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Novel Mutations in Msx1 and Kremen1 are Responsible for Non-syndromic and Syndromic Hypodontia in the Palestinian Population

By

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"Novel Mutations in Msx1 and Kremen1 are Responsible for Non-syndromic and Syndromic Hypodontia in the Palestinian Population"

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"Novel Mutations in Msx1 and Kremen1 are Responsible for Non-syndromic and Syndromic Hypodontia in the Palestinian Population"

by Yasmin A. Issa, BDS

ABSTRACT

Background: Hypodontia is the most common developmental anomaly in humans. It occurs as a consequence to disruption of genes involved in the signaling pathways of tooth development. Mutations in candidate genes such as Msx1, Pax9 and Axin2 have been associated with isolated tooth agenesis. Syndromic hypodontia involves numerous syndromes where hypodontia is part of the clinical spectrum of anomalies. The aim of this study was to investigate the genetic determinants of syndromic and non-syndromic tooth agenesis in five Palestinian families.

Methods: Five Palestinian families displaying variable forms of hypodontia were recruited for blood sampling. A combination of homozygosity mapping, whole exome sequencing and Sanger sequencing were used to identify the causative mutations. The identified mutations were validated by testing 200 normal controls. Functional validation using reverse transcriptase PCR was conducted where applicable on selected tissues.

Results: Direct sequencing identified two novel heterozygous mutations in the coding sequence of Msx1 gene to segregate with non-syndromic autosomal dominant hypodontia in one family. The first is a missense mutation (c.306 C>T, p.P24L). The second is a loss-of-function splicing mutation (c.704 A>T) that has been functionally shown to produce aberrant splicing in adult oral periosteal tissue, with a shift in the reading frame and a premature stop codon yielding a truncated Msx1 sense transcript. Whole exome sequencing revealed that a novel homozygous variant of Kremen1 gene (c.679 T>C, p.F209S) is responsible for autosomal recessive syndromic oligodontia alongside findings of ectodermal dysplasia in four families. Two hundred Palestinian controls were negative for the Msx1 mutations and gave a heterozygosity value of 1/200 for the Kremen1 variant.

Conclusion: Our results expand the genetic spectrum of syndromic and non-syndromic tooth agenesis with the introduction of Kremen1 as a novel candidate gene for a distinct form of ectodermal dysplasia.

Keywords: tooth agenesis, hypodontia, oligodontia, ectodermal dysplasia, Msx1, Kremen1

"طفرات جديدة في جين Msx1 وجين Kremen1 تسبّب نقص عدد الأسنان التلازمي وغير التلازمي في المجتمع الفلسطيني"

د. ياسمين علي عيسى

مُلخّص

خلفية: يعتبرنقص عدد الأسنان من أكثر التشوهات الخَلْقية شيوعاً في الإنسان حيث يحدث نتيجة اختلال في الجينات التي تعمل ضمن المسارات الباعثة للإشارات خلال تخلُّق الأسنان. وقد ارتبطت الطفرات في الجينات المرشّحة Msx1، Pax9 و Axin2 بغياب الأسنان المنعزل. قد يكون نقص الأسنان مرتبطاً بمتلازمة ويشكّل عارضاً من الطيف السريري للتشوهات المصطحبة لها. الهدف من هذه الدراسة هو استقصاء المحدّدات الجينية لغياب الأسنان التلازمي وغير التلازمي في خمس عائلات فلسطينية.

الطرق المستخدمة: سُحبت عيّنات دم من خمس عائلات فلسطينية تعاني أشكالاً مختلفة من النقص في عدد الأسنان. تمّ استخدام مجموعة من الوسائل من أجل الكشف عن الطفرات المسبّبة مثل Homozygosity Mapping إضافة إلى Whole Exome Sequencing و Sanger Sequencing. تم التحقّق من صحة الطفرات عن طريق فحص 200 شخص ضابط. وقد تم التحقّق من الطفرات وظيفياً على أنسجة مختارة حسب الحاجة باستخدام تقنية Reverse Transcriptase PCR.

النتائج: باستخدام Direct Sequencing تم تحديد طفرتين جديدتين مختلفتي الزيجوت في التسلسل الترميزي للجين Msx1 في إحدى العائلات، حيث فُصلت مع صفة نقص الأسنان الجسمية الذاتية القاهرة. الطفرة الأولى مغلطة (c.306 C>T) من المتوقع أن تؤدي إلى استبدال الحمض الأميني p.P24L. الطفرة الثانية طفرة ربط (c.704 A>T) قد تبيّن وظيفياً أنها تسبب تحريكاً في إطار القراءة وكودون توقّف مبكّر يؤدي إلى نسخة مبتورة من ال RNA الخاص ب Msx1 في النسيج الفموي السمحاقي.

باستخدام تقنية Whole Exome Sequencing تم تحديد طفرة نادرة متماثلة الزيجوت (c.679 T>C, p.F209S) في الجين Kremen1 مسؤولة عن التوريث الجسمي الذاتي المتنحي لنقص الأسنان التلازمي المصاحب لعسر التصنّع الأديمي، حيث ثبتت مسؤوليتها عن التشوهات الأديمية في أربع عائلات. غابت طفرتا Msx1 عن 200 فرد ضابط فلسطيني في حين كان 1/200 من الأفراد الضابطة مختلف الزيجوت لطفرة Kremen1.

الاستنتاجات: إنّ نتائجنا توسّع الطيف الجيني لنقص عدد الأسنان التلازمي و غير التلازمي وتلقي ضوءاً على دور جين Kremen1 باعتباره مرشّحاً جديداً لصنف محدّد من عسر التصنّع الأديمي.

الكلمات الدالّة: نقص الأسنان الوراثي، عسر التصنّع الأديمي، جين Msx1، جين Kremen1

DECLARATION

I declare that the Master Thesis entitled "Novel Mutations in Msx1 and Kremen1 are Responsible for Non-syndromic and Syndromic Hypodontia in the Palestinian Population " 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.

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Dedication

I dedicate this thesis to my husband, daughter and parents. You are my source of inspiration. Thank you for putting up with me throughout these years and I apologize for all the inconvenience that I caused. You watched over me and I hope I did not disappoint you.

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Finally, this thesis would not have been possible without the cooperation of the patients and their families.

Abbreviations

Abl	Abelson
AER	Apical ectodermal ridge
APC	Adenomatous polyposis coli
AS	Antisense
Axin2	Axis inhibition 2
Barx1	BarH-Like homeobox 1
BMP	Bone morphogenetic protein
Cbfa1	Core-binding factor A1
Cdk4	Cyclin-dependent kinase 4
CDS	Coding sequence
CUB	Complement proteins C1r/C1s, uEGF and Bmp1 domain
Disp1	Dispatched1
DKK1, 2	Dickkopf 1, 2
Dlx1, 2	Distal-less 1, 2
DMSO	Dimethyl sulfoxide
EDA	Ectodysplasin A
EDAR	Ectodysplasin A receptor
EDARADD	EDAR-associated death domain
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
Fgfr1, r2b	Fibroblast growth factor receptor1, receptor2b
FRZb	Frizzled related protein
Fz	Frizzled
Gli1, 2, 3	Glioma associated 1, 2, 3
Gsc	Goosecoid
GSK3	Glycogen synthase kinase 3
Hox	Homeobox gene
IKK	IκB kinase
Kremen1, 2 (Krm)	Kringle-coding marking the eye and the nose 1, 2

Lef1	Lymphoid enhancer-binding factor 1
Lhx6, 7	LIM homeobox 6, 7
LRP5, 6	Low density lipoprotein receptor-related protein 5,6
MSX1, 2	Muscle segment homeobox homolog 1, 2
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation
NAT	Natural antisense transcript
NF-KB	Nuclear factor kappa B
NFKBIA	Nuclear Factor of Kappa Light Chain Gene Enhancer in B Cells Inhibitor, Alpha
NGS	Next generation sequencing
Pax3, 9	Paired box 3, 9
PCR	Polymerase chain reaction
PIAS1	Protein inhibitor of activated STAT 1
Pitx1, 2	Paired-like homeodomain transcription factor 1
PRC2	Polycomb repressive complex 2
Ptc	Patched
RUNX2	Runt-related, 2
sFRP	Secreted frizzled related protein
SHH	Sonic hedgehog
SNP	Single nucleotide polymorphism
SDS	Sodium dodecyl sulfate
TAE	Tris base, acetic acid and EDTA buffer
TGFβ	Transforming growth factor beta
TNF	Tumor necrosis factor
WIF	Wnt-inhibitory factor
Wnt	Wingless-related integration
WSC	Cell wall integrity and stress response component, domain

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CHAPTER 1

Introduction

1.1 Hypodontia: Definition, Developmental Value and Classification

The normal human dentition comprises 20 primary and 32 permanent teeth (Appendix 1). Dental agenesis is the developmental lack of teeth that can occur to the primary or permanent dentition. It is often described as *hypodontia* when up to 6 teeth are missing or *oligodontia* when more than 6 permanent teeth are absent excluding the third molars. Hypodontia, as its name implies, is commonly used as a broader term denoting the condition of having fewer teeth than normal. It is considered the most common craniofacial developmental anomaly in humans with arguments that there is an evolutionary trend leading to its increased prevalence in the recent decades (Mattheeuws et al., 2004).

Teeth are considered an attractive developmental model system for several reasons. The dental pattern and morphology are species-unique and the alterations of tooth types and their organization are indicative of adaptations linked to exploitation of new feeding niches. Despite that, there is a considerable morphological variation within each species that has been used by dental anthropologists to clarify population history (Irish and Guatelli-Steinberg, 2003). Teeth develop in a similar manner to other ectodermal organs like hair, feathers and mammary glands. Only two embryonic cell types interact during odontogenic development, providing valuable information of positional information, reciprocal tissue interactions and cell commitment (Weiss et al., 1998). The embryonic tooth primordia can be further cultured in vitro which enables experimental manipulation and investigation to be carried out. The disruption of the signaling genetic pathways during development causes abnormalities in tooth number, size and shape and the causative mutations allow for elucidation of the specific roles of ligands and receptors (Thesleff et al., 1995).

Although hypodontia can be the result of environmental factors influencing dental development such as infection or radiation, its causes are genetic in the majority of cases with two main forms. The first one is non-syndromic because it presents as the only familial developmental abnormality. The affected individuals generally inherit the disorder in an

autosomal dominant manner but tend to display variable penetrance and expressivity in addition to a complex phenotype in terms of the number and location of the missing teeth. Genetic linkage analysis of affected families and gene knockout experiments in mice identified developmental transcription regulatory genes to be responsible for the non-syndromic phenotype. Examples of candidate genes include Msx1 and Pax9. In addition to that, mutations in Axin2 gene have been found responsible for non-syndromic autosomal dominant oligodontia and predisposition to colorectal cancer (Lammi et al., 2004; Mostowska et al., 2006a). The second form of hypodontia is syndromic and is generally an autosomal recessive disorder, but can be also autosomal dominant and X-linked. It comprises many syndromes where tooth agenesis in affected individuals is part of their clinical spectrum of anomalies. On the molecular level, it is a reflection of mutations that occur in genes involved in the signaling pathways of multiple developmental processes involving dental and other tissues. Cleft lip and palate patients, for example, have a higher prevalence of tooth agenesis. Ectodermal dysplasia is a syndrome characterized by abnormal ectodermal structures including hair, skin, sweat glands, nails and teeth (De Coster et al., 2009).

1.2 Problem Statement and Objectives

The consequences of dental agenesis vary depending on the number and location of missing teeth and are sometimes associated with the presence of other dental anomalies including microdontia, taurodontism and rotation in addition to ectopic and delayed eruption of the remaining teeth. Masticatory and speech problems, in addition to loss of function and esthetics influence the psychological development of the growing patient. Oligodontia is often associated with disturbances in the facial appearance as well as abnormalities in the maxillofacial skeleton such as maxillary and mandibular retrognathism, lower anterior facial height and reduced thickness and height of the alveolar process. Occlusal disturbances like deep bite, cross bite, attrition and steep inclination of the maxillary incisors accompany the altered dentoalveolar dimensions. The remaining teeth may be smaller in size or different in shape than normal teeth (Worsaae et al., 2007). The management of these problems often involves a combination of orthodontic, prosthetic and surgical treatment approaches including the insertion of dental implants which presents a financial burden to the family and requires regular dental follow-up of the patients through their puberty and adulthood years.

The molecular investigation of the causes of dental agenesis is advantageous on many levels. Oral clefts and syndromic dental agenesis can serve as suitable genetic models that will allow for the localization and quantification of the effects of specific gene mutations on teeth formation. Therefore, the mutational investigation of affected families, whether non-syndromic or syndromic, will further our understanding of dental and craniofacial development.

The prevalence of dental agenesis has been addressed in many studies. The incidence of missing permanent teeth in the Asian and European population varies from 2.6% in Saudi Arabia (Salama and Abdel-Megid, 1994) to 11.3% in Ireland (O'Dowling and McNamara, 1990) and Germany (Behr et al., 2011). The prevalence of hypodontia in the primary dentition is lower and ranges from 0.5% in Icelandic children (Magnusson, 1984) to 2.4% in Japanese children (Yonezu et al., 1997). In the Jordanian population, the prevalence of hypodontia in the permanent dentition is reported to be 5.5% in which the lower second premolar is the most commonly missing tooth excluding the third molars (Albashaireh and Khader, 2006). More than 80% of permanent tooth agenesis cases present with one or two missing teeth, whereas only less than 1% present with oligodontia, missing 6 or more teeth (Larmour et al., 2005).

Although the majority of studies on hypodontia in the countries neighboring to Palestine are concerned with the prevalence and description of various affected groups, some mutational analyses have been conducted on affected families in which genotype-phenotype correlations have been drawn. A missense mutation of the Eda gene has been found in a Jordanian family with X-linked hypohidrotic ectodermal dysplasia (Khabour et al., 2010). Syndromic hypodontia has been also reported in Arab Muslim families residing in Israel (Spiegel et al., 2010; Zlotogora, 2014).

Still, the molecular investigation of dental agenesis is a theme that is understudied in the Middle Eastern countries. No studies, so far, have tackled the clinical manifestation and the inheritance of this anomaly in affected families in Palestine. In this study, five Palestinian families from the south of Hebron displaying various forms of oligodontia are investigated. Despite the shared lack of teeth between the families, they vary in their phenotypic

manifestations, suggesting different underlying mutational etiologies that affect downstream odontogenic developmental processes.

The main goal of this study is to identify the causative mutations that lead to the development of oligodontia in five Palestinian families and to deduce their developmental consequences.

More specifically the study aims to:

1. Screen genes previously associated with hypodontia
2. Combine homozygosity mapping, exome sequencing and/or candidate gene sequencing to identify novel genetic determinants of syndromic and non-syndromic hypodontia
3. Investigate the functional consequences of detected mutations if applicable
4. Relate the molecular presentation of the probands to their clinical picture
5. Provide consultancy to affected families regarding consanguineous marriages

CHAPTER 2

Literature Review of Tooth Genesis/Agenesis

2.1 Evolutionary Origins

Teeth are considered complex structures composed of two hard tissues (enamel and dentin) that envelop the innervated and vascularized pulp cavity (Carlson, 2013). They are thought to have originated as electro-sensory pores on early chordates that were later modified by mineralization into exoskeletal scales with feeding and defensive functions (Smith and Hall, 1990). Denticles have been found in fossils from over a half billion years ago. The evolution of teeth is linked to the appearance of the neural crest; a feature only found in vertebrates. Vertebrates share a great similarity in the events that take place in early tooth development and vary in the organization and function of the dentitions (Weiss et al., 1998).

The human ancestral tooth formula comprises three primary incisors, one primary canine and four primary post-canine teeth developing in each jaw quadrant. These are replaced by successional teeth. Three or more molars develop posterior to those teeth and have no primary predecessors. In modern man the formula is modified; two primary incisors, a primary canine and two primary postcanine teeth (primary molars) are replaced with two permanent incisors, a permanent canine and two permanent premolars. Posterior to those, three permanent molars develop without deciduous predecessors (Weiss et al., 1998) (see Appendix1).

2.2 Models of Dental Development

The most recently adapted dental developmental model is proposed by Mitsiadis and Smith to integrate three earlier different models that explain dental patterning (Mitsiadis and Smith, 2006).

The first model is the morphogenetic field model. It reasons that the teeth of higher vertebrates are multiple classes, e.g. incisors, molars, premolars, and are organized into morphogenetic fields corresponding to each class. According to Butler, teeth are not individual organs but rather a whole system that is influenced by two effects: a ‘meristic’ effect and a ‘field’ effect. The first one affects the spacing and number of teeth and shows graded differences between teeth of a particular class due to the role of their position. The

order of teeth development, their differentiation and end form are the consequence of substances and signals that comprise the 'field' (Butler, 1995).

Secondly, the clone model proposed by Osborn, suggests that all teeth of a particular class arise from a single clone of pre-programmed cells according to their positional identity in the neural crest. As those cells migrate, tooth buds are formed surrounded by zones that inhibit the development of other teeth before the migrating clone advances and so different tooth families arise from the clones of stem cells (Osborn, 1973).

Another model is the odontogenic homeobox code model that shows a combinatorial role of both theories in which the ectoderm-derived neural crest cells migrating to the first branchial arch create signals that induce specific overlapping spatial domains of homeobox gene expression in the ectomesenchyme. As a result, different morphogenetic fields and dental patterns are established (McCollum and Sharpe, 2001). The code explains how the four different classes: incisors, canines, premolars and molars result from homeobox gene expression domains and how tooth number, size, shape and differentiation can be modified if certain signaling molecules are modulated (Plikus et al., 2005).

A single model of dental patterning unifies the afore mentioned models by encompassing the signaling molecules generated by the dental epithelium, the clones of the migrating neural crest cells and homeobox gene expression of the mesenchymal cells, thus respecting the multifactorial etiology of dental development (Townsend et al., 2009).

2.3 Overview of Tooth Development: Mice versus Humans

The development of teeth is under strict genetic control in terms of the position, number, size, and shape. Over 300 genes are involved in dental development (Thesleff, 2006). It is multidimensional and takes place in the three spacial dimensions, the *x*, *y*, *z* axes and the fourth dimension of time (Brook, 2009). Interactions of the signaling pathways between the embryonic ectodermal and neural crest-derived mesenchymal-cell layers occur over a long period that contains critical developmental stages. For this purpose, animal models have been used revealing common developmental events.

Odontogenesis has been studied extensively in mice. The migration of murine neural crest cells occurs around embryonic day 8.5 (E8.5). Tooth type and position determination occurs at E10.5 (Fig. 2.1A). The dental placode forms from epithelial thickening and polarization at

E11.5. The bud stage at E12.5 and E13.5 describes the proliferation and invagination of the thickened dental epithelium into the underlying mesenchyme. This is accompanied by the change of the basal layer cells from cuboidal to columnar. The enamel knot is a transient cluster of non-dividing epithelial cells that serves as a signaling center that controls tooth cusp patterning around the cap stage at E14.5. In the bell stage, the tissues start to look more like a tooth with the continuous differentiation of the epithelially derived ameloblasts and the mesenchymally derived odontoblasts. The sum of all those tissues is known as the tooth germ. The mesenchyme surrounding the tooth germs differentiates into bone (Fig. 2.1A).

Although mice provide informative developmental models, they differ from the human dentition. Mice have a single dentition whereas humans shed their primary teeth to give rise to a permanent dentition. Moreover, unlike the modern human formula, mice develop one incisor and three molars in each quadrant. In spite of that, rudimentary tooth buds are found during murine embryogenesis in the region that lacks teeth between the incisor and molars (Tureckova et al., 1995).

Initiation of dental genesis in humans occurs as early as the sixth embryonic week. Epithelial thickening forms invaginations that form dental placodes in the upper and lower jaws. This is followed a week later by the formation of 10 buds of the primary teeth in each jaw. All primary teeth erupt by the first postnatal 29 months. The first primary teeth to develop are the central upper and lower incisors. The buds of the permanent teeth develop in the eighth to tenth week from superficial buds in the dental epithelium lingually and distally to the germs of the primary teeth. They pause in the bell stage in the eleventh to twelfth embryonic week and resume development after primary teeth are replaced (Peters and Balling, 1999). All permanent crowns (except third molars) are completed between 5 and 7 years with root development taking 6 to 7 years. The last permanent teeth (excluding third molars) erupt at the age of 12 or 13. The third molars develop late and erupt around 21 years with large variations (Pirinen and Thesleff, 1995). Eruption timeline can be seen in Appendix 1.

2.4 Epithelio-mesenchymal Interactions during Tooth Development

The epithelio-mesenchymal interactions take place between tissues of odontogenic potential and responding tissues of odontogenic competence (Fig.2.1B). The dental epithelium possesses the odontogenic potential during pre-bud dental development at E11.5 in mice, i.e. can induce gene expression and tooth formation in the mesenchyme. Only the mesenchyme that is derived from the cranial neural crest is of odontogenic competence and can respond to odontogenic signals and develop into dental tissues. Dental epithelium has been combined with non-neural crest derived mesenchyme such as limb mesenchyme and demonstrated failure of tooth formation. At the bud stage, the odontogenic potential is switched to the mesenchyme which is potentially capable of inducing both dental and non-dental epithelium to form tooth structures. Around E14.5, the signaling epithelial cells within the enamel knot take over the odontogenesis. Thus, the epithelium and mesenchyme shift roles in their potential to dominate tooth development. The precise nature of the molecules in charge of transferring the induction potential between those two compartments is poorly understood (Lumsden, 1988; Mina and Kollar, 1987; Kollar and Baird, 1970).

