-catenin dephosphorylation and activation of the signaling (Zeng et al, 2008). On the other hand, LRP6 inhibits Wnt/β-catenin pathway by binding to DDKs. Dkk1 and Dkk2 bind in high affinity to the last two YWTD–epidermal growth factor (EGF) repeat domains of LRP6. This binding disrupts the Wnt-induced Fz–LRP6 complex formation and induces LRP6 endocytosis in the presence of its coreceptor Kremen (Cruciat & Niehrs, 2013).

LRP6 dual role in WNT signaling pathway implies its significance in this pathway. Thus LRP6 protein disruption will change its structure and consequently affecting its ability to bind to its ligand WNT protein or antagonist DDK. This results in cessation of Wnt/β-catenin pathway.

In humans, only one gene involved in Wnt/β-catenin pathway was found to cause hypodontia. This gene as mentioned earlier is Axin2 (Lammi et al, 2004). However, in mice, knocking out Wnt/β-catenin pathway components has shown tooth agenesis disorders. For instance, LEF1 knockout mouse results in teeth missing. These teeth were arrested at the bud stage (Matalova et al, 2008; Thesleff et al, 1995). LEF1 is a target gene for Wnt/β-catenin pathway and it is an essential regulatory molecule for tooth development (Thesleff et al, 1995). Inactivation of β-catenin or epithelial expression of Dkk1 will arrest tooth developmental at the early bud stage (Chen et al, 2009). Additionally, homozygous mutation for LRP6 in knock out mouse exhibited absence of the third molars and small/or unerupted lower incisors (MGI, 2014).

The LRP6 mutation that was identified in this research is inherited in an autosomal dominant fashion. This means that the phenotype is expressed even if the gene is heterozygous. Haploinsufficiency is the proposed mechanism responsible for the dominant phenotype. The hypodontia affected family displays missing permanent incisors and premolars with spacing and large diastema. This could be explained by the "odontogenic homeobox code" concept. This means that a combination of homeobox genes expressed in a particular region of the jaw will determine teeth class and patterning (Cobourne, 1999). In other words, tooth agenesis occurs as a consequence of a qualitatively or quantitatively impaired function of genetic networks, which regulate tooth development. This results in signaling reduction or impaired signal regulation, thus affecting cell proliferation, migration, commitment and differentiation. All these together will affect the "tooth forming potential" (Nieminen, 2007). Based on the above, LRP6 protein as a coreceptor in WNT signaling pathway might has more important role in incisors and premolars development. However, I-2 and II-2 individuals have developed premolars. This could be explained by the functional redundancy between the two receptors LRP6 and LRP5 due to the extensive similarities between them structurally and biochemical properties. Since they share 73% sequence identity in their extracellular domain and 64% sequence identity in their intracellular domain (Goel et al, 2012). Further, the variable expression of hypodontia phenotype among the three generations could be referred to the modifying factors containing the molecular, environment, genetic background, environmental factors and antisense transcriptional regulation (Issa, 2014)

In conclusion, the novel mutation R675G in LRP6 that resulted in non-syndromic hypodontia could contribute to the deep cognition of the teeth development as well as epithelial-mesenchymal interaction. However, developing assays such as gene knockdown functional assays using mouse models are required to test the consequences of the missense mutation of LRP6 on protein function and how it changes it and additionally studying the 3D structure by using bioinformatic tools to understand the mutation impact on LRP6 structure.

Chapter 5

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Chapter 6

Appendix 1

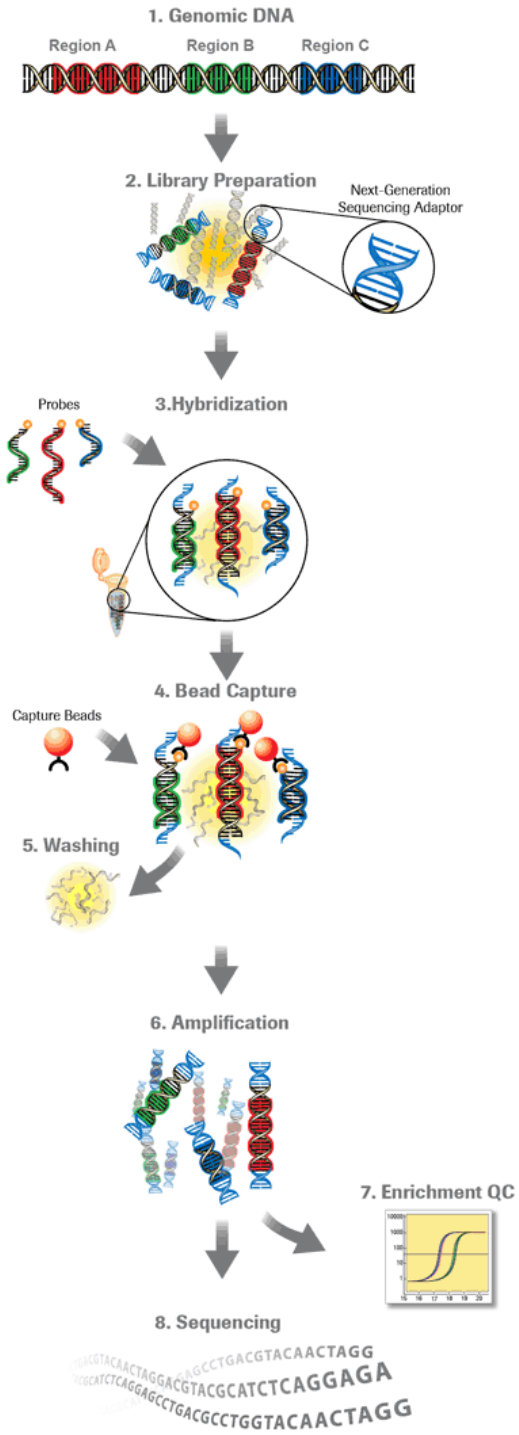

Figure 5: Whole exome sequencing protocol:
Standard shot-gun sequencing library is made from genomic DNA. Then the sequencing library is hybridized to the Oligo pool that was previously made against target regions in the genome.
Capture beads are used to pull down the complex of capture oligos and genomic DNA fragments. Unbound fragments are removed by washing. Enriched fragment pool is amplified by PCR.
The success of enrichment is measured by qPCR at control loci. Finally, the end product is a sequencing library enriched for target regions, ready for high throughput sequencing (NimbleGen, 2014).