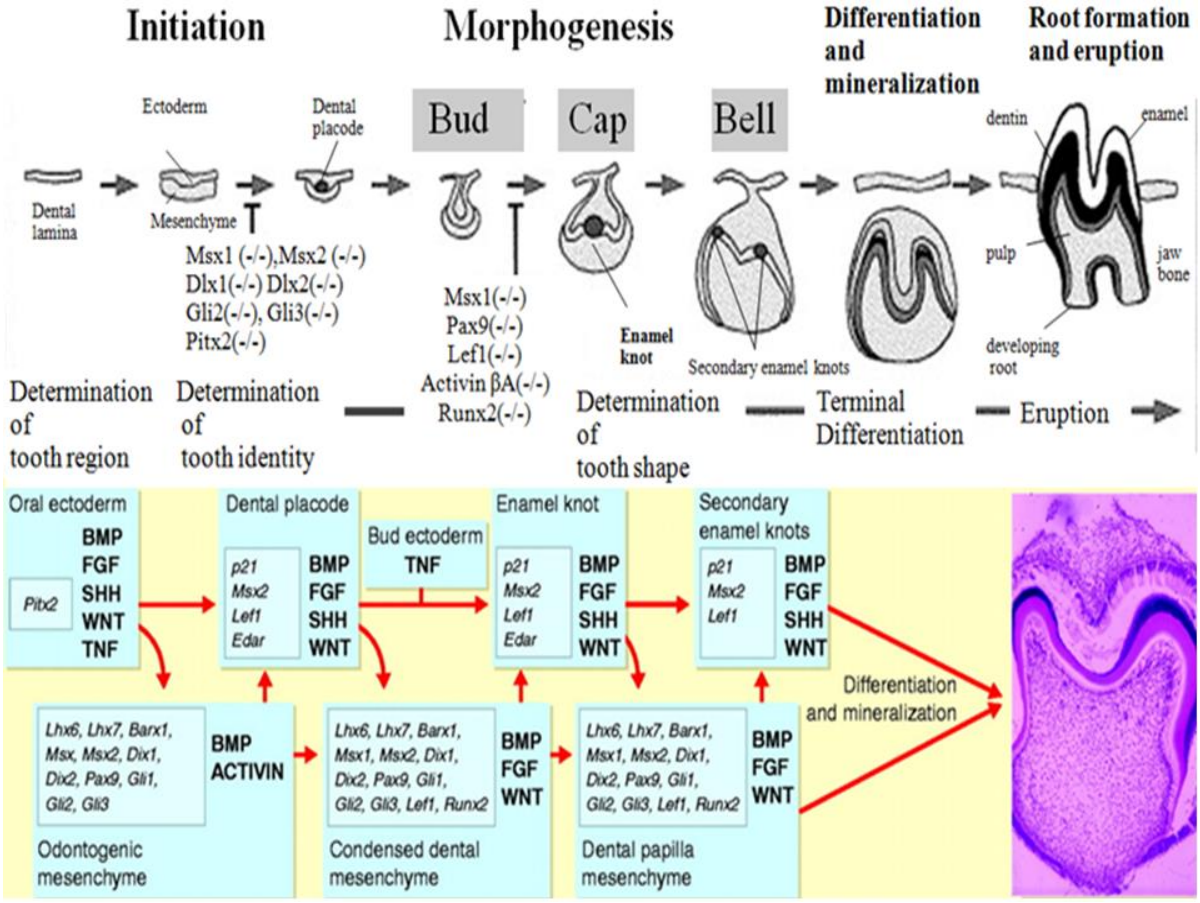


Fig.2.1: Reciprocal signaling between epithelium and mesenchyme during odontogenesis. **Upper panel:** The morphological stages and knockout mouse experiments that arrest development at the corresponding stage.

Lower panel: The signaling pathways (in bold) and the transcription factors in each stage. The upper and lower boxes represent ectodermal and mesenchymal interactions, respectively (Jernvall and Thesleff, 2000).

2.5 Signaling Pathways of Odontogenesis

Signaling molecules belong to different families of growth factors and play key roles in the odontogenesis. Complex processes of interactions take place on the extracellular level between them and receptors as they reciprocate between the epithelium and the mesenchyme (Fig.2.1B). Intracellularly, signaling molecules play a sigificant role in the regulation of key transcription factors such as LEF1, PITX2, MSX1 and PAX9. The signals belong to families of growth factors such as the epidermal growth factor (EGF) family, the fibroblast growth factor family (FGF), the hedgehog (Hh) family, the wingless (Wnt) family and the transforming growth factor β (TGFβ) family which includes the bone morphogenetic proteins (BMPs) (Thesleff, 2000).

2.5.1 Bone Morphogenetic Proteins

BMPs belong to the Transforming Growth Factor β superfamily of cytokines and have been identified as osteo-inductive active components of bone. Several Bmp genes are expressed during tooth development.

BMP4 plays a critical role around E10 in the dental epithelium by restricting Pax9 expression to the mesenchyme and Pitx2 expression to the dental epithelium (Fig. 2.1B). It is also expressed in the distal region of the mandible. This expression on the one hand causes restriction of mesenchymal Barx1 expression to the molar anlage which is essential for molar tooth type specification. On the other hand, it induces Islet1 expression in the future incisor mesenchyme. Other Bmp genes are expressed in the dental epithelium at E11.5 such as Bmp2 and Bmp7. Their products in addition to other epithelial growth factors such as FGF8, FGF9, WNT10a and WNT10b are responsible for inducing the expression of genes in the underlying mesenchyme that include Msx1, Msx2, Lef1, Dlx1, Dlx2, Patched (Ptc), Gli1, and Syndecan-1 (Zhang et al., 2005).

In the following bud stage the mesenchyme gains an odontogenic potential as it starts expressing genes that encode signaling molecules like Bmp4 and Fgf3 (Zhang et al., 2005). In addition to giving feedback signals to the epithelium, those growth factors also regulate tooth development in the mesenchyme. Epithelial BMP4 provides a positive feedback loop that maintains mesenchymal Msx1 and Bmp4 expression. To maintain this cycle,

mesenchymal MSX1 interacts physically with PAX9. Therefore it is considered an amplifier of the BMP4 signal as it shifts Bmp4 expression from the epithelium to the mesenchyme. Loss of expression of either Msx1 or Pax9 will break this cycle and arrest the tooth in the bud stage (De Coster et al., 2009).

BMPs play a role during tooth morphogenesis as well. Differentiated odontoblasts express Bmp2 and Bmp4 in addition to Tgfβs 1-3 which are potential inducers of ameloblast differentiation (Vaahtokari et al., 1991). The expression of Bmp4, Bmp2 and Bmp7 in the cap stage is responsible for the apoptosis in the enamel knot cells (Jernvall et al., 1998).

2.5.2 Fibroblast Growth Factors

The fibroblast growth factors are heparin binding proteins that are involved in growth and development of cells from different developmental origins. The expression of these signals is often redundant. Fgf8 and Fgf9 are expressed in the primitive oral epithelium and dental epithelium up to the start of the bud stage and play an important role in the initiation of tooth development. Fgf4 and Fgf8 are expressed only in the epithelium and are upregulated in the following cap stage where they mediate cuspal and coronal morphology. LEF1 from the Wnt pathway activates Fgf4, which is expressed only in the knot, causing proliferation of enamel epithelium and dental papilla (Zhang et al., 2005).

FGF signaling induces mesenchymal cell proliferation and the mesenchymal expression of Pax9, Pitx1, and Pitx2. Epithelial FGF8 induces Barx1 in the molar mesenchyme. It also induces Lhx6 and Lhx7 expression before and during tooth formation. Lhx6/7 expression activates mesenchymal homeobox genes and maintains expression of Msx1 in molar patterning. In addition to that, epithelial FGF8 induces mesenchymal Fgf3 expression in an Msx1-dependant manner. Therefore FGF8 is often considered a part of the epithelial odontogenic potential because mice that lack epithelial FGF8 fail to develop most first branchial arch structures except incisors which are rescued by the presence of FGF9. On the other hand, functional redundancy in tooth development is proposed for FGF3 (expressed only from the mesenchyme) and FGF10 (expressed only from the epithelium); mice deficient in either Fgf3 or Fgf10 do not exhibit major tooth defects (Zhang et al., 2005; Cudney and Vieira, 2012).

FGFs signal to receptors that are differentially expressed by mesenchymal and epithelial cells. The elimination of FGFR2b, which is a tyrosine kinase receptor for both FGF3 and FGF10, causes arrest of tooth development at the bud stage (Zhang et al., 2005).

2.5.3 Sonic Hedgehog

Sonic hedghehog (SHH) signaling is required for the development of the early tooth germ. SHH expressed in the odontogenic epithelium stimulates its proliferation and regulates its invagination to form an epithelial tooth bud. It is also expressed by the enamel knot and induces mesenchymal signals and FGFs both in the epithelium and the mesenchyme (Cobourne et al., 2001).

SHH acts as a negative-feedback regulator of Wnt signaling during the bud-to-cap transition (Ahn et al., 2010). The three Gli genes encode the transcription effectors of SHH signaling. The Gli2 and Gli3 genes show redundancy in tooth development because single knockout mice exhibit almost normal tooth phenotype. However, double mutants show an arrest of tooth development before the bud stage (Zhang et al., 2005).

Dispatched1 (Disp1) regulates the transduction of SHH signals. Hypomorphic mutants of this gene have a reduced SHH signal and missing maxillary incisors, which is a finding also associated with human holoprosencephaly (HPE) that results from reduced SHH signaling (Zhang et al., 2005).

2.5.4 The Wingless-Int

2.5.4.1 Wnt Signaling General Overview and Role in Odontogenesis

Wnt signals in vertebrates represent a large variety of molecules involved in the patterning, proliferation and differentiation of different tissues and organs. (Cadigan and Nusse, 1997). They play a role in increasing bone mass through renewal of stem cells, stimulation of pre-osteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis (Nakamura and Matsumoto, 2008).

The Wnt ligand is a secreted glycoprotein that is rendered hydrophobic as a result of fatty acylation and can bind to two types of receptors: the N-terminal extra-cellular cysteine-rich domain of the Frizzled (Fz), and the low-density lipoprotein (LDL) receptor known as LRP5/6. Binding of Wnt to any of those receptors forms a functional complex that

transduces the signal to the cytoplasmic phosphoprotein Dishevelled (Dsh/Dvl). This is capable of activating three pathways: the canonical/ β-catenin pathway, the planar cell polarity pathway which recruits small GTPases of the rho/cdc42 family to activate Jun kinase (JNK) and the Wnt/Ca2+ cascade (Komiya and Habas, 2008).

Canonical Wnt signals play an important role in odontogenesis. In addition to their involvement in hair and mammary placode development, they are strong candidates for an ectodermal placode initiator (Sarkar and Sharpe, 1999). In the canonical pathway, the Wnt protein binds to a complex of receptors. Those include both a member of the frizzled family of seven-transmembrane receptors (encoded by 10 genes in humans) and either LRP5 or LRP6. As a result, the intracellular cytoplasmic tail of LRP5 or LRP6 is phosphorylated, creating a binding site for the AXIN protein. This inhibits the activity of GSK3 kinase, yielding increased levels of cytoplasmic and nuclear β-catenin (He et al., 2004). Consequently, the formation of active transcription complexes between β-catenin and LEF1 is triggered in the nucleus, which activates the expression of target genes. When there is no Wnt, cytosolic β-catenin is phosphorylated by an AXIN/GSK3/APC complex and targeted for rapid degradation by the proteasome (He et al., 2004).

Concerted Wnt signaling, along with BMP and FGF signals from the dental epithelium to the mesenchyme are crucial during early tooth development. Bmp4 functions downstream of Wnt activation to promote Msx gene expression, rather than acting synergistically with Wnt (Chen et al., 2009; Liu et al., 2008). During odontogenesis, Wnt genes 4, 6, 10a and 10b create signals that are necessary for dental lamina to bud transition. Wnt signals induce Eda expression, which promotes epithelial placode formation (Laurikkala et al., 2001).
Epithelium-specific inactivation of β-catenin or epithelial expression of Dkk1, an inhibitor of canonical Wnt signaling, causes arrest at the early bud stage. Also β-catenin function has been shown to play a role in the developing dental mesenchyme for the transition of tooth morphogenesis from the bud to cap stage (Chen et al., 2009).
At E12.5, reduction in Wnt signaling leads to reduced Msx1 and Msx2 expression in dental mesenchymal cells as well as reduced SHH expression, whereas Pax9 and Pitx2 are regulated independently of Wnt signaling. Furthermore, Eda expression in the dental epithelium is independent of Wnt/β-catenin activity at E12.5 and E13.5. β-catenin function

is required for the transition of tooth morphogenesis from the bud to cap stage (Liu et al., 2008). Lef1 expression in the epithelium at the bud stage triggers epithelial Fgf4 to generate an inductive signal to the mesenchyme by triggering mesenchymal Fgf expression. This in turn, causes SHH expression in the future enamel knot. Blocking of Wnt co-receptors as well as the knocking out of Lef1 causes absence of all teeth. Lef1 null mutant mice lack Eda expression in the early dental epithelium (Kratochwil et al., 1996; Laurikkala et al., 2001).

2.5.4.2 Regulation of Wnt Signaling

An intricate regulatory mechanism is involved in the induction/limitation of Wnt pathway signaling. Wnt inhibitors antagonize Wnt signaling by preventing ligand–receptor interactions or Wnt receptor maturation. In contrast, Wnt activators promote Wnt signaling by binding to Wnt receptors or releasing a Wnt-inhibitory step (Cruciat and Niehrs, 2013).

A number of secreted proteins in the extracellular matrix have been found to antagonize Wnt signaling by binding to either Fz or LRP5/6 and thus limiting the Wnt signal-gradient. (Bejsovec, 2005; Gordon and Nusse, 2006). Members of the secreted Frizzled-related protein (sFrp) family can compete with Frizzled receptors for binding of secreted Wnt (Moon et al., 1997).

DKK1 and DKK2 inhibit Wnt signaling by binding to LRP5/6 and the KREMENS (KRM1/KRM2) (Mao et al., 2002). Expression of the secreted inhibitor DKK1 has been shown to block signaling in epithelial and underlying mesenchymal cells in mouse embryos (Liu et al., 2008). Examples of other inhibitors include Wnt-inhibitor protein (WIF), FRZb, WISE and Sclerostin.

On the other side, factors such as NORRIN and R-SPONDIN2 are capable of binding to the LRP5/6 receptor to activate Wnt signaling independent of a Wnt ligand (Komiya and Habas, 2008).

Axin2 gene is expressed in the dental mesenchyme, odontoblasts and enamel knots. It codes for the Wnt receptor AXIN2, which functions as an intracellular antagonist and negative feedback regulator of Wnt signaling by inducing intracellular β-catenin degradation (Lammi et al., 2004; Swinnen et al., 2008).

2.5.4.3 KREMEN1 is the Gatekeeper of Wnt Signaling

KREMENS (KRM1 and KRM2) are shown to promote Wnt inhibition. They have a high affinity for Dickkopf1 (DKK1) and form a ternary complex with LRP6, therefore blocking

Wnt/β-catenin signaling by inducing rapid endocytosis of LRP6 from the plasma membrane (Nakamura et al., 2001) (Fig. 2.2). Along with DKK1, they play an important regulatory role in the AP patterning of the central nervous system through their functional cooperation in Wnt inhibition in *Xenopus* (Davidson et al., 2002). Moreover, Kremen1 expression plays a role in Wnt inhibition associated with the formation of thymic architecture (Osada et al., 2006).

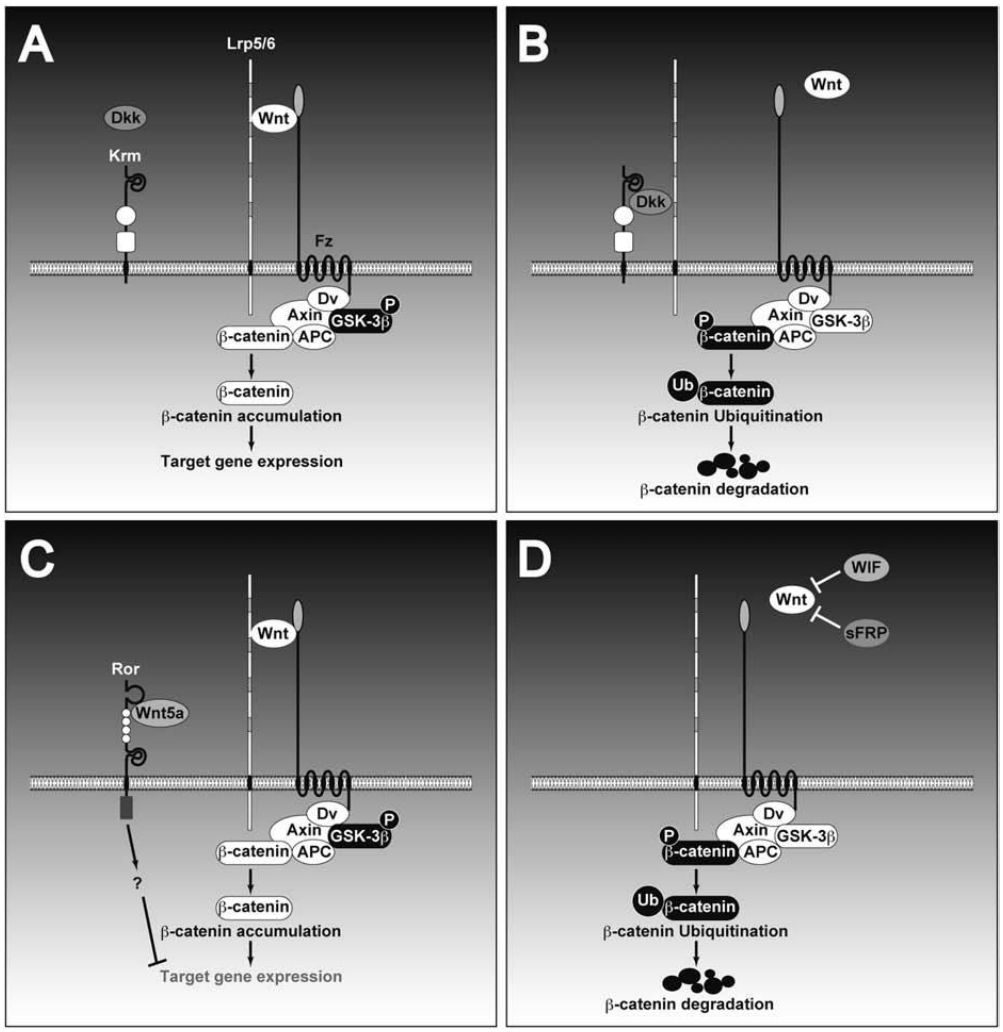


Fig. 2.2: Regulation of Wnt/ β-catenin signaling pathway. **(A)** Ternary complex of Wnt, Fz and LRP5/6, promotes stabilization of β-catenin and activation of the pathway. **(B)** Ternary complex of DKK1, LRP5/6 and KRM blocks Wnt signal transduction by causing endocytosis of LRP5/6. **(C)** Ror is the receptor for Wnt5a and blocks Wnt/ β-catenin signaling. **(D)** WIF and sFRP antagonize the binding of Wnt to Fz (Nakamura and Matsumoto, 2008).

KREMEN1 or KRM1 protein is a type-I transmembrane protein that plays a role in the inhibition of Wnt signals. It is composed of an extracellular region of 372 amino acids and a cytoplasmic region of 60 amino acids. In addition to those, it contains two hydrophobic stretches: the N-terminal signal sequence (amino acids 1-20) and the transmembranous

domain (amino acids 393-413), respectively. The extracellular region of KRM contains a kringle domain (amino acids 31-114), a WSC domain (amino acids 116-210) and a CUB domain (amino acids 214-321) (Fig. 2.3). The CUB domain was originally identified in the embryonic sea urchin protein Uegf, and bone morphogenetic protein-1. It has also been identified in proteins that regulate development such as tolloid (a dorso-ventral patterning molecule), Bp10 and Span (blastula specific proteins) and neurophilin. The WSC domain was originally identified in WSC1/SLG1 yeast protein which plays a role in cell wall integrity and stress response. The kringle domain is a triple-disulfide-linked domain and is found in other important proteins such as tissue plasminogen activator tPA, hepatocyte growth factor and Ror transmembrane receptor tyrosine kinase. The intracellular region of KRM on the other side, is not homologous to other proteins and does not have a specific motif involved in signal transduction (Nakamura and Matsumoto, 2008; The Uniprot Consortium, 2014).

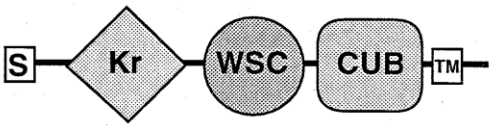


Fig.2.3: Schematic representation of the modular organization of KREMEN. S: signal peptide; Kr: kringle domain; WSC: WSC domain; CUB: CUB domain; TM: transmembrane domain (Nakamura et al., 2001).

In murine studies, Kremen expression was found in adult skeletal muscles, lung and brain. Its expression level is also increased during embryonic development. Whole mount in situ hybridization in embryonic mouse tissue at day 10.5 showed localized Kremen mRNA expression in the apical ectodermal ridge (AER) of limb buds, telencephalon, and the first branchial arch in addition to the myotome and sensory tissues such as nasal pit, optic vesicle, and otic vesicle. AER signaling regulates mesenchymal cell numbers and the number of forming digit condensations. Krm loss causes increased Wnt3/β-catenin signaling and AER expansion, giving rise to polydactily phenotypes. In contrast, when AER and mesenchymal mass are reduced, fewer digits develop (Nakamura et al., 2001).

It has been proven that KREMENS are not universally required for DKK1 function, but rather this interaction is physiologically relevant only in certain tissues. Mice knockout experiments imply functional redundancy of the KREMENS. Single Krm mutant mice display normal bone formation and mass, compared to double mutants that show increased bone volume and bone formation parameters emphasizing KREMENS' role in the negative

regulation of bone formation. On the other hand, Dkk1 mutants display embryonic lethality, head truncations, and vertebral and limb defects. In addition to that, triple mutant Krm1$^{-/-}$ Krm2$^{-/-}$ Dkk1$^{+/-}$ mice show enhanced growth of ectopic digits which proves the interaction of the mutated genes during limb development in negative regulation of bone formation (Ellwanger et al., 2008). Effective DKK1-mediated Wnt antagonism can be accomplished independent of KREMENS by preventing Wnt-LRP6 interaction and disrupting the Wnt-induced Fz8–LRP6 complex (Semenov et al., 2001). In another study, KREMENS play a role in facilitating DKK-mediated antagonism when the level of LRP5/6 is high (Wang et al., 2008). The colipase fold in DKK1 can interact with LRP6 and is sufficient for KREMEN binding (Mao and Niehrs, 2003).

On the other side, recent evidence suggests a context-dependent role for KREMENS in Wnt-β-catenin signaling. In the absence of DKK1, KREMENS were shown to stimulate Wnt-β-catenin-mediated transcription by binding to LRP6 and enhancing LRP6 protein levels at the plasma membrane. Still, it is not known whether this occurs through canonical or noncanonical Wnt signaling. In the presence of DKK1, Krm1 overexpression sensitized cultured mammalian cells to DKK1 mediated inhibition of Wnt signaling (Hassler et al., 2007). In view of that, a model for the action of KREMENS was proposed. Assuming a uniform concentration of Wnt ligands and in the presence of both KREMENS and quantities of DKK1 above a threshold concentration, Wnt inhibition is promoted. Below this DKK1 threshold, Wnt signal transduction is potentiated (Cselenyi and Lee, 2008).

2.5.4.4 Dysregulation of Wnt signaling Causes Non-neoplastic and Neoplastic Disorders

Loss of function mutations in the negative Wnt regulator AXIN2 triggers the activation of Wnt signaling and causes autosomal dominant oligodontia. The same disorder has been associated with predisposition to colorectal cancer, which sheds a light on commonalities between developmental pathways and tissue homeostasis degradation (Lammi et al., 2004; Swinnen et al., 2008).

On the other hand, mutations that promote Wnt transduction and prevent β-catenin degradation may lead to skin, gastrointestinal, ovarian and hepatocellular tumors as well as familial colorectal polyposis accompanied by extracolonal cysts, osteomas and odontomas (small teeth-like structures) (Lammi et al., 2004).

The fact that activated Wnt-signaling may cause tooth agenesis at times and be associated with odontomas at others deserves further attention and suggests that an intricate control of Wnt-signal activity is necessary for normal tooth development. It could be deduced that the suppression of Wnt function is necessary during specific morphogenetic stages of tooth development in the dental mesenchyme and/or in the enamel knot (Lammi et al., 2004).

2.5.5 Tumor Necrosis Factor Signaling Pathway

Key genes in this pathway namely Ectodysplasin A1 (EdaA1), which is a member of the TNF family, and two TNF receptors: EDAR and EDAR death domain adaptor (EDARADD) are expressed in the outer enamel epithelium and enamel knot, (Tucker et al., 2000; Headon et al., 2001). *Tabby* mice mutants are devoid of the Ectodysplasin (EDA) function and have frequently absent incisors and third molars (Pispa et al., 1999). Mice mutants of the other two receptors are called *Downless* and *Crinkled*, respectively and they, along with *Tabby* mice show abnormal enamel knots with reduced molar-cusp number and shallow depth (Laurikkala et al., 2001). The Ectodysplasin would normally bind to its receptor EDAR which recruits the adapter protein EDARADD to transduce a signal and activate NF-kB via the IKK complex. EDA signaling is regarded a modulator of the effects of Wnts to activate SHH and antagonize BMPs (Pummila et al., 2007). Mutations in the genes Eda, Edar and Edaradd in humans are associated with anhidrotic ectodermal dysplasia. In addition to hypodontia, this condition is also characterized by scanty hair and failure of sweat gland development (Mikkola and Thesleff, 2003).

2.5.6 Odontogenic Homeobox Code

The first branchial arch comprises mesenchymal cells that migrate from the midbrain region. Unlike the posterior branchial arches and the main body axis, it does not express Hox genes but instead other homeobox genes including Msx1, Dlx1, -2, -3, -5, -6, -7, Barx1, Lhx6 and -7 (Mackenzie et al., 1991b; Qiu et al., 1997; Tissier-Seta et al., 1995; Grigoriou et al., 1998).

According to the overlapping expression domains of the homeobox genes, the jaw is subdivided into different regions. Barx1 domains determine the molar tooth type and are restricted to the posterior molar mesenchyme. The expression of the Distalless genes Dlx1 and Dlx2 in the ectomesenchyme is a mirror image of Msx1 and Msx2. Moreover, the

expression of Msx2 and Dlx2 overlap in the ectoderm of the dental field corresponding to the position of the dental placode where Msx2 and Bmp4 are subsequently expressed. The Goosecoid gene Gsc is expressed in the mandibular ectomesenchyme more medial that Dlx1 and Dlx2. The overlapping expression domains of Msx-1, Msx-2, Dlx-2 and Gsc subdivide the mandible into homeobox code regions that could specify the developing tooth type (Sharpe, 1995) (Fig. 2.4).

2.6 Overview of Msx Genes

Msx genes are muscle segment homeobox-containing genes that diverged from the Drosophila msh (muscle segment homeobox) genes. They emerged with the evolution of the vertebrate head and are highly conserved and tightly regulated due to their involvement in the development of important sensory and feeding structures. They are not involved in segmentation but rather play an important role in organogenesis and cell differentiation.

Mammals express three Msx genes: Msx1 (previously known as Hox7), Msx2 (previously known as Hox8) and Msx3 (only expressed in the dorsal neural tube). They modulate the development of craniofacial structures, limbs and the nervous system by the transduction of cellular signaling events to changes in gene expression (MacKenzie et al., 1991a; MacKenzie et al., 1992).

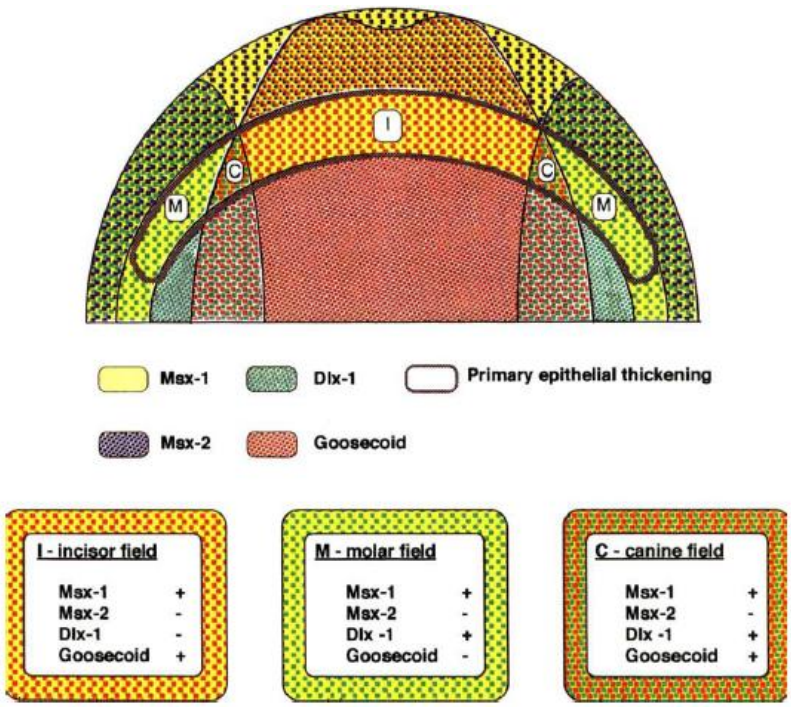


Fig. 2.4: Odontogeic homeobox gene code. Expression of homeobox genes creates spatial domains in the ectomesenchyme of the developing mandible with the specified tooth type in each region (Sharpe, 1995).

Msx1 and Msx2 genes are highly homologous, showing similar DNA-binding site preference and transcriptional repression. They are first expressed in the primitive streak mesoderm and the neural crest cells. Later in development, they are detected in regions of epithelio-mesenchymal interactions. Various regions show expression of both genes including the suture mesenchyme of the forming skull, the meninges, dura mater, the facial structures and teeth (MacKenzie et al., 1991a; MacKenzie et al., 1992).

MSX proteins act as transcriptional repressors through protein-protein interactions with other transcription factors, with components of the core transcription complex (Zawel and Reinberg, 1993) or through their DNA-binding homeodomains. This structure is a common conserved component of regulatory proteins including MSX proteins. Still, it is presumed that it serves mainly as a scaffold for selective protein-protein interactions and that it does not drive selective sequence-specific DNA-protein interactions (Catron et al., 1995). Cases exist where homeodomains are dispensable for in-vivo activity (Ananthan et al., 1993).

2.6.1 Msx1 Gene and Gene Product: Structure, Regulation and Function

The functional properties of MSX1 are attributed to its structural components. MSX1 in humans is encoded by the two-exon Msx1 gene. At the DNA coding level Msx1 is 80% homologous to the murine gene (Hewitt et al., 1991; Padanilam et al., 1992). The protein is composed of 303 amino acid residues arranged into domains (GenBank accession number NP_002439.2).

The most prominent functional structure is the homeodomain (residues 166-225), which is encoded by the second exon. It is highly conserved and a 100% homologous to the murine homeodomain at the nucleotide coding level which is a reflection of its involvement in crucial functional processes (Hewitt et al., 1991; Padanilam et al., 1992). It is held accountable for the nuclear localization of MSX1 (Wang et al., 2011). It can bind a multiprotein transcriptional complex containing TATA-binding protein (TBP), Sp1, or the cAMP-response-element-binding protein (CBP/p300) to mediate its repressive function. Participation of the homeodomain in this complex would relieve MSX1's transcriptional autorepression (Shetty et al., 1999). It can also interact with other homeodomain-containing

proteins. Upon heterodimer formation with DLX2, DLX5, LHX2 and PAX3, mutual antagonism can be accomplished (Alappat et al., 2003).

Besides its homeodomain, MSX1 shares homologous regions (MHRI-III) with other MSX and regulatory proteins that may be more directly associated with its repressive function. The N-terminal part contains two such regions that are considered elementary for its repressive function, whereas the C-terminal homozygosity region contributes to its stability (Hu et al., 1998) (Fig. 2.5). Both terminals contain a high percentage of hydrophobic residues (Catron et al., 1995). Furthermore, the N-terminal region is involved in the inhibition of differentiation of mesenchymal progenitor cells by indirectly upregulating cyclin D1 expression and Cdk4 activity. This encourages the active division of cells, and prevents their exit from the cell cycle (Hu et al., 2001). Two structures of MSX1 are involved in the repression of MyoD gene and myogenic differentiation. The N-terminal region of MSX1 (residues 105 to 139) has been found necessary to interact with the linker histone H1b which gets concentrated near the core enhancer region (CER) of MyoD. In addition to that, the homeodomain binds a consensus site at the CER (Lee et al., 2004).

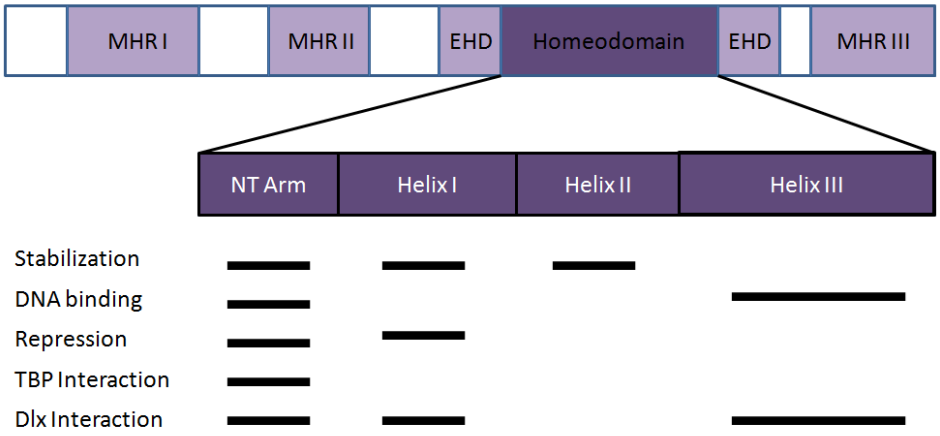


Fig. 2.5: Conserved regions in MSX proteins and contributions made by homeodomain subdivisions. **MHRI to –III**: the MSX homology regions I to III; **EHD:** the extended homeodomain**; NT Arm:** the N-terminal arm (Hu et al., 1998).

The expression of Msx1 gene is regulated by different mechanisms. The 5´ flanking region of human Msx1 gene contains an enhancer element that responds to retinoic acid. The same region also contains multiple enhancer elements that include three sites that potentially bind NFkB in addition to an MSX1 consensus binding site that contributes to transcriptional autorepression (Alappat et al., 2003). Epigenetic control of Msx1 by imprinting is proposed

to play a role in non-syndromic cleft lip and palate with a parent-of-origin effect showing a higher risk of paternal than maternal transmission (Suazo et al., 2010). An antisense Msx1 transcript is produced from a TATA promoter located in the 3' end of exon2. With a length of more than 2 kb, it belongs to the long cis antisense (AS) transcripts subclass and is fully complementary to part of the Msx1 genomic DNA sequence. Similarly to other natural antisense transcripts (NAT), it may be involved in a wide range of gene regulatory mechanisms including DNA replication interference, chromatin remodeling, transcriptional interference and translation interference (Li and Ramchandran, 2010). The amount of functional MSX1 protein can be determined by balancing the sense and antisense transcripts. The sense RNA maintains cells in a proliferative state. On the other hand, the antisense RNA encourages the differentiation and mineralization of craniofacial structures (Alappat et al., 2003).

The expression of Msx1 during embryogenesis and after birth encourages cell proliferation and/or apoptosis and represses differentiation. Postnatally, Msx1 plays a role in maintaining the proliferative capacity of tissues that are capable of renewal (Orestes-Cardoso et al., 2001).

MSX1 protein is involved in crucial developmental processes. Numerous growth factor signaling pathways such as BMP, FGF, Endothelin and SHH can induce mesenchymal Msx1 expression. Mesenchymal Msx1 protein product changes the expression of several growth and transcription factors in the mesenchyme like Bmp4, Fgf3, Dlx2, syndecan-1, and Ptc. Moreover, it appears to have a role in palatal development as evidenced by its expression in the palatal mesenchyme (Alappat et al., 2003). One of the major sites for Msx1 expression is the limb bud where it is essential for limb morphogenesis (Houzelstein et al., 1999; Hill et al., 1989). Recent evidence has shown that MSX1 binds the DKK1 promoter and inhibits the transcription of DKK1 which is an important inhibitor of Wnt signaling (Menezes et al., 2012). One mechanism proposed for the repressive function of MSX1 involves recruiting the PRC2 complex from genes not regulated by MSX1 to its own target genes and redistributing the H3K27me3 repressive mark from non-targets to MSX1 target genes in a gene-specific way which is mediated by the homeodomain (Wang and Abate-Shen, 2012). PIAS1 has been shown to confer DNA-binding specificity on MSX1 in myoblast cells by regulating its subnuclear localization and proximity to target genes at the

nuclear periphery such as MyoD and Myf5 (Lee et al., 2006). Msx1, Msx2 and Dlx5 genes are involved in the regulation of genes involved in mineralized tissue formation in osteoblasts and odontoblasts. Cbfa1 expression is necessary for osteocalcin gene expression and differentitation of those cells. Cbfa1 is inhibited by MSX1 and DLX5 which may involve a negative control of the Msx1 AS RNA by DLX5 and a negative control of Cbfa1 expression by MSX1. Along with the balancing between the sense-antisense Msx1 transcripts, these processes are capable of controlling the transition from proliferation toward terminal differentiation (Blin-Wakkach et al., 2001).

In adulthood, Msx1 expression is found in a wide range of tissues. It is expressed in the basal epithelium of the epidermis (Stelnicki et al., 1997), in stem cells of uterine epithelium (Pavlova et al., 1994) and mammary gland epithelium (Friedmann and Daniel, 1996). It might determine local pools of bone cells in the osteoprogenitor department which is important for bone growth and homeostasis as it is expressed in the cranio-facial sutures, growth plate cartilages and periosteum (Orestes-Cardoso et al., 2001; Kim et al., 1998). Furthermore, its expression is associated with mammalian fracture repair (Gersch et al., 2005).

2.6.2 Msx1 Function during Odontogenesis

Throughout odontogenesis, Msx1 is expressed in the dental mesenchyme; in the dental papilla and dental follicle, throughout the lamina, bud, cap and bell stages (Zhang et al., 2005). Its expression can be driven in response to epithelial BMP4, which is necessary for reciprocal epithelio-mesenchymal signaling. In addition to that, it is required for the mesenchymal expression of Fgf3, Bmp4, the SHH receptor Patched and the transcription factors LEF1 and RUNX2 (Catron et al., 1995).

However, the expression of Msx1 solely is insufficient and other factors must be coordinately induced to express signaling mesenchymal molecules. For instance, it has been shown that Msx and Dlx gene products inhibit each other's transcriptional properties through their homeodomains (Zhang et al., 1997). PAX9 interacts with Msx1 on the transcriptional level by means of its conserved paired box domain. In addition to that, heterodimeric interaction between PAX9 and MSX1 enhances the transcriptional activation of both the Msx1 and Bmp4 promoters in the mesenchyme. This interaction determines the transition of

the tooth from the bud to the cap stage and the enamel knot induction in the late cap stage (Ogawa et al., 2006).

Epithelial FGF1, FGF2 and FGF8 can induce Fgf3 expression in the dental wild-type mesenchyme but are not capable of doing so in Msx1-mutant dental mesenchyme. This emphasizes the role of MSX1 as a mediator for epithelial BMP4 and FGF signals (Catron et al., 1995; Bei and Maas, 1998). Epithelial BMP4 and FGFs are responsible for the regulation of other mesenchymal transcription factors such as PAX9, DLX and LHX (Neubuser et al., 1997).

2.6.3 Msx1 Mutations

Mutations in this gene are responsible for the development of syndromes such as Witkop and Wolf- Hirschhorn syndromes, non-syndromic cleft lip with or without cleft palate and non-syndromic autosomal dominant hypodontia (Jumlongras et al., 2001; Jezewski et al., 2003; Mostowska et al., 2003b).

The selective tooth agenesis tends to affect certain teeth, particularly the second premolars and third molars whereas the other flanking teeth are variably affected. Msx1 null mutant mice display a cleft palate, abnormal craniofacial development and arrest of tooth development at the bud stage (Satokata and Maas, 1994). They also lack alveolar bone as a result of downregulation of Bmp4 expression in the dental mesenchyme (Zhao et al., 2000).

Increased cancer risk has been linked to developmental instability, developmental anomalies and evolutionary change (Galis and Metz, 2003). Loss-of-function mutations in Msx1 lead to developmental anomalies. Furthermore, reduced Msx1 expression is a feature that has been associated with cervical carcinoma cells. In addition to that, MSX1 protein has a role in inducing the apoptosis of cancerous cells by way of stabilizing the tumor suppressor protein P53 (Park et al., 2005).

2.7 Diagnosis of Dental Agenesis

Dental agenesis can be detected both clinically and radiographically. If a certain tooth has not erupted in the oral cavity and is not visible in a radiograph at a particular age, then it is considered absent. For the diagnosis to be reliable, accurate recording of history of dental treatment, a trauma and tooth extraction is essential. Radiographic imaging is critical for the

diagnosis of young subjects but is not always possible and is sometimes unreliable at a very young age as the late developing teeth are sometimes scored as developmentally missing. All primary teeth are mineralized by birth and can be visualized radiographically at a young age. Permanent tooth crowns except third molars are mineralized by the age of 6. Third molars are first noted radiographically at ages 8 to 10 years but can be delayed to the age of 14 to 18 years. The variation of the onset of mineralization of these teeth coupled with their common extractions makes them subject to being falsely diagnosed as developmentally absent and are therefore excluded in the majority of studies. Hence the radiographic diagnosis of hypodontia in the permanent dentition should be made after the age of 6 if third molars are excluded. If diagnosis is made based on clinical observation solely, agenesis of permanent teeth excluding the third molars can be determined by the age of 14. Depending on clinical examination alone, hypodontia of primary teeth can be diagnosed at age 3 to 4 years as all primary teeth are supposed to have erupted before then (Pirinen and Thesleff, 1995).

2.8 Prevalence of Hypodontia

Non-syndromic hypodontia of one or a few permanent teeth is considered the most common developmental anomaly in man. It is argued that hypodontia has increased in the recent decades (Mattheeuws et al., 2004). This could be an evolutionary trend or the result of improved screening of affected cases. The teeth most commonly missing are the third molars. At least one third molar fails to develop in more than 20% of Caucasian populations (Grahnen, 1956). If the third molar is excluded, the incidence of permanent tooth agenesis ranges between 1.6% and 9.6%. The wide range could be attributed to different racial group derivations, sampling size and methodologies in addition to diagnostic criteria. More than 80% of persons with hypodontia lack only one or two teeth (Vastardis, 2000; Lidral and Reising, 2002). The teeth most commonly absent are the last teeth in each class to develop. It is therefore not surprising that the permanent teeth most predominantly affected are the third molars, second premolars and upper lateral incisors. The following missing teeth in order are lower incisors, maxillary first premolars, mandibular first premolars, maxillary canines and mandibular second molars (Polder et al., 2004). Oligodontia is present in 1% or less of the population (Schalk-van der Weide et al., 1992). A summary of hypodontia findings in different populations can be seen in Table 2.1. The hypodontia of deciduous teeth is reported to be 0.5% to 0.9% (Vastardis, 2000) and even 2.4% in the Japanese

population (Yonezu et al., 1997). The teeth most commonly missing in the primary dentition are the maxillary lateral incisors and the mandibular incisors (Whittington and Durward, 1996). In most of the cases, agenesis of the primary tooth leads to the absence of its successor (Lidral and Reising, 2002).

2.9 Etiology of Dental Agenesis

Failure of tooth development can be caused by a number of factors including environmental factors during dental development as well as genetic factors influenced by environmental and spaciotemporal determinants. The 'epigenetic' influences of such determinants stems from the interactions between cells at the local tissue level in addition to processes that affect DNA directly and account for the third source of developmental differences (Molenaar et al., 1993).

Country	Prevalence of hypodontia	Prevalence of oligodontia	Most frequently missing tooth	Reference
Saudi Arabia	2.6%		Lower second premolar	(Salama and Abdel-Megid, 1994)
Jordan	5.5%		Lower second premolar	(Albashaireh and Khader, 2006)
Turkey	2.8%	0.13%	Upper lateral incisor	(Altug-Atac and Erdem, 2007)
Iran	5.21%		Upper lateral incisor	(Amini et al., 2012)
Hong Kong	6.9%		Lower central incisor	(Davis, 1987)
Malaysia	2.8%	0.19%	Upper lateral incisor	(Nik-Hussein, 1989)
Britain	4.3%	0.17%	Lower second premolar	(Rose, 1966)
Ireland	11.3%		Lower second premolar	(O'Dowling and McNamara, 1990)
Sweden	7.4%	0.19%	Lower second premolar	(Bergstrom, 1977)
Norway	6.5%	0.10%	Lower second premolar	(Aasheim and Ogaard, 1993)
Iceland	7.9%	0.18%	Lower second premolar	(Magnusson, 1977)
Denmark	7.8%		Upper second premolar	(Rolling, 1980)
USA	3.5%	0.05%	Lower second premolar	(Muller et al., 1970)
Canada	7.4%	0.08%	Lower second premolar	(Thompson and Popovich, 1974)
Australia	6.3%		Upper lateral incisor	(Lynham, 1990)

Table 2.1: Prevalence of hypodontia in the permanent dentition according to different studies.

2.9.1 Environmental Factors

Numerous environmental factors can negatively affect tooth development. They can be physical and invasive such as extraction of the preceding primary tooth, trauma to the dentoalveolar process and surgeries or disturbances to the innervations of the jaw (Schalk-van der Weide et al., 1992). It has been shown that hypodontia occurs mostly in regions that are innervated last (Kjaer et al., 1994). Chemical factors such as drugs and chemotherapy in addition to irradiation may adversely affect dental development in a dose and age-dependent manner and cause lack of teeth. One example is Thalidomide (N-phthaloylglutamimide) taken by pregnant mothers that induces hypodontia in the children (Nasman et al., 1997; Maguire et al., 1987). Dioxin accidents have been reported to be associated with a number of anomalies including agenesis of permanent teeth (Alaluusua et al., 2004). Biological causes like rubella infection may be responsible for failure of tooth development (Gullikson, 1975).

2.9.2 Genetic Factors

Segregation analyses of families affected with tooth agenesis is mandatory for the determination of the mode of inheritance. Grahnen (1956) analyzed 171 affected families and found out that in 73 % of the families at least one of the parents was affected. His data was in favor of the autosomal dominant inheritance with reduced penetrance and variable expression. Schalk-van der Weide (1994) made similar conclusions on cases of severe agenesis. In Grahnen's studies penetrance of hypodontia (i.e. the percentage of individuals with a particular gene combination displaying hypodontia at a particular degree) was found to be 86%. Unilateral agenesis may be a result of reduced penetrance. Variable expression (the degree of phenotypic expression in an individual) meant that teeth can be agenetic, or modified in shape or size such as peg lateral incisors (Grahnen, 1956).

On the other hand, Chosack and colleagues suggested a polygenic inheritance based on segregation that treats agenesis of distinct tooth classes, for example agenesis of incisors and premolars, as separate traits. Polygenic inheritance is commonly found in continuous traits and involves a number of genes each with a relatively small effect (1975).

A multi-factorial model has been described by Brook, where genetic factors and epigenetic effects are included. Both tooth number and size are located on a continuous scale. At both ends of this normal distribution are hypodontia and supernumerary teeth, respectively. They

are considered the threshold points of microdontia (or small teeth) and megadontia, respectively (Brook, 1984) (Fig. 2.6).

The differences in phenotype between monozygous twin pairs are attributed to environmental factors at times (Lapter et al., 1998) and suggest a relatively low penetrance of agenesis with high variability of phenotypic expression at others (Gravely and Johnson, 1971).

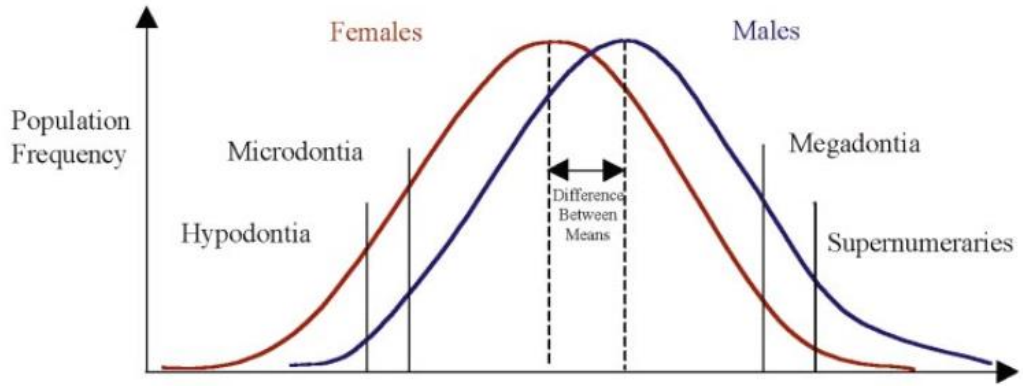


Fig. 2.6: Brook's multi-factorial model of the etiology of dental anomalies of tooth number and size. The curves display the distribution of females and males (Brook, 1984).

According to Svinhufvud, there are 'fragile lamina sites' that are more sensitive to epigenetic factors during tooth maturation. Examples include the area of the fusion between the lateral maxillary process and the medial nasal process in which upper lateral incisors develop which explains the frequent agenesis of these teeth. The second lower premolar is also very prone to agenesis as it originates in another fragile area of the dental lamina. Lower central incisors are commonly missing and are located in the initial fusion area of the jaw (Svinhufvud et al., 1988). This hypothesis is highly supported especially after affirmation by Kjaer of the existence of sensitive areas where innervations develop the last (Kjaer, 1997). As a consequence, it is a common finding to find the last developing teeth in each tooth family to be most frequently missing (Clayton, 1956).

2.10 Classification of Hypodontia

A conduction of thorough systemic examination is necessary to differentiate non-syndromic from syndromic forms of hypodontia. Physical examination of hairs, sweat glands, eyes and other body parts is often necessary to make such a distinction. On the molecular level, no

regulatory gene has been identified to solely affect tooth development (Schalk-van der Weide et al., 1992).

2.10.1 Non-syndromic Hypodontia Mutational Spectrum

Non-syndromic hypodontia is very common and involves a variable number of teeth. Subclasses include sporadic and familial forms. It is usually autosomal dominant in inheritance and is at times considered part of normal variation in its mild forms (Arte et al., 2001). It can also follow autosomal recessive and X-linked patterns of inheritance. Variability in its penetrance and expressivity is very common (Burzynski and Escobar, 1983).

Defects in various genes are responsible for the development of selective tooth agenesis (Table 2.2). Examples include transcription factor encoding genes such as Msx1 and Pax9 in addition to genes coding for proteins that regulate important signaling processes or receptors for growth factors such as Axin2 and Fgfr1, respectively (Chhabra et al., 2013). It is worth mentioning that defects in Msx1 are also responsible for the development of nail dysplasia and oral clefts with oligodontia as a common finding (Jumlongras et al., 2004; van den Boogaard et al., 2000).

The consequences of Msx1 mutations are twofold. They either result into substitution within the MSX1 protein product or introduction of a stop codon that prematurely truncates the protein (Table 2.2). The location of the identified missense mutations (in relation to the homeodomain) and their effect on MSX1 protein does not correlate clearly with the severity of the hypodontia. The mechanism proposed for such an anomaly is haploinsufficiency resulting from the susceptibility of the particular tooth family to the reduced dose of MSX1 protein in the genetic background (Vastardis et al., 1996). Unlike humans, mice that are heterozygous for Msx1 display normal dental development. The reason could be that they normally lack premolars (Satokata and Maas, 1994).

Most of the Pax9 frame-shift, deletion and substitution termination mutations cause hypodontia in both the permanent and the primary dentitions. Missense mutations in this gene, on the other hand affect the permanent dentition only. Furthermore, the reduced

amount of PAX9 protein seems to mostly affect molars. In addition to that, Pax9 is located upstream of Msx1 and they are both intimately involved in regulating tooth development through their interaction at the gene and protein level. Consequently, when mutated, PAX9 displays reduced binding to sites that regulate Msx1 expression levels which produces deficient Msx1 downstream processes. As a result, it will show an impaired ability for activation of transcription from the Msx1 and Bmp4 promoters (Ogawa et al., 2006; Wang et al., 2009).

Gene and its protein	**Mutations in gene/protein**	**Type of disorder**	**Mode of inheritance**	**Reference**
Msx1gene (GenBank protein accession no: AAH67353.1)	M61K,	Hypodontia	Autosomal dominant	(Lidral and Reising, 2002)
	S104X,	Hypodontia and/or CLP	Autosomal dominant	(van den Boogaard et al., 2000)
	Q187X	Hypodontia	Autosomal dominant	(De Muynck et al., 2004)
	R31P	Hypodontia	Autosomal dominant	(Vastardis et al., 1996)
	A194V	Hypodontia	Autosomal dominant	(Mostowska et al., 2006b)
	A219T	Hypodontia	Autosomal recessive	(Chishti et al., 2006)
	G22RfsX168	Oligodontia (mostly premolars)	Autosomal dominant	(Kim et al., 2006)
	R151S	Hypodontia +/- CLP	Autosomal dominant	(Kamamoto et al., 2011)
	L224P	Hypodontia	Autosomal dominant	(Mostowska et al., 2012)
	R196P	Hypodontia	Autosomal dominant	(Vastardis et al., 1996)
	A221E	Oligodontia	Autosomal dominant	(Xuan et al., 2008)
	R176W	Hypodontia	Autosomal dominant	(Bergendal et al., 2011)
	p.Q216QfsX	Oligodontia	Autosomal dominant	(Bergendal et al., 2011)

	125			
	N222KfsX118	Oligodontia	Autosomal dominant	(Arte et al., 2013)
	K237SfsX2	Oligodontia	Autosomal dominant	(Arte et al., 2013)
	W139X	Oligodontia	Autosomal dominant	(Kimura et al., 2013)
Pax9gene (GenBank protein accession no: NP_006185.1)	R26W	Molar hypodontia	Autosomal dominant	(Lammi et al., 2003)
	ΔPax9 (1A>G)	Severe oligodontia	Autosomal dominant	(Klein et al., 2005)
	S43K	Oligodontia	Autosomal dominant	(Wang et al., 2009)
	G6R	Mild hypodontia	Autosomal dominant	(Wang et al., 2009)
	G51S	Oligodontia	Autosomal dominant	(Mostowska et al., 2003a)
	G51A	Oligodontia	Autosomal dominant	(Bergendal et al., 2011)
	R28P	Oligodontia	Autosomal dominant	(Jumlongras et al., 2004)
	G73fsX316,	Molar oligodontia	Autosomal dominant	(Stockton et al., 2000)
	L21P	Molar oligodontia +/- CLP	Autosomal dominant	(Das et al., 2003)
	L27P	Molar oligodontia	Autosomal dominant	(Liang et al., 2012)
	I29T	Molar oligodontia	Autosomal dominant	(Liang et al., 2012)
	K91E	Molar oligodontia	Autosomal dominant	(Das et al., 2003)
	F15I	Molar oligodontia	Autosomal dominant	(Wang et al., 2012)
	R47P	Molar oligodontia	Autosomal dominant	(Arte et al., 2013)
	I56T	Molar oligodontia	Autosomal dominant	(Arte et al., 2013)
	Q136X	Molar oligodontia	Autosomal dominant	(Arte et al., 2013)
	K114X	Molar oligodontia	Autosomal dominant	(Nieminen et al., 2001)
	V265fsX315	Molar oligodontia	Autosomal dominant	(Frazier-Bowers et al., 2002)
	R59fsX177	Molar oligodontia	Autosomal dominant	(Das et al., 2003)

	I87F	Molar oligodontia	Autosomal dominant	(Kapadia et al., 2006)
	Y143C	Oligodontia	Autosomal dominant	(Bergendal et al., 2011)
	T80A	Oligodontia	Autosomal dominant	(Bergendal et al., 2011)
	p.Arg77_Pro 81delfsX4	Oligodontia	Autosomal dominant	(Bergendal et al., 2011)
	Y160X	Oligodontia	Autosomal dominant	(Zhu et al., 2012)
Axin2	R656X	Oligodontia	Autosomal dominant	(Lammi et al., 2004)
	1994-1995insG	Oligodontia	Autosomal dominant	(Lammi et al., 2004)
	A684V	Oligodontia	Autosomal dominant	(Bergendal et al., 2011)
	A758T	Hypodontia	Autosomal dominant	(Bergendal et al., 2011)
Ltbp3	Y774X	Oligodontia	Autosomal recessive	(Noor et al., 2009)
Eda	T338M	Hypodontia	X-linked recessive	(Kurban et al., 2010)
	V365A	Hypodontia	X-linked recessive	(Mues et al., 2010)
	Q358E	Hypodontia	X-linked recessive	(Kurban et al., 2010)
	D316G	Hypodontia	X-linked recessive	(Li et al., 2008)
	T338M	Hypodontia	X-linked recessive	(Li et al., 2008)
	M364T	Hypodontia	X-linked recessive	(Rasool et al., 2008)
	G255C	Hypodontia	X-linked recessive	(Kurban et al., 2010)
	G291R	Hypodontia	X-linked recessive	(Kurban et al., 2010)
	A259E	Hypodontia	X-linked recessive	(Kurban et al., 2010)
	R289C	Hypodontia	X-linked recessive	(Kurban et al., 2010)
	R334S	Hypodontia	X-linked recessive	(Kurban et al., 2010)
	S374R	Hypodontia	X-linked recessive	(Kurban et al., 2010)
Edaradd	S103F	Hypodontia	Autosomal dominant	(Bergendal et al., 2011)

Table 2.2: Genes and mutations responsible for non-syndromic hypodontia. CLP: Cleft lip and palate.

2.10.2 Syndromic Hypodontia

Syndromic hypodontia affects multiple organ systems and reveals common genetic pathways of dental and crananiofacial development as well as other ectodermal organs. It tends to be inherited in an autosomal recessive (Pirinen et al., 2001) or X-linked fashion (Erpenstein and Pfeiffer, 1967). There are over 60 different syndromic conditions in the

Online Mendelian Inheritance in Man (OMIM), that display hypodontia as part of the anomalies (Online Mendelian Inheritance in Man, 2014).

2.10.2.1 Ectodermal Dysplasia: Classification

Ectodermal dysplasias are disorders characterized by changes in two or more ectodermal structures, at least involving one in hair, teeth, nails, or sweat glands. They are divided into two categories: Group A in which two or more of the upper structures are involved and Group B involving one of the upper structures in addition to another ectodermal defect. Group A contains over 180 EDs (Freire-Maia and Pinheiro, 1988).

A clinical-functional classification of EDs has been proposed by Priolo et al. (2001) assuming the causative genes act through two different pathogenic mechanisms that place the disorders in two groups; group1 is characterized by defects in the epithelial-mesenchymal interaction, with genes that are involved in differentiation and apoptosis localized in the nucleus and group2 that includes disorders characterized by defects in the ectodermal structural proteins that are usually localized in the plasma membrane domains and cytoplasm with roles in cell membrane and cytoskeleton stability (Priolo and Lagana, 2001).

2.10.2.1.1 Causative Mutations of Ectodermal Dysplasia

Ectodermal dysplasia (ED) includes heterogeneous clinical groups that are genetically diverse. The mutational spectrum involves so far more than 60 genes. Mutations in one gene can cause clinically different EDs and mutations in different genes can determine the same ED entity and/or display the same clinical features.

Hypohydrotic dysplasias refer to conditions where there is a remarkable reduction in sweat production. Three hypohidrotic EDs (HED) show identical clinical manifestation but different inheritance patterns and are classified as separate entities: X-linked HED (OMIM 305100), autosomal dominant (ADHED, OMIM129490) and autosomal recessive (ARHED, OMIM 224900). All the HED forms present with abnormal teeth, sparse hair, and thin and dry skin. The observations reflect a common etiology involving the same signaling pathway. Mutations in Eda gene cause X-linked dominant ED. Edar gene codes for a TNF receptor (EDAR) and may cause autosomal dominant and recessive forms of ED when mutated (OMIM Entry 604095). Another protein that works in the same pathway is the

Ectodysplasin-A receptor adapter (EDARADD). Mutations in the Edaradd gene cause autosomal dominant and recessive ED (Visinoni et al., 2009). Treatments to EDs have been proposed. Fetal mice with ED were treated with a recombinant EDA protein through intravenous injection into the pregnant mother, reverting to a wild-type-like phenotype. Similar results were obtained in dogs with ED treated intravenously after birth (Cudney and Vieira, 2012).

Homozygous mutations in Wnt10A cause various EDs that combine classic ectodermal developmental anomalies with additional cutaneous features. They are displayed in odontoonychodermal dysplasia (OODD) and Schöpf-Schulz-Passarge syndrome (Adaimy et al., 2007; Cluzeau et al., 2011). Mutations in the Nfkbia gene are responsible for autosomal dominant anhidrotic ectodermal dysplasia with T-cell immunodeficiency (Courtois et al., 2003).

Mutations in the genes associated with EDs can result into isolated features. Wnt10A heterozygotes may display isolated ectodermal defects (Bohring et al., 2009) and have been associated with cases of non-syndromic autosomal dominant tooth agenesis (Kantaputra and Sripathomsawat, 2011). Eda mutations were also associated with non-syndromic hypodontia (Kurban et al., 2010) (Table 2.2). Digenic mutations in Wnt10A and Eda were found to result in either isolated oligodontia or syndromic tooth agenesis in the Chinese population (He et al., 2013).

2.11 SNP Array Homozygosity Mapping

SNP arrays are a subgroup of DNA microarrays that contain collections of microscopic DNA spots attached to a solid surface. Each spot contains picomoles of probes or oligos. These have the possibility to hybridize the target DNA in question under high stringency conditions. The convergence of DNA hybridization, fluorescence microscopy, and solid surface DNA capture are the basic principles of SNP arrays.

SNP arrays have been used extensively for surveying of genomic polymorphic sites. The principle of homozygosity mapping has been applied to the genome of affected individuals and their parents/siblings in an attempt to define regions that are identical by descent i.e. contiguous SNPs on the array that share the same genotype. Identifying such regions that are shared by affected siblings enables the exclusion of regions that are shared by unaffected

relatives of the same family. After identifying large shared regions of homozygosity blocks, they are inspected for the disease/phenotype-causing gene (Bell et al., 2011).

2.12 Whole Exome Sequencing

Whole exome sequencing is an approach that belongs to the next generation sequencing strategies and has been developed to study the approximately 1% of the human genome that is protein-coding (the exome). As the majority of genetic variants that underlie Mendelian disorders disrupt protein-coding sequences, this approach has become one of the main tools for studying the genetic causes of Mendelian disease. Furthermore, it is relatively cost-efficient because it enables deep coverage with relatively few reads thus allowing for the detection of variants. Academic groups can use this approach to study the exomes of hundreds of patients with Mendelian diseases per year. When compared to whole genome sequencing, it presents with modest bioinformatic challenges.

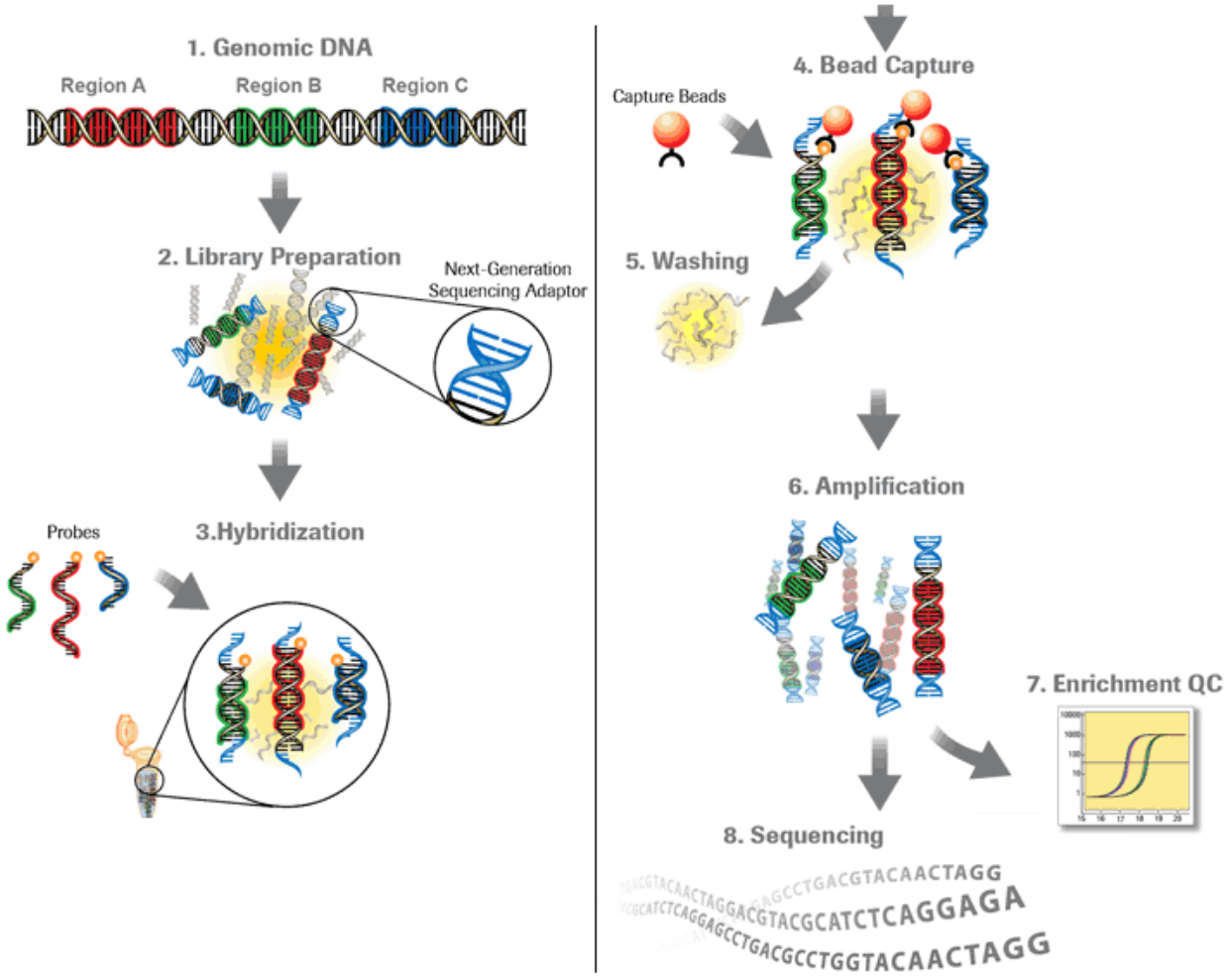


Fig. 2.7: Whole exome sequencing protocol © Copyright 2002 - 2014 Roche NimbleGen, Inc.

CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Buffers

Red blood cell lysis buffer
155 mM NH4Cl (Amresco Cat# 12125-02-9)
10 M NH4HCO3 (Sigma-Aldrich Cat# 1066-33-7)
0.1 mM EDTA (Sigma-Aldrich Cat# 6381-92-6) with (pH=7.4)

1X WBC lysis buffer
50 mM Tris (Amresco Cat# 77-86-1) HCL with (pH=7.5)
100 mM NaCl (Amresco Cat# 7647-14-5)
1 mM EDTA with (PH=8)

50X TAE buffer
2M Tris (pH= 8.0)
1M Acetic acid
0.05M EDTA (pH=8.0)

3.2 Methods

3.2.1 Recruitment and Phenotypic Analysis

Dental practitioners from south of Hebron were asked to refer families that display moderate to severe hypodontia to me for further clinical examination and genetic analysis. I recruited probands from 5 Palestinian families and informed them of the purpose and procedures of the research. Their relatives were later asked to participate in the study. All participants, or in case of individuals under 18 years, their parents were asked to sign an informed consent in accordance with the guidelines of Bethlehem University IRB Committee.

Diagnosis of hypodontia was confirmed by clinical and radiographic examination and by family interviews and medical history. All relevant information regarding the number and location of missing teeth and extraoral characteristic features was recorded carefully. In addition to that, intraoral and facial photographs were taken to serve as records for documentation and further evaluation. Eventually, and based on comprehensive evaluation, pedigrees were constructed.

3.2.2 Isolation of DNA by Salting-out Technique

The methods in this section were conducted by Yasmin Issa at the Hereditary Research Lab/Bethlehem University.

About 5 ml of peripheral blood was drawn from each participant and transferred to a sterile EDTA vacutainer tube. Forty-five ml of Red Blood Cell lysis buffer was added to each tube. Tubes were kept on ice for 30 minutes and then centrifuged at 2000 rounds per minute (rpm) for 10 minutes at 4°C. The supernatant was carefully removed and the pellet was re-suspended in 10 ml Red Blood Cell lysis buffer and centrifugation was repeated. After breaking the pellet, it was suspended in a mix of 3ml of 1X WBC lysis buffer,100ul of 20% sodium dodecyl sulfate SDS (Amresco-Cat#1328-M112) and 100ul of 5mg/ml Proteinase K (Amresco-Cat#E195), vortexed until foamy and incubated at 37°C overnight. After incubation, 1ml of 6M NaCl was added to the lysate and vigorously vortexed. Then it was centrifuged at 3000 rpm for 20 minutes at room temperature. The supernatant (the clear upper phase) was transferred gently into a 15ml-tube, avoiding the salt protein deposit. Absolute cold ethanol (EtOH) was poured slowly on the inner walls of the tube in twice the volume of the supernatant and gently moving the tube from side to side. DNA threads were removed with a closed-end glass Pasteur pipette and washed in 70% EtOH. After air-drying for a few minutes on the Pasteur pipette, DNA was transferred to a microfuge containing 500ul (or 200-1000ul depending on the amount of DNA) of 0.02% Sodium Azide (Sigma-Aldrich- Cat#S2002) and left to dissolve at room temperature overnight.

3.2.3 Mutational Analysis of the Candidate Genes for Dental Agenesis

All methods in this section were conducted by Yasmin Issa at the Hereditary Research Lab/Bethlehem University.

3.2.3.1 Primers

- Axin2 primers source: (Lammi et al., 2004).
- Rest of the primers: designed with Primer3 (Untergrasser et al., 2012; Koressaar and Remm, 2007).

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 R: ATCAACAGCCACCCAGTAGC	283 bp
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	684 bp

		R: TCCACCCATCCACCATACTT	
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
Homozygosity mapping candidates			
Gene	**Template**	**Primer sequences**	**Size of amplicon**
Nfkbia			
Exon1part1	DNA	F: GGTCTGACTGGCTTGGAAAT R: CTCCTTGACCATCTGCTCGT	344 bp
Exon1part2	DNA	F: CCAGCCAGCGTTTATAGGG R: CCAGCCAGCGTTTATAGGG	692 bp
Exon2	DNA	F: AATAACCTCGCGGAAAACAC R:CGAATGCAAGAGAGACCAGA	570 bp
Exon3	DNA	F: ACCTGGAGCCTCTGCTATTTG R: TAGGCACTTTGCACACATATTTT	500 bp

Exon4	DNA	F: TACACCTTGCCTGTGAGCAG R: CCCCACACTTCAACAGGAGT	676 bp
Exon5	DNA	F: TTTGGTGTCCTTGGGTGCT R: TTTGCCTTTAATGCTTCTCTTT	600 bp
Exon6	DNA	F: TTTGGGCTATGGAGAATGGA R: TACCTCCTCCCTCACACTGC	766 bp
NGS candidates			
Gene	**Template**	**Primer sequences**	**Size of amplicon**
Pmm1			
Exon6	DNA	F: GGGCAGATGGTTTGAGGAG R: GTCTCCTCGCCCTTCCTACT	432 bp
Aldh6			
Exon11	DNA	F: TCCTCAAAACTTTGTTGCTTTG R: GAGGATAGGGTAGAAGGTGGGTA	422 bp
USP20			
Exon13	DNA	F: CGTTTTGCAGAGGATGACAC R: CCTCCCCTGGATAAAAGCAG	397 bp
Kremen1			
Exon5	DNA	F: TTGGCATGACTGTAGCTAATCC R: GAAGGGACCCTGGGAAGTT	497 bp
Exons 4, 5, 6	cDNA	F: AACCTTGGCTGCTACAAGGA R: TGACGAAGTCCAGAGAGACG	544 bp

Table 3.1: Primers used for amplifying candidate Msx1, Pax9 and Axin2 CDS in addition to the homozygosity mapping candidate Nfkbia and the NGS candidate coding regions.

3.2.3.2 PCR Amplification Protocol and Program

Basic PCR touchdown protocol

2X PCR ReddyMix Master Mix (Abgene-Cat# AB-0575-DC-LD)	12.5ul
Primer F (10pM working soln.)	0.5ul
Primer R (10pM working soln.)	0.5ul
DNA(100ng/ul)	1ul
ddH_2O (Ambion®Cat#AM9932)	10.5ul

Total volume per sample (1X) = 25ul

A reaction mix was prepared by multiplying each volume minus DNA template by the number of samples tested +1 (for control). This was followed by aliquoting 23ul of the mix per sample and adding the DNA template.

Amplification protocol for Msx1 exon1 was modified by the addition of 2ul of DMSO (BDH laboratory reagent-product# 28216). The volume of ddH_2O was altered accordingly for a total of 25ul per sample.

Basic PCR touchdown program

The used PCR touch down 60 program:

		Annealing	**Extension**
Step1:	**95** ºC for 4 min.		
Step2 (3 cycles):	**94** ºC for 30 sec,	**68** ºC for 30 sec,	**72** ºC for 30 sec
Step3 (3 cycles):	**94** ºC for 30 sec,	**66** ºC for 30 sec,	**72** ºC for 30 sec
Step4 (3 cycles):	**94** ºC for 30 sec,	**64** ºC for 30 sec,	**72** ºC for 30 sec
Step5 (3 cycles):	**94** ºC for 30 sec,	**62** ºC for 30 sec,	**72** ºC for 30 sec
Step6 (35 cycles):	**94** ºC for 30 sec,	**60** ºC for 30 sec,	**72** ºC for 30 sec
Step7:	**72** ºC for 5 min,	**4**ºC for 10 min.	

The reaction was carried out in the thermocycler Geneamp PCR System 9700 (Applied Biosystems).

Amplification program for Msx1exon1 was modified; extension was performed for 1 min.

3.2.3.3 Electrophoresis of PCR Products Using Agarose Gel

One and a half percent agarose gel containing 0.01% ethidium bromide (Amresco-Cat# E406-5ML) was usually used, prepared using agarose (SeaKem® LE Agarose CAT≠ 50004) and 1XTAE running buffer. Three µl of PCR product were loaded onto the gel along with 100bp DNA ladder GeneRuler (Thermo Scientific-Cat# SM0241) and run in 1X TAE buffer at 100V for 15-20 minutes, depending on the fragment size. DNA fragments were observed and documented using ultraviolet light and photographed using the Molecular Imager®, Gel DOC ™ Imaging System, BioRAD).

3.2.3.4 DNA Sequencing

3.2.3.4.1 PCR Product Cleaning Protocol and Program

PCR products that showed clearly visible bands on agarose gels were cleaned using equal amounts of the enzymes Antarctic Phosphatase (New England Biolabs, Cat# M0289L) and Exonuclease I (New England Biolabs, Cat# M0293L).

PCR cleaning protocol

Antarctic Phosphatase enzyme	0.25ul
Exonuclease I enzyme	0.25ul
ddH_2O	1.5ul
PCR product	5ul
Total volume per sample (1X=7ul)	

A reaction mix was prepared by multiplying each volume minus PCR product by the number of samples to be tested. This was followed by aliquoting 2ul of the mix per sample and adding the clean PCR product volume.

PCR cleaning program

Step 1: 37 ºC for 30 min.

Step 2: 80 ºC for 20 min.

Step 3: 4 ºC for ∞

3.2.3.4.2 Sequencing of the Purified PCR Product Protocol and Program

Sequencing PCR protocol

DNA samples were prepared for sequencing PCR using approximately 10ng per 100bp of clean PCR product with 1ul of 10pM primer adding up ddH_20 to a total of 16ul.

5X buffer (Applied Biosystems, CAT#4336697)	1.5ul
64X buffer (MCLAB, CAT# BDX-100)	0.8ul
Big dye (Applied Biosystems, CAT#4336768)	0.2ul
Primer (forward or reverse)	1ul
Clean PCR product	2ul
ddH_2O	10.5ul

Total volume per sample (1X) = 16ul

A reaction mix was prepared by multiplying each volume minus PCR product by the number of samples to be tested. This was followed by aliquoting 14ul of the mix per sample and adding the clean PCR product volume.

Sequencing PCR program

Step1 (1 cycle): 96 ºC for 3 min.

Step2 (30 cycles): 96 ºC for 10 sec, **50** ºC for 5 sec, **60** ºC for 2 min.

Step 3: 4 ºC for ∞

3.2.3.4.3 Cleaning of the Sequenced PCR Product using EDTA/Ethanol Precipitation Method and Capillary Electrophoresis

Each sequenced PCR reaction was precipitated and cleaned by adding 100ul of Absolute Ethanol and 5ul of 125mM EDTA (Amresco-Cat # 0720). This was followed by incubating the reactions at -20°C for 30 minutes, and centrifugation for 30 minutes at 3800 RPM and 4°C. The supernatant was discarded and 60ul of 70% Ethanol were added to each reaction, and centrifuged again for 20 minutes. Then the samples were inverted and centrifuged for 1 minute at 500 RPM to get rid of any residual supernatant. Drying the samples was performed at 95°C for 5 minutes. 16ul of Hi-Di Formamide (Applied Biosystems, Cat# 4311320) were then added, and the samples were dried again at 95°C for 2 minutes. Finally, the reactions were put on ice for 5 minutes before loading 13ul of each sample on the sequencing plate and proceeding to capillary electrophoresis using the ABI 3130 Genetic Analyzer (Applied Biosystems, S/N:20355-023).

The sequences were aligned against GRCh37/hg19 human genome browser (Kent et al., 2002).

3.2.4 SNP Array Homozygosity Mapping

The methods used in this section were conducted by the staff at the Hereditary Research Lab/Bethlehem University.

The protocol used was based on the GeneChip Mapping 500K Assay Manual. The GeneChip Human Mapping 250K Nsp assay was conducted. Basic steps included:

Stage 1: Genomic DNA plate preparation

Stage 2: Restriction enzyme digestion with NspI enzyme

Stage 3: Ligation: DNA is ligated to a common adaptor with T4 DNA ligase

Stage 4: PCR: using TITANIUM™ *Taq* DNA polymerase

Stage 5: PCR product purification and elution

Stage 6: Quantitation and normalization

Stage 7: Fragmentation: with fragmentation reagent (DNAse I)

Stage 8: Labeling: end-labeling of the cleaned PCR product using terminal deoxynucleotidyl transferase

Stage 9: Target hybridization

Affymetrix Equipment and Software Required	**Part Number**
GeneChip® Fluidics Station 450	00-0079
GeneChip® Hybridization Oven 640	800139
GeneChip® Scanner 3000 7G	00-0205
GeneChip® Operating Software version 1.4	690031
GeneChip® Genotyping Analysis Software 4.0	690051

Materials and their companies	**CAT#**
GeneRuler 1Kb DNA ladder **(Thermo SCIENTIFIC)**	SM0314
T4 DNA Ligase 100,000U/ Buffer **(Affymetrix)**	NEB-M0202L
Nsp I,250 units/BSA/Buffer **(New England Biolabs)**	NEB-R0602L
GC-melt, 1ml **(Fisher Scientific)**	639238
Titanium Taq DNA Polymerase **(Clontech Laboratories)**	639209
dNTP mixture **(Clontech Laboratories)**	IM 4030
DNA Amplification Clean-up Kit/RB buffer **(Clontech Laboratories)**	636974
TempPlate® Sealing Foil Sheets	2923-0100
EDTA, diluted to 0.1M (working stock is 0.5 M, pH 8.0)**(Ambion, Applied Biosystems)**	AM9260G
pUC mix marker, 0.5ug/ul **(Fermentas Life Sciences)**	SM0301
Denhardts' Solution 50X Concentrate **(Sigma-Aldrich)**	D2532
Tetramethylammonium Chloride (TMACL) **(Sigma-Aldrich)**	T3411
Dimethyl Sulfoxide (DMSO) 500ml **(Sigma-Aldrich)**	D5879
MES Hydrate SigmaUltra **(Sigma-Aldrich)**	M5287-50G
MES Sodium Salt **(Sigma-Aldrich)**	M5057-100G
Surfact-Amps (Tween-20, 10%) **(Thermo Scientific)**	28320
Herring Sperm DNA Promega (HSDNA) **(Promega)**	D1815
Human Cot-1 DNA® 500ug **(Invitrogen)**	15279011

R-Phycoerythrin Streptavidin **(Molecular probes by Life technologies)**	S866
Biotinylated Anti-Streptavidin, 0.5mg **(Vector laboratories)**	VE-BA-0500
AccuGENE™ 20X SSPE Buffer	51214
5 M NaCl **(Ambion, Life technologies)**	AM9760G
Human Mapping 250K Nsp array (CHIPS) **(Affymetrix)**	900768
Human Mapping 250K Nsp assay kit (Reagents) **(Affymetrix)**	900766
Barrier tip ART 10ul (with filter) **(Molecular Bioproducts)**	MBP-2140
Barrier tip ART 10ul (NO filter) **(Molecular Bioproducts)**	MBP-3511
Barrier tip ART 20ul (with filter) **(Molecular Bioproducts)**	MBP-2149
Abgene strips with domed caps (colored) **(Thermo Scientific)**	AB-0490
Abgene strips of Flat caps only **(Thermo Scientific)**	AB-0784
MicroAMP optical 96-well plate **(Applied Biosystems)**	N801-0560
Quality Tubes 1.5ml Flat cap,Brown 1000/pkg **(Sarstedt)**	72.690.004
1.5 ml Copolymer Microcentrifuge Tubes, Natural **(USA Scientific)**	1415-2500
1.5ml Blue centrifuge tubes **(SealRite)**	1615-5501
MBP RNase AWAY® spray 475ml	E-3070-4
StrataCooler® LP benchtop cooler **(Agilent Technologies)**	401349
CoolSafe CHAM-1000 Aluminum Cooling Chamber for 0.2ml Tubes **(Diversified Biotech)**	DB CHAM-1000
Multiscreen HTS vacuum manifold **(Merck Millipore)**	MSVMHTS00
Stericup-GP Filter Unit **(Merck Millipore)**	SCGPU05RE

3.2.5 Whole Exome Sequencing

The methods used in this section were based on the HiSeq 2000 Illumina platform and were conducted at the Department of Medicine, Division of Medical Genetics, University of Washington, Seattle, WA 98195, USA.

3.2.5.1 Genomic DNA Sample Preparation

Paired-end 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).

1µg of genomic DNA extracted from blood was fragmented by sonication. This was followed by end-repair of the fragments and addition of AMPure XP beads to the end-repaired mix An ‘A’- base was then added to the 3’ends, preparing them for ligation to the sequencing adapters. Single-index adapters were ligated to the fragments.

3.2.5.2 Hybridization, PCR and Sequencing

The DNA libraries were hybridized to biotinylated DNA oligonucleotide baits from the SeqCap EZ Human Exome Library v.2.0 (Roche NimbleGen). Capture streptavidin-bound

magnetic beads were then used to pull down the complex of capture oligos and genomic DNA fragments. After washing away the unbound fragments, the enriched fragment pool was amplified by PCR and sequenced on a HiSeq 2000 instrument (Illumina). The exome sequenced contains 44 Mb of the human genome, corresponding to the exons and flanking intronic regions of ~300,000 coding exons, 700 miRNAs from miRBase v.14, and 300 noncoding RNAs.

3.2.6 Functional mRNA Assay for Msx1 and Kremen1

All methods under this section were performed by Yasmin Issa. Tissue sampling was conducted at dental clinics' settings. The remaining methods under this section were conducted at the Hereditary Research Lab/Bethlehem University.

3.2.6.1 Isolation of RNA from Intraoral Tissues

3.2.6.1.1 Harvesting Mucoperiosteal Tissue

A semi-surgical procedure was performed for harvesting periosteal tissue from selected probands. After obtaining informed consents, alveolar mucoperiosteal specimens were obtained from selected probands in addition to a healthy donor undergoing implant placement surgery. Following administration of a local anesthetic, a minimal semilunar incision was performed, followed by excavation of periosteal tissue (about $1cm^2$) under sterile conditions. Harvested tissues were immediately placed in microfuges containing RNAlater® RNA Stabilization Reagent (Cat#76104). The healthy donor's sample was obtained from the lower anterior region and the probands were operated on a posteriofacial site (Fig. 3.1). The surgical sites were closed using sutures and postoperative medicaments were prescribed to minimize the possibility of infection and relieve any pain and/or swelling.

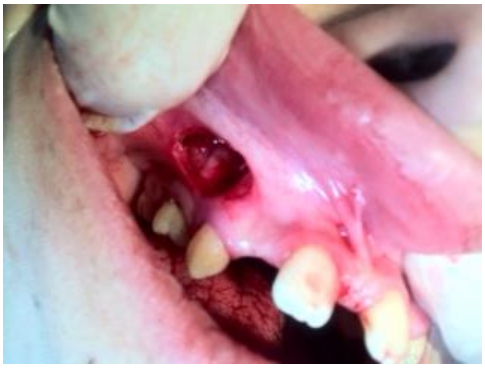


Fig. 3.1: Semilunar incision of the upper right quadrant of individual III-8 in family1 and mucoperiosteal tissue sampling.

3.2.6.1.2 RNA Extraction from Harvested Tissues

Mucoperiosteal samples stabilized in RNALater were placed in liquid nitrogen and ground thoroughly using a mortar and a pestle (previously sterilized). Homogenization was performed using a syringe and a 20-gauge needle. After homogenization, 10µl of 20 mg/ml proteinase K solution (Amresco Cat# E195-25ML) was added to disrupt connective tissues and proteins followed by incubation at 55°C for 10 minutes and centrifugation for 3 minutes at 10,000g and 25°C. RNeasy Protect mini kit (Qiagen Cat# 74124) was used to proceed to RNA extraction according to the RNeasy® Mini Handbook Protocol: Purification of Total RNA from Animal Tissues.

3.2.6.2 Isolation of RNA from Blood

The protocol involved the use of Trizol for RNA extraction:

- Five ml of blood was taken from each proband and kept in EDTA tubes.
- 45 ml of RBC lysis buffer was added to each tube, kept on ice for 30 min. and centrifuged at 2000 rpm and 4°C for 10 min.
- The supernatant was carefully removed and the pellet was re-suspended in 10 ml RBC lysis buffer and centrifugation was repeated.
- After discarding the supernatants, 1ml of Trizol (Invitrogen Cat# 15596-018) was added to each tube to re-suspend the pellets and samples were transferred to 1.5-ml-tubes
- After vigorous hand shaking for 1 minute the samples were incubated at room temperature for 5 min.
- 200µl of Chloroform (Biolabs Cat# 67-66-3) was added per tube, followed by shaking for 15 sec and incubation for 3 minutes.

- Samples were spun at full speed for 15 min. at 4°C.
- The colorless phase was transferred to new tubes and 500µl of isopropanol was added, followed by incubation at room temperature for 10 min.
- Samples were spun at full speed for 10 min. at 4°C.
- Supernatants were discarded and pellets were washed once with 1ml of 75% ethanol.
- Samples were spun again at full speed and 4°C for 10 min.
- Supernatants were removed completely and tubes were left open for 5 min. to let the pellets dry.
- 25µl of pure water was added to each pellet to redissolve it.

3.2.6.3 Reverse Transcriptase-PCR

Extracted RNA was used as a template for reverse transcription, using the qScript™ cDNA Synthesis Kit (Quanta Biosciences-Cat#95047-500) according to the manufacturer's protocol and program.

3.2.6.4 Quantitative Real Time PCR

Quantitative PCR was applied for the detection and quantification of Kremen1 cDNA using the Power SYBR® Green PCR Master Mix (P/N 4367659) on the 7300 Real Time PCR System (Applied Biosystems). Equal cDNA amounts (100ng) were used and relative quantitation of the target Kremen1 cDNA was performed against an internal standard (Abl gene). The results were displayed using the Sequence Detection Software (SDS) Version 1.4 (Applied Biosystems).

CHAPTER 4

Results

4.1 Clinical Examination of Family1

Family1 from Yatta region (south of Hebron) was recruited and a comprehensive oral exam was conducted on family members for pedigree construction (Fig. 4.3). First generation phenotypes were deduced by family interviews. The rest of the individuals were examined and diagnosed for hypodontia. For most individuals, intraoral photographs were used to record presence/absence of hypodontia. Panoramic X-rays confirmed the presence of permanent tooth germs in the youngest individuals IV-1, IV-2 and IV-3

Affected individuals display non-syndromic oligodontia/hypodontia of the permanent dentition with variable severity (no systemic conditions were observed). It can be deduced from family history that this form of hypodontia spares the primary teeth. The most commonly missing teeth are third molars followed by the upper second premolars, lower first and second premolars, upper lateral incisors and lower incisors. Number of total missing permanent teeth ranges from 5 to 22 per individual. Phenotypic observations of the number and location of missing teeth for the examined affected individuals are presented in Table 4.1.

Individual	Gender	Age	Number of Missing Teeth in each jaw including third molars	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
II-4	M	50y	11 7	18	Max Man	* * * * * * * * *	* * * * * * * * * *
III-3	F	25y	6 7	13	Max Man	* * * * * *	* * * * * * *
III-5	F	24y	10 12	22	Max Man	* * * * * * * * * * *	* * * * * * * * * * * *
III-7	M	21y	3 2	5	Max Man	* * *	* *
III-8	F	16y	8 5	13	Max Man	* * * * * *	* * * * * * *

Table 4.1: Phenotypes of the examined affected family1 members. Missing teeth are indicated with an asterisk. In dentition: (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.

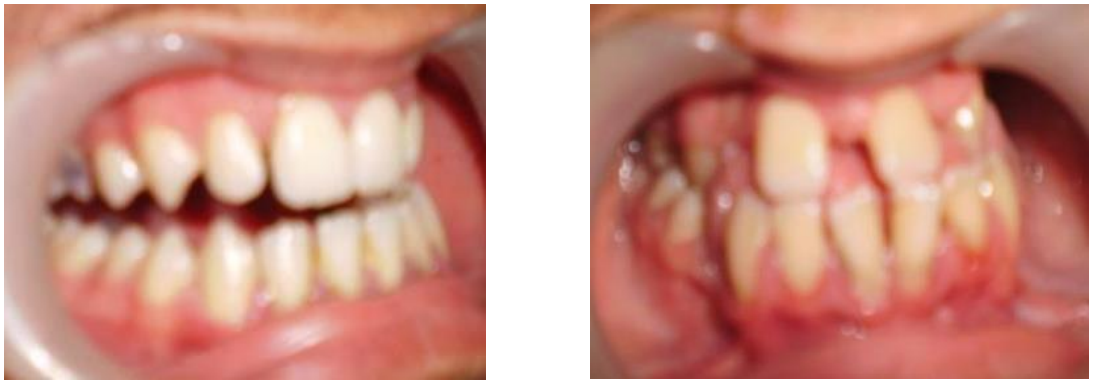


Fig. 4.1: Variability of the expression of hypodontia in affected individuals of family1. **Left:** intraoral view of affected individual III-8 shows a missing upper right first premolar, minimal spacing and esthetically acceptable teeth. **Right:** intraoral view of affected individual III-7 shows a more severe form of hypodontia with missing upper lateral incisors and almost all premolars, large diastema and spacings and hypoplastic darker teeth with square-shaped upper central incisors.

4.2 Sanger Sequencing of the Candidate Genes in Family1

4.2.1 Sanger Sequencing of Msx1 Gene

DNA template of the individual II-4 was PCR amplified for the coding sequences of Msx1 gene using the primers in Table 3.1. Sanger sequencing of the PCR product revealed two mutations in the first exon (Fig.4.2).

The first is a heterozygous point substitution (c.306 C>T) leading to p.Pro24Leu. According to the prediction tool PolyPhen-2 it is probably damaging with a score of 1.000 (Adzhubei et al., 2010). A second heterozygous mutation (c.704 A>T) is located at the 3' end of exon1 and is expected to affect the splicing machinery (based on NM_002448.3).

Both mutations are found to segregate with hypodontia in the family in an autosomal dominant manner. All the affected individuals are heterozygous for both mutations, whereas all the unaffected are wild-type.

4.2.2 Sanger Sequencing of Pax9 and Axin2 Genes

The sequencing of the coding sequences of individual II-4 using the primers in Table 3.1 revealed a wild-type genotype.

4.3 Sequencing of the Coding Msx1 Exon1 Sequence and its Non-coding Boundaries in 200 Palestinian Controls

To confirm the pathogenicity of Msx1 mutations in family1, 200 Palestinian control subjects were sequenced using the Msx1 DNA primers in Table 3.1. Both point substitutions (c.306 C>T and c.704 A>T) were absent in all controls.

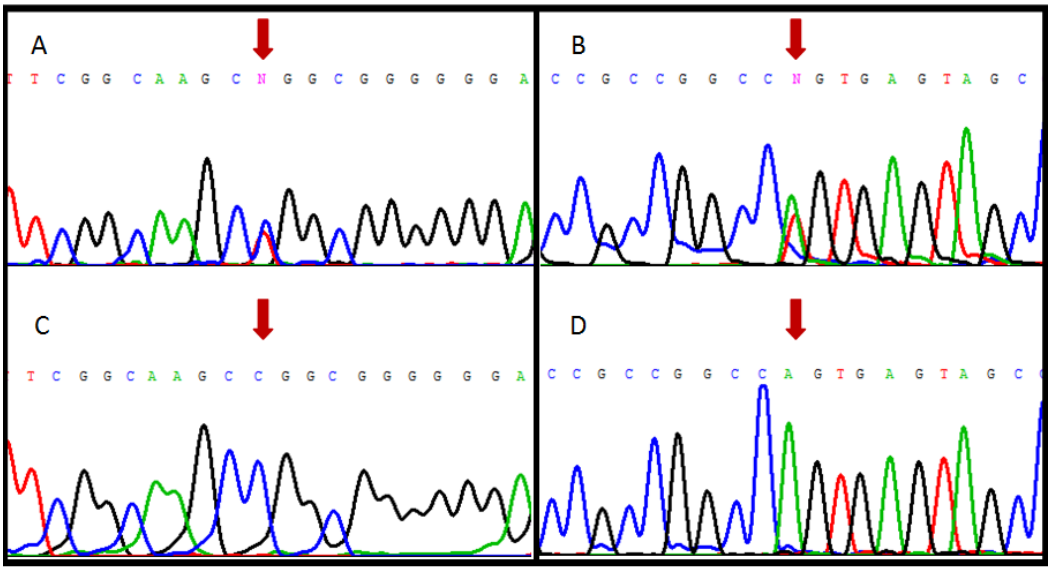


Fig. 4.2: DNA sequencing chromatograms showing the location of both mutations of Msx1 gene in family1. **(A)** First mutation found in all affected individuals is a heterozygous c.306 C>T that is predicted to result in p.P24L **(B)** Second mutation that segregates in affected individuals is a heterozygous c.704 A>T which produces aberrant mRNA splicing. **(C)** and **(D)** Unaffected individuals are wild-type.

4.4 Functional mRNA Splicing Analysis of Msx1

To determine the influence of the second Msx1 mutation on mRNA splicing, mRNA was harvested from intraoral mucoperiosteal tissue of a control and of individual III-8. Examination of the surgical site (Fig. 3.1) revealed a thin alveolar ridge and poor bone quality of the affected individual when compared to the normal control. Msx1 cDNA of both was PCR-amplified (primers in Table 3.1).

Only one band could be seen after running the PCR product of III-8 on the agarose gel, corresponding to the mutant paternal allele and it is shorter than the normal control band (Fig. 4.4). The absence of a maternal band of a normal size could be attributed to a silencing mechanism of the maternal allele.

Sequencing of the mutant paternal band revealed a 166-bp deletion (Fig. 4.5, Fig. 4.6). This causes a shift in the reading frame that changes the coding of amino acids after the residue P106 introducing 54 foreign amino acids followed by a premature stop codon (Fig. 4.6). The resultant truncated MSX1 protein is a composite that contains a few of the native N-terminal domains and is devoid of the homeodomain.

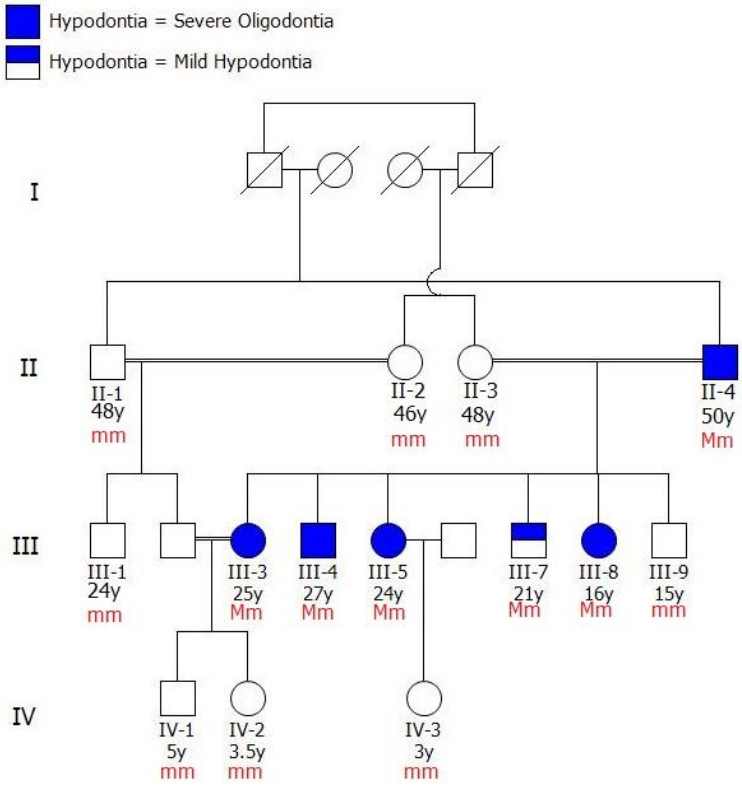


Fig.4.3: Pedigree of family1 showing affection status of sampled pedigree individuals, their ages and Msx1 genotypes. Dental agenesis is inherited in an autosomal dominant manner. Variable expression of the phenotype can be noticed as the least affected individual (III-7) has mild hypodontia and the rest of the affected individuals display different forms of oligodontia.

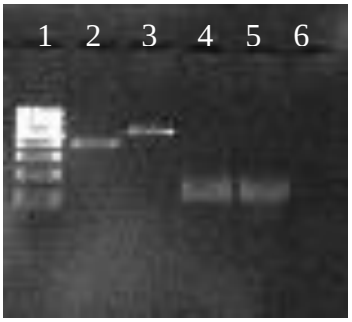


Fig. 4.4: Agarose gel electrophoresis of Msx1 and Abl gene cDNA from mucoperiosteal tissue of family1 individual III-8 and a healthy control. **(1)**100-bp ladder. **(2)** Msx1 gene expression of III-8 shows only the mutant Msx1 paternal allele and this expressed allele is truncated compared to the control. **(3)** Control Msx1 cDNA band of the expected 548-bp-size. **(4), (5)** Positive control: Abl housekeeping gene expression in mucoperiosteal tissue of III-8 and a healthy control, respectively. **(6)** Negative control (ddH_2O).

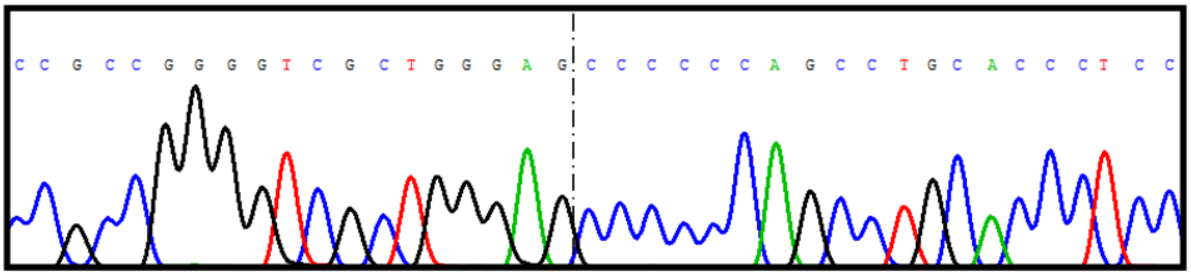


Fig. 4.5: cDNA sequencing chromatogram of Msx1 PCR product in individual III-8 in family1. Partial coding sequence loss of the first and the second exon totals 166bp (the mRNA region 548-704 and 705-714, respectively). The vertical dashed line demarcates the point where both exons meet.

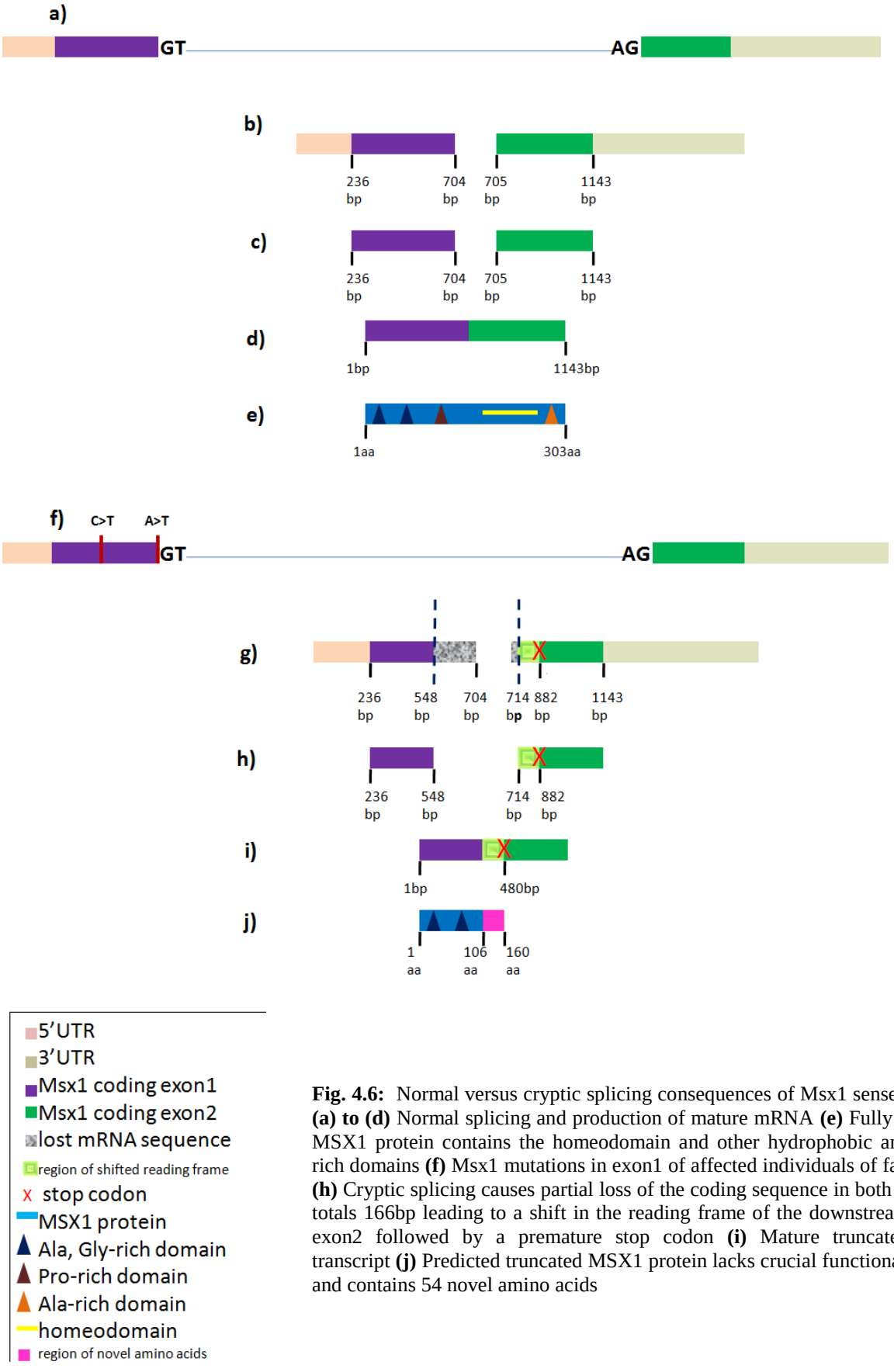


Fig. 4.6: Normal versus cryptic splicing consequences of Msx1 sense transcript **(a) to (d)** Normal splicing and production of mature mRNA **(e)** Fully functional MSX1 protein contains the homeodomain and other hydrophobic amino acid-rich domains **(f)** Msx1 mutations in exon1 of affected individuals of family1 **(g), (h)** Cryptic splicing causes partial loss of the coding sequence in both exons that totals 166bp leading to a shift in the reading frame of the downstream CDS of exon2 followed by a premature stop codon **(i)** Mature truncated mRNA transcript **(j)** Predicted truncated MSX1 protein lacks crucial functional domains and contains 54 novel amino acids

4.5 Clinical Examination of Families 2, 3, 4, 5

Four families displaying oligodontia as the major symptom were seen from Al-Dahriyyeh (Hebron). Families 2 and 3 share the same clan, as do families 3 and 4. Upon interviewing, they did not deny the possibility of sharing a common ancestor but could not however, trace the relatedness despite the frequent consanguineous marriages within each family. Diagnosis was based mainly on clinical examination and panoramic x-rays when present. Similar findings in all four families suggested a common etiology. Syndromic oligodontia is a common finding in severely affected individuals and tends to be inherited in an autosomal recessive manner. They are usually born to unaffected parents with the exception of a few parents displaying a mild form of non-syndromic hypodontia.

The most severely affected individuals displayed systemic findings suggesting they display a special kind of ectodermal dysplasia. The most prominent features of those individuals were:

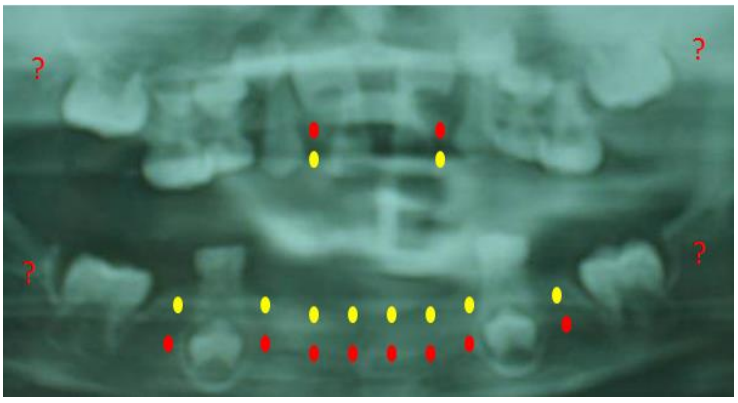
- Variable degrees of oligodontia affecting the primary and permanent dentition. Number of missing teeth ranges from 8-20 missing permanent teeth. The most common missing teeth are upper primary and permanent lateral incisors and lower primary and permanent anterior teeth. The remaining teeth are not conical, but tend to be square-shaped. The alveolar ridges are hypoplastic with an increased palate depth.
- Abnormal hair distribution of the scalp (brittle hair). In addition to that, a prominent fuzzy forehead is a common finding where some individuals display a very prominent widow's peak. Facial hair fuzziness is common. Eyelashes and eyebrows are present but thin.
- Tendency to have markedly protruded lips, a depressed nasal bridge and broad nose associated with hypertelorism and downward slanting of the palpebral fissures.
- One of the most severely affected individuals complained of dry skin that required frequent ointment application. Abnormal sweating was described in other affected individuals.
- A general slender bony structure, where limbs and fingers appear slim. X-rays, however show the bone density to be within normal limits.
- Hypogonadism was reported in one affected male which was corrected with hormonal supplements.

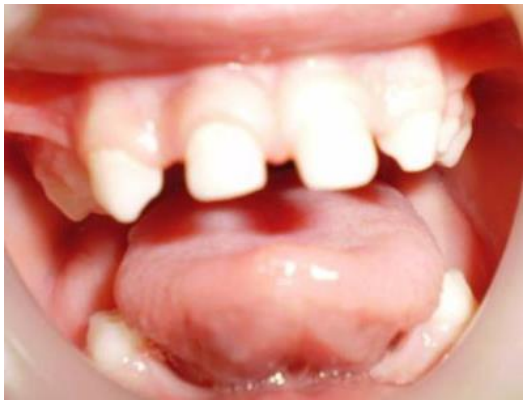
Facial characteristics and the number and location of missing teeth were recorded and pedigrees were constructed accordingly (Fig. 4.16-4.19, Table 4.3).

4.5.1 Clinical Assessment of Family2

Careful examination of family members reveals variable degrees of oligodontia ranging from 6 to 10 permanent teeth. Dental agenesis seems to affect primary teeth as well, as can be noticed in younger individuals (Fig. 4.7). Most commonly absent permanent teeth are the upper lateral incisors and lower anterior teeth, followed by the lower second premolars. Similarly, in the primary dentition upper lateral incisors and lower incisors are most frequently missing (Table 4.3).

All affected individuals except IV-19 display signs of ectodermal dysplasia. Besides the severe lack of teeth and delayed eruption, they have a lower height and width of the alveolar ridges, contributing to a lower vertical dimension of occlusion. In addition to that, most of them have abnormal scalp hair distribution and thin eyebrows. Despite that, they seem to have short, but denser hair on both sides of the forehead. Moreover, low nose bridges, thick lips and slender limb bone structure are not uncommon. Individual V-4 complains of an exceptionally dry skin that requires frequent ointment application. Consanguineous marriages generate severely affected offspring. Individual III-10 is the only parent who is not closely related to his spouse. However, he belongs to the clan of families 3 and 4 which has severely affected individuals. Individual IV-19 shows a less severe form of hypodontia missing only 5 permanent teeth without any systemic abnormalities. The constructed pedigree can be seen in Fig. 4.16.





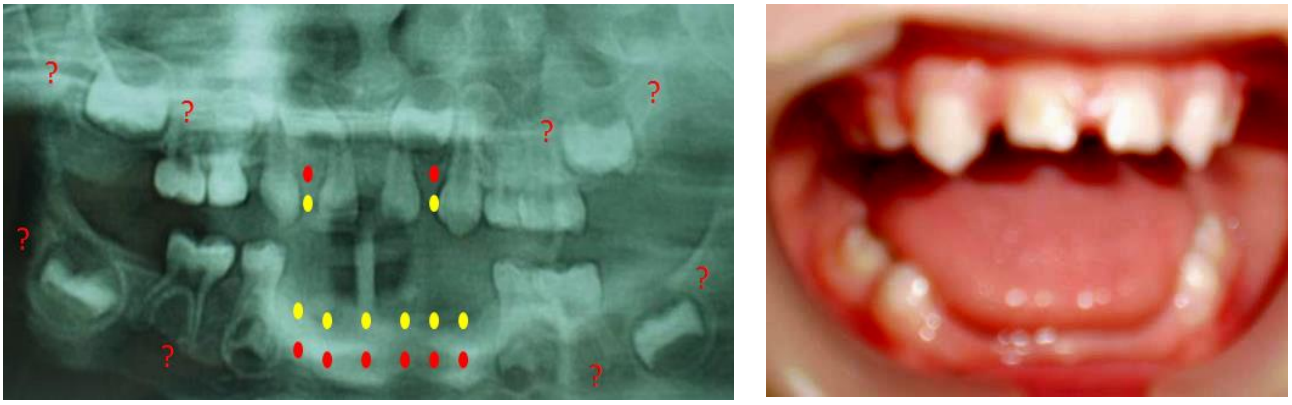


Fig. 4.7: Panoramic x-rays and intraoral views of family 2 affected siblings VI-1 and VI-2. **Upper left panel:** VI-1 presents with 10 missing primary (yellow) and at least 10 missing permanent (red) teeth. **Lower left panel:** VI-2 is missing 8 primary and at least 8 permanent teeth. **Right panel:** Intraoral views of both siblings show spacings and low alveolar bone height. Question marks on X-rays symbolize uncertain presence statuses of the corresponding permanent tooth germs.

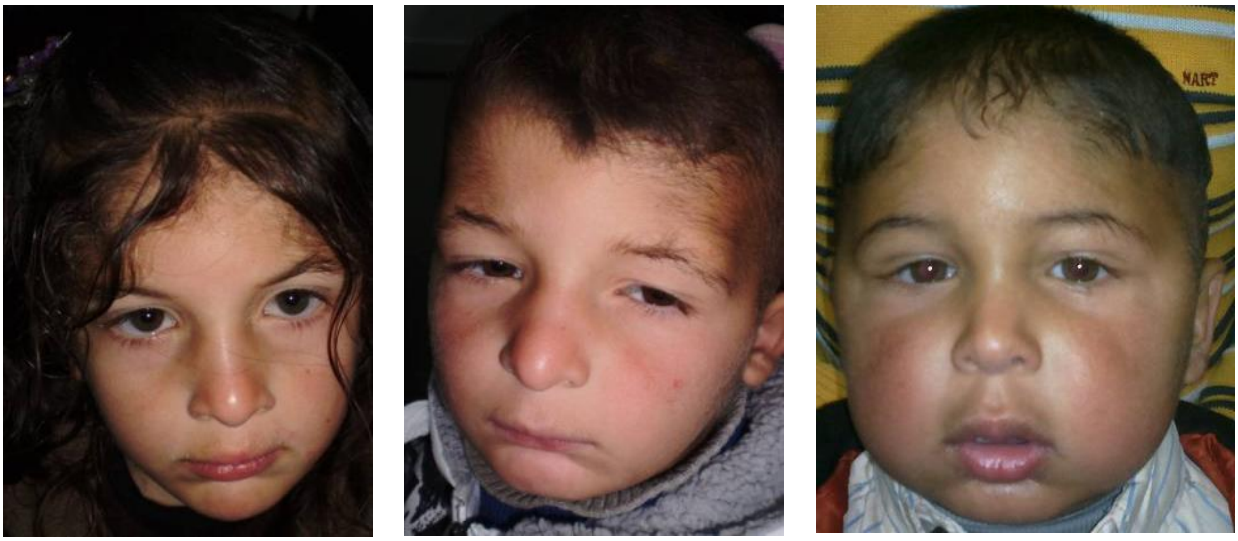


Fig. 4.8: Facial views of affected individuals in family2 VI-1, VI-2 and V-4. **Left to right:** VI-1, VI-2 and V-4. Syndromic ectodermal dysplasia features of abnormal scalp hair, broad nose and thick lips in addition to the feature of forehead and facial fuzziness.

4.5.2 Clinical Assessment of Family3

Number of missing teeth in affected individuals of family3 ranges from 4 to 12 permanent teeth affecting primarily the lower anterior teeth and the upper lateral incisors.

Individuals II-6, II-7 and III-1 have variable forms of hypodontia ranging from 4 to 6 permanent teeth but do not show any abnormally distinguishable facial or bodily signs. III-3 and III-5 on the other hand, (offspring of II-7) present with oligodontia affecting both the primary and permanent dentition in addition to abnormal ectodermal signs including abnormal scalp hair distribution, broad nose, thick everted lips, hair growth on the forehead and hypertelorism (Fig. 4.9 and Fig. 4.17).

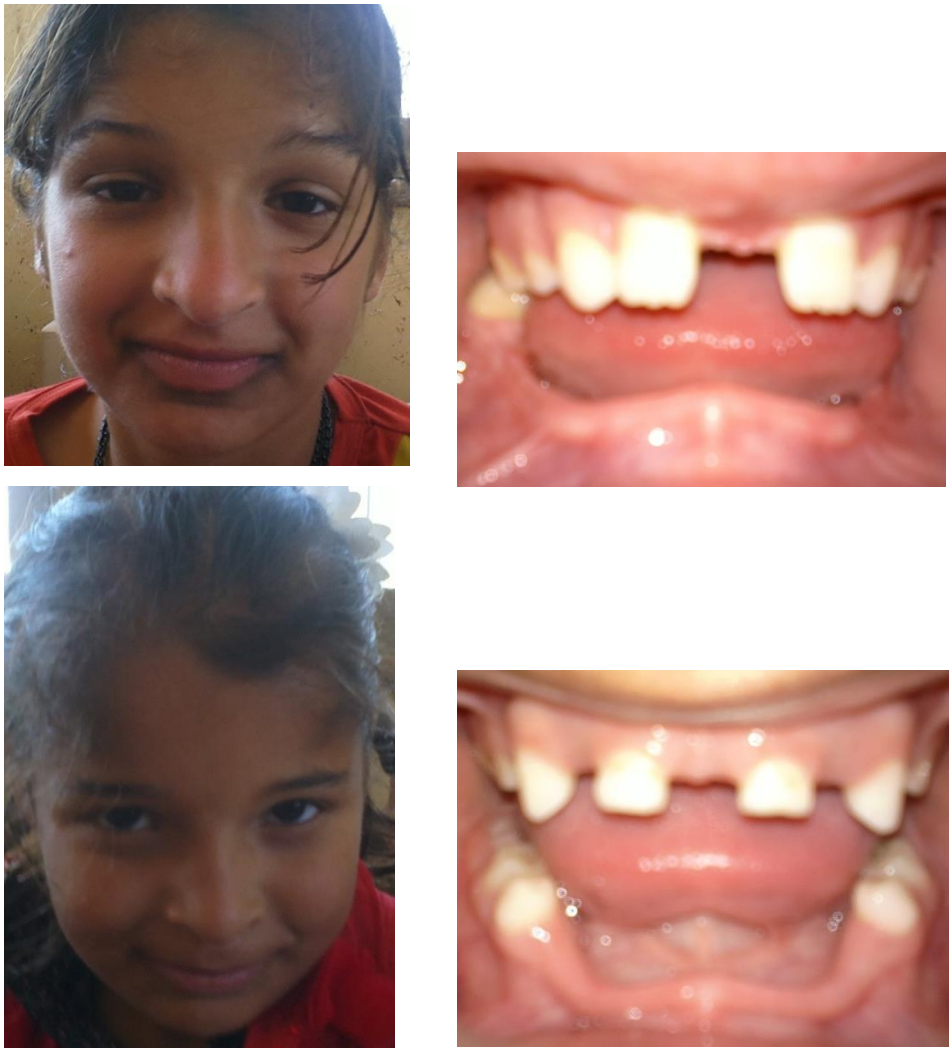


Fig. 4.9: Phenotypic presentation of family3 syndromic features in affected individuals III-3 and III-5. **Upper panel:** 9-year-old individual III-3 presents with ectodermal signs of abnormal distribution of scalp hair along with fuzzy forehead, broad nose and thick lips. This is accompanied by intraoral severe hypodontia. Upper permanent lateral incisors are missing and so the central incisors (which appear square-shaped and are narrower than usual) drifted to their place yielding a large diastema. Other missing teeth involve lower anterior teeth and premolars. **Lower panel:** III-5 is 6 years old and shares characteristic extraoral ectodermal features with III-3. Intraorally, she lacks 8 primary teeth including the upper lateral incisors and lower anterior teeth and shows huge gaps and deficient bone height.

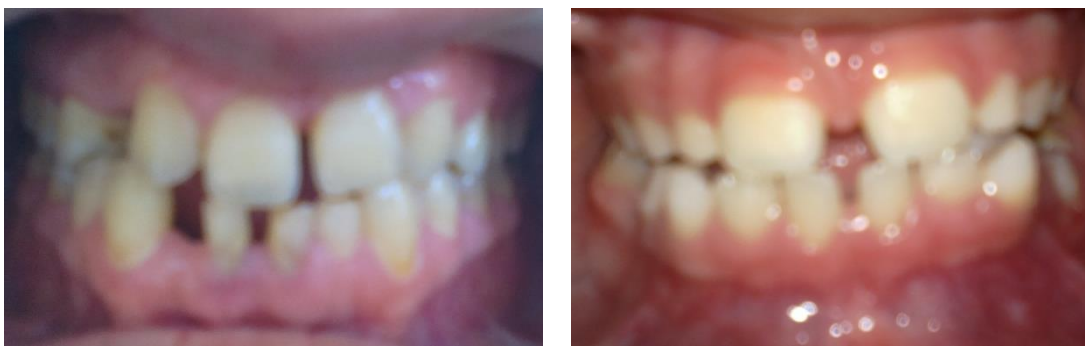


Fig. 4.10: Intraoral views of family3 individuals II-7 and III-1. **Left:** individual II-7 is 28 years old and lacks upper permanent lateral incisors and lower permanent incisors, having 3 retained primary lower incisors**.**

Right: individual III-1is 8 years old and lacks at least 4 permanent lower incisors (the 4 retained primary lower incisors are in place) with probable agenesis of the upper permanent lateral incisors as well. The intraoral view does not contain spacings or bony deficiencies.

4.5.3 Clinical Assessment of Family4

Four individuals were seen from family4, two of which displayed oligodontia along with abnormalities in the ectodermal structures (Fig. 4.17). Common observations include scalp hypotrichosis in certain regions, fuzzy forehead, broad nose, everted lips and hypertelorism (Fig.4.11). Both the primary and permanent dentition of affected individuals is devoid of the upper lateral incisors and lower anterior teeth.

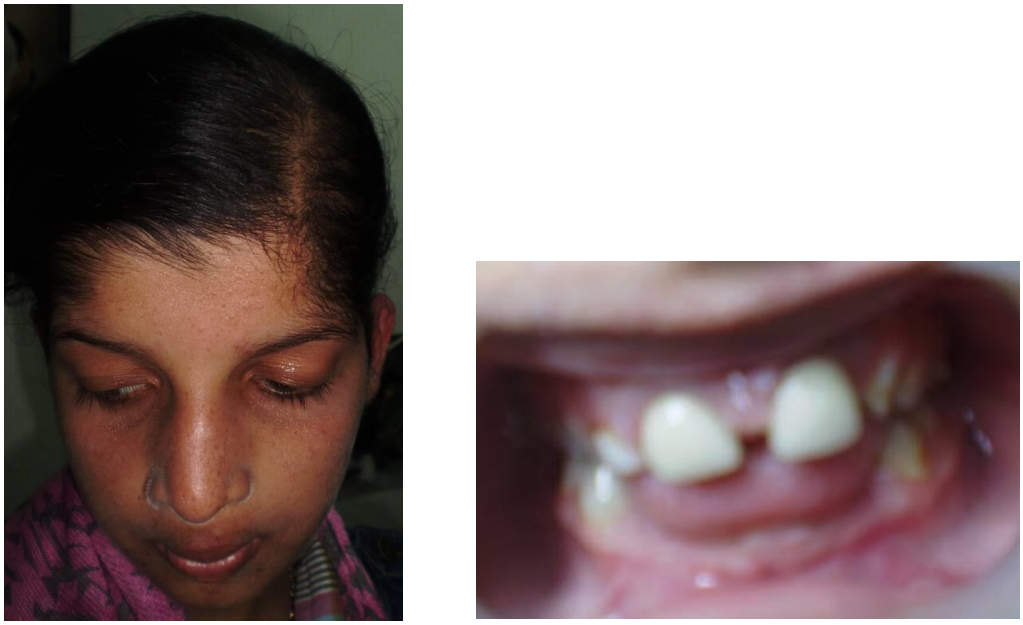


Fig. 4.11: Facial and intraoral view of individual IV-5 in family4. Ectodermal dysplastic features accompany intraoral oligodontia where at least 8 teeth are missing including permanent upper lateral incisors and lower anterior teeth.

4.5.4 Clinical Assessment of Family5

Only the syndromic form of hypodontia is manifested in family5. Two affected members show signs of syndromic oligodontia with similar observations to the previous families. A striking severity of oligodontia is manifested in individual III-2 who lacks all but first molars and second premolars. The severe oligodontia is accompanied by reduced alveolar dimensions (Fig. 4.12). A careful medical history of individual II-8 indicates a loss of at least 8 permanent teeth: upper lateral incisors and lower anterior teeth. On intraoral examination, he presents with an implant work and fixed prosthesis replacement treatment (Table 4.3).

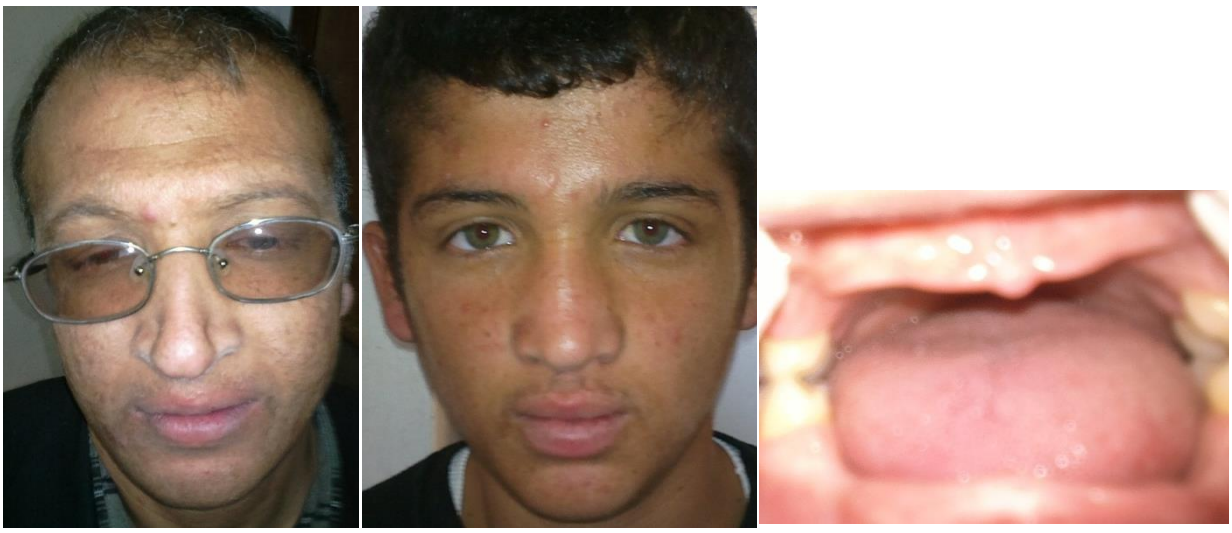


Fig. 4.12: Phenotypic presentation of family5 affected members. **Left to right:** extraoral views of individuals II-8, III-2, respectively showing common signs of ectodermal dysplasia such as thin hair, broad nose and thick lips and intraoral presentation of III-2 showing severe oligodontia with 20 missing permanent teeth including all upper and lower anterior teeth, upper and lower first premolars and upper and lower second molars in addition to absence of their primary predecessors. This is accompanied by alveolar bone deficiency in the maxilla and mandible.

4.6 Genotyping of Family2 for Candidate Genes: Msx1, Pax9 and Axin2

Sanger sequencing of the coding sequences of Msx1, Pax9 and Axin2 candidate genes in the affected individual VI-1 showed no abnormalities.

4.7 SNP Microarray Homozygosity Mapping Results in Family2

Clinical examination of the affected individuals of family2: VI-1 and VI-2, indicates they share a homozygous mutant allele that is responsible for inheriting ectodermal dysplasia. Their parents: IV-8 and V-3 show a normal clinical presentation. The 4 individuals were picked to undergo homozygosity mapping.

4.7.1 Regions of Shared Homozygosity in Affected Family2 Individuals VI-1 and VI-2

Table 4.2 summarizes the shared homozygous regions of the affected individuals of family2 VI-1 and VI-2 as compared to their parents IV-8 and V-3.

4.7.2 Sequencing of the Candidate Nfkbia Gene in Family2

The largest region of shared homozygosity is found on chromosome14. Gene distiller2 has shown it contains 435 genes (Seelow et al., 2008). It contains a candidate gene for

ectodermal dysplasia: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (Nfkbia). However, Sanger sequencing of the 6 coding exons of Nfkbia in both VI-1 and VI-2 individuals revealed no abnormalities.

Chromosome number	Coordinates of homozygous region	Physical length of homozygous region	Number of SNPs in homozygous region
Chr. 14	**32,534,703 – 74,980,816**	**42,446,113**	**3690**
Chr. 1	**55,352,701 – 117,231,902**	**21,879,201**	**1836**
Chr. 22	**27,384,762 – 44,816,607**	**17,431,845**	**1139**
Chr. 13	**27,263,750 – 43,894,796**	**16,631,046**	**1745**
Chr. 6	**166,384,301–170,874,759**	**4,490,458**	**383**
Chr. 6	**8,909,840 – 10,574,312**	**1,664,472**	**467**
Chr. 5	**79,665,635 – 83,334,641**	**3,669,006**	**301**
Chr. 10	**45,419,333 – 47,640,757**	**2,221,424**	**56**

Table 4.2: Homozygosity mapping results of family2 affected individuals VI-1 and VI-2.

4.8 Exome Sequencing Analysis Results in Family2

The four previously selected individuals from family2: IV-8, V-3, VI-1 and VI-2 underwent whole exome sequencing under the HiSeq 2000 Illumina platform. DNA variants were classified by predicted function: nonsense mutations, frameshift mutations, variants within 1 bp of a splice site, and putatively damaging missense variants. Four homozygous variants were shared among both VI-1 and VI-2. They are: PMM1_p.E160stop at chr22:41974882, C>A; KREMEN1_p.F209S at chr22:29521399 T>C; USP20_p.V457M at chr9:132631681 G>A and ALDH6A1_p.F497L at chr14:74531537 G>C.

The pathogenicity potential of the KREMEN1_p.F209S variant was evaluated using PolyPhen-2 and was predicted to be probably damaging according to the HumVar model with a score of **0.997** (sensitivity: **0.27**; specificity: **0.98**). In addition to that, F209 is highly conserved in vertebrates (Adzhubei et al., 2010) (Fig. 4.13).

4.9 Sanger Sequencing Results of Candidate Kremen1 Gene in Family2

To assess the causality of the homozygous c.T679C KREMEN1 p.F209S variant that was identified by NGS in the affected individuals, the target DNA base positions (Kremen1 isoform2 mRNA accession number NM_032045) and their flanking sequences were amplified from DNA samples of family members using the primers in Table 3.1. The PCR products were later sequenced in both forward and reverse directions.

All affected individuals that display the combination of characteristic ectodermal appearance with severe oligodontia are homozygous for the mutation. Parents who lack ectodermal abnormalities are heterozygous carriers. Individual IV-19 presents with a non-syndromic form of hypodontia and lacks 5 permanent teeth (Fig. 4.14), (Table 4.3). The results suggest the presence of both non-syndromic and syndromic genetic determinants (Fig. 4.16).

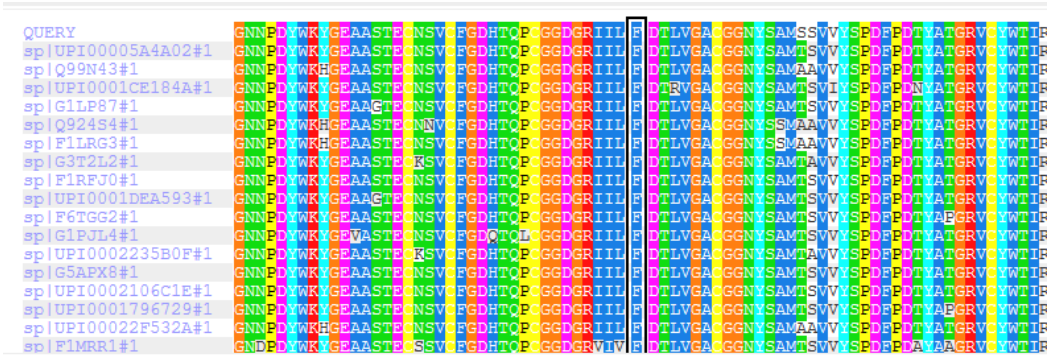


Fig. 4.13: Multiple protein sequence alignment (PolyPhen-2 embedded) of KREMEN1 harboring F209. The 75 amino acids surrounding the mutation site F209 indicate the conservation of this sequence in vertebrates (Adzhubei et al., 2010).

4.10 Sanger Sequencing Results of Candidate Kremen1 Gene in Families 3, 4, 5 and Palestinian Controls

The coding Kremen1 region harboring the mutation c.T679C was sequenced for all recruited members of families 3, 4 and 5. The results obtained were similar to family2. The most severely affected individuals were homozygous for the mutation. Individuals who displayed non-syndromic hypodontia were heterozygous as well as most of the individuals who appeared unaffected. The respective pedigrees are shown in figures 4.17, 4.18 and 4.19. A summary of the Kremen1 genotype/phenotype findings for families 2, 3, 4 and 5 can be found in Table 4.3.

To confirm the pathogenicity of the mutation, Palestinian controls were sequenced for the Kremen1 mutation site. Heterozygosity was found to be 1/200.

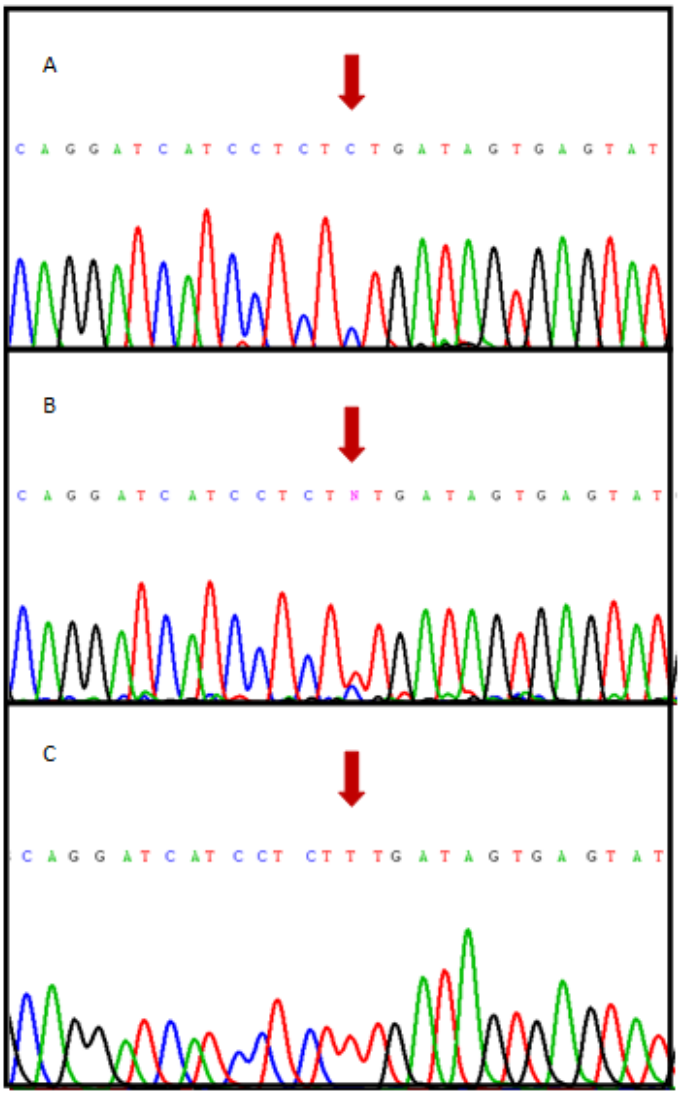


Fig. 4.14: Sanger sequencing results of the NGS candidate Kremen1 gene in families 2-5**(A)** The segregation of the homozygous mutant allele c.T679C with the syndromic ectodermal observations and severe hypodontia **(B)** All parents of affected individuals are heterozygous at the mutation site **(C)** Wild-type genotype of unaffected individuals: IV-2 of family2 and III-3, III-4, III-5, III-6 from family5.

4.11 Functional Kremen1 mRNA Analysis

The expression of Kremen1 was evaluated in selected individuals from families 2 and 5.

4.11.1 Kremen1 mRNA Analysis in Blood

4.11.1.1 PCR and Sequencing of Kremen1 cDNA (Blood)

Total RNA was isolated from the peripheral blood of family2 individuals: IV-8, IV-19. VI-1and IV-2 and converted to cDNA.

Kremen1 cDNA band harboring the mutation was produced in the 4 individuals using the exonic Kremen1 primer pair in Table 3.1.

A single PCR amplicon band representative of a mutant Kremen1 transcript was produced per individual. Sequencing results per individual replicated the genomic findings (Fig. 4.15).

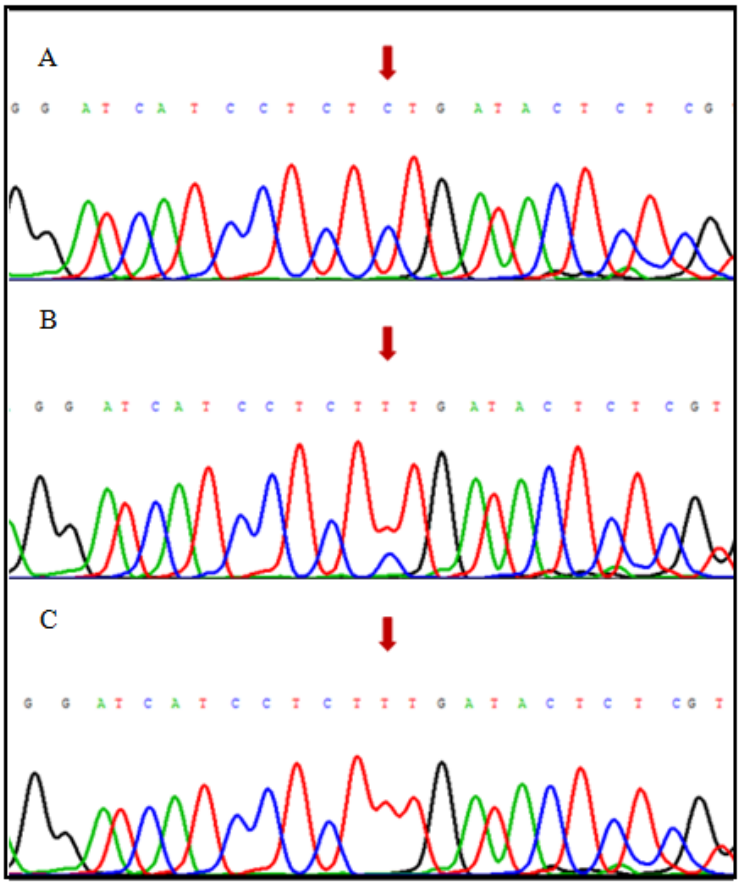


Fig. 4.15: Sanger sequencing results of Kremen1 cDNA in family2 **(A)** Individual VI-1is homozygous for c.T679C **(B)** Individuals IV-8 and IV-19 are heterozygous for the mutation **(C)** Wild-type genotype of the individual IV-2.

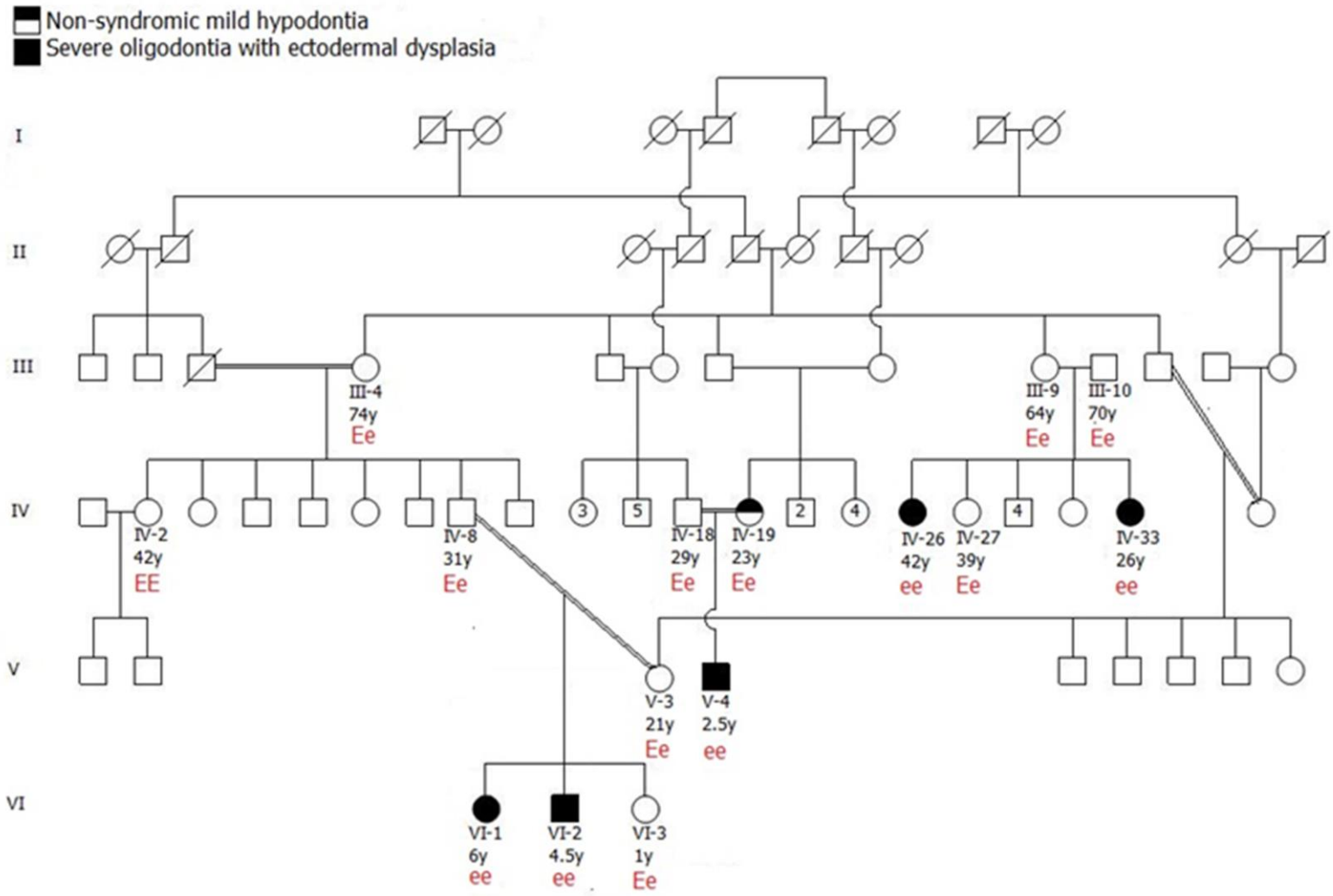


Fig. 4.16: Pedigree of family2 displaying age, phenotypic presentation and Kremen1 genotype of sampled individuals. Highly consanguineous marital relationships produce offspring with two copies of the mutant Kremen1 allele that segregate with ectodermal abnormalities and severe hypodontia in the primary and permanent dentition in individuals IV-26, IV-33, V-4, VI-1 and VI-2. IV-19 presents with non-syndromic hypodontia.

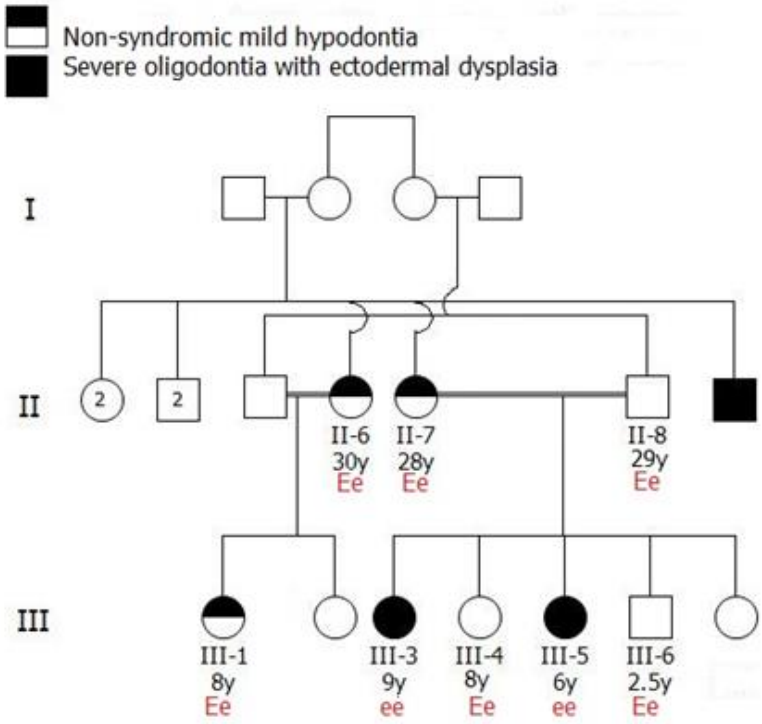


Fig. 4.17: Pedigree of family3 displaying age, phenotypic presentation and Kremen1 genotype of sampled individuals. Homozygous Kremen1 mutants III-3 and III-5 display syndromic oligodontia. Heterozygous Kremen1 carriers display no syndromic ectodermal findings. Non-syndromic hypodontia is observed in individuals II-6, II-7 and III-1.

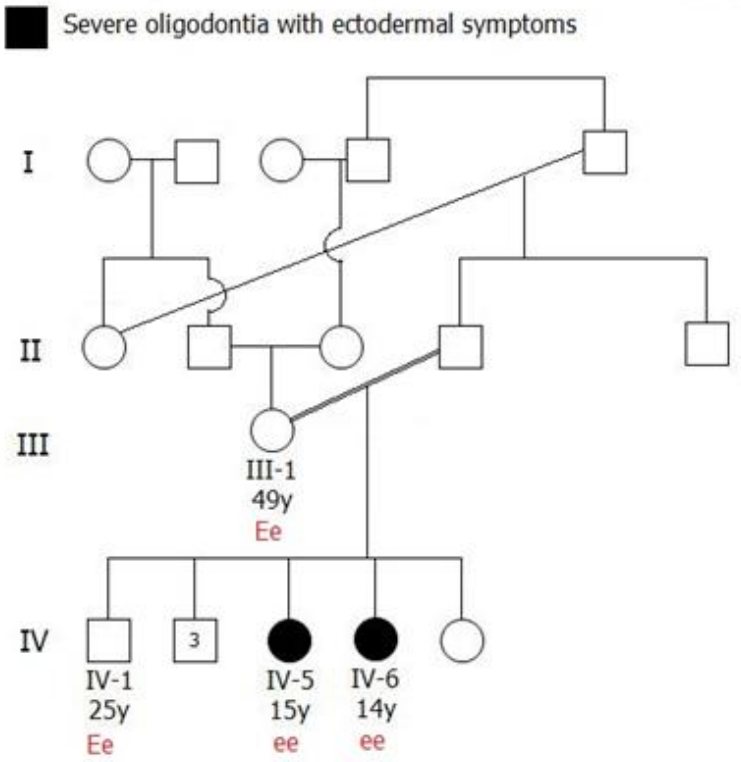


Fig. 4.18: Pedigree of family4 displaying age, phenotypic presentation and Kremen1 genotype of sampled individuals. All affected members are homozygous for Kremen1 mutation and inherit severe oligodontia with ectodermal dysplasia in an autosomal recessive manner.

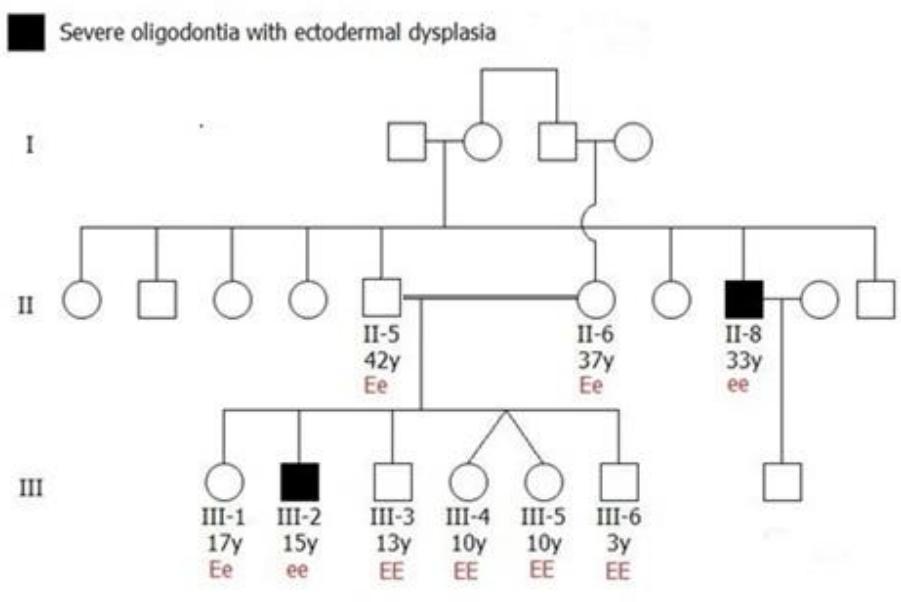


Fig. 4.19: Pedigree of family5 displaying age, phenotypic presentation and Kremen1 genotype of sampled individuals. Ectodermal dysplasia along with severe tooth agenesis accompanies mutant Kremen1 homozygotes II-8 and III-2. None of the heterozygotes displays any syndromic abnormalities.

4.11.1.2 Real Time Quantitative PCR (Blood)

Relative quantification of Kremen1 cDNA PCR products using the dsSYBR green dye did not show any significant differences between the 4 individuals (α=0.005).

4.11.2 Kremen1 Expression in Oral Mucoperiosteum

Mucoperiosteal tissue samples were collected from 3 individuals: 2 heterozygous individuals from family2: IV-8 and IV-19 and the homozygous individual III-2 from family5.

4.11.2.1 Kremen1 qPCR (Mucoperiosteum)

Relative quantification of the Kremen1 gene expression did not reveal notable variation between the 3 individuals (α=0.005).

Individual	Age	Kremen1 F209S genotype	Gender	Presence of Hypodontia (Yes/No)	Presence of ectodermal abnormalities (hair, lips, nose) (Yes/No)	Total number of primary genetically missing teeth	Number of permanent genetically missing teeth	Jaw	**Right Side** ◊ 7 6 5 4 3 2 1	**Left Side** 1 2 3 4 5 6 7 ◊
Fam2 III-4	74y	Hetero	Female	No	No	Not known (exfoliated)	None			
Fam2 III-9	64y	Hetero	Female	No	No	Not known (exfoliated)	None			
Fam2 III-10	70y	Hetero	Male	Not known	No	Not known (exfoliated)	Not known (all extracted)			
Fam2 IV-2	42y	WT	Female	No	No	Not known (exfoliated)	None			
Fam2 IV-8	31y	Hetero	Male	No	No	Not known (exfoliated)	None			
Fam2 IV-18	29y	Hetero	Male	No	No	Not known (exfoliated)	None			
Fam2 IV-19	23y	Hetero	Female	Yes	No	Not known (exfoliated)	5	Upper Lower	* *	* * *
Fam2 IV-26	42y	Homo	Female	Yes	Yes	Not known (exfoliated)	6	Upper Lower	* * *	* * *
Fam2 IV-27	39y	Hetero	Female	No	No	Not known (exfoliated)	None			
Fam2 IV-33	26y	Homo	Female	Yes	Yes	Not known (exfoliated)	8	Upper Lower	* * * *	* * * *
Fam2 V-3	21y	Hetero	Female	No	No	Not known (exfoliated)	None			
Fam2 V-4	2.5 y	Homo	Male	Yes	Yes	6	Not known (not	Upper Lower	* * *	* * *

							erupted)		
Individual	Age	Kremen1 F209S genotype	Gender	Presence of Hypodontia (Yes/No)	Presence of ectodermal abnormalities (hair, lips, nose) (Yes/No)	Total number of primary genetically missing teeth	Number of permanent genetically missing teeth	Jaw	**Right Side** ◊ 7 6 5 4 3 2 1 / **Left Side** 1 2 3 4 5 6 7 ◊
Fam2 VI-1	6y	Homo	Female	Yes	Yes	10	At least 10	Upper Lower	* * * * * / * * * *
Fam2 VI-2	4.5 y	Homo	Male	Yes	Yes	8	At least 8	Upper Lower	* * * * / * * *
Fam2 VI-3	1y	Hetero	Female	Not known	No	Not known (not erupted)	Not known (not erupted)		
Fam3 II-6	30y	Hetero	Female	Yes	No	Not known (exfoliated)	6	Upper Lower	* * * / * *
Fam3 II-7	28y	Hetero	Female	Yes	No	Not known (exfoliated)	6	Upper Lower	* * * / * *
Fam3 II-8	29y	Hetero	Male	No	No	Not known (exfoliated)	None		
Fam3 III-1	8y	Hetero	Female	Yes	No	2?	4 or 6	Upper Lower	? * * / ? * *
Fam3 III-3	9y	Homo	Female	Yes	Yes	12 (expected)	12	Upper Lower	* * * * * * / * * * * *
Fam3 III-4	8y	Hetero	Female	No	No	None	None		

Individual	Age	Kremen1 F209S genotype	Gender	Presence of Hypodontia (Yes/No)	Presence of ectodermal abnormalities (hair, lips, nose) (Yes/No)	Total number of primary genetically missing teeth	Number of permanent genetically missing teeth	Jaw	Right Side ◊ 7 6 5 4 3 2 1	Left Side 1 2 3 4 5 6 7 ◊
Fam3 III-5	6y	Homo	Female	Yes	Yes	8	Not known (too young)	Upper Lower	* * * *	* * * *
Fam3 III-6	2.5 y	Hetero	Male	No	No	None	Not known (too young)			
Fam4 III-1	49y	Hetero	Female	No	No	Not known	None			
Fam4 IV-1	25y	Hetero	Male	No	No	Not known	None			
Fam4 IV-5	15y	Homo	Female	Yes	Yes	8	At least 8	Upper Lower	* * * *	* * * *
Fam4 IV-6	14y	Homo	Female	Yes	Yes	8	At least 8	Upper Lower	* * * *	* * * *
Fam5 II-5	42y	Hetero	Male	No	No	Not known	None			
Fam5 II-6	37y	Hetero	Female	No	No	Not known	None			
Fam5 II-8	33y	Homo	Male	Yes	Yes	Not known (exfoliated)	At least 8 (implants and fixed prosthesis in place)	Upper Lower	* * * *	* * * *
Fam5 III-1	17y	Hetero	Female	No	No	Not known	None			
Fam5 III-2	15y	Homo	Male	Yes	Yes	All primary (20)	20	Upper Lower	* * * * * * * * * * * *	* * * * * * * * * * * *
Fam5 III-3	13y	WT	Male	No	No	None	None			
Fam5 III-4	10y	WT	Female	No	No	None	None (expected)			

Fam5 III-5	10y	WT	Female	No	No	None	None (expected)		
Fam5 III-6	4y	WT	Male	No	No	None	Not known (not erupted)		

Table 4.3: Kremen1 F209S genotype/phenotype relationships of families 2, 3, 4 and 5. *Homo:* homozygous*; Hetero:* heterozygous*; WT:*wild-type.
◊: third molars are excluded on examination
?: unknown status of tooth
Asterisks in yellow: missing primary teeth
Asterisks in red: missing permanent teeth
Individuals in green: heterozygous carriers displaying non-syndromic hypodontia
Individuals in red: homozygous showing severe hypodontia and ectodermal abnormalities
Individuals in black: heterozygous carriers/or wild-type individuals showing no hypodontia and no ectodermal abnormalities

CHAPTER 5

Discussion

In this thesis, we report novel mutations that are responsible for the development of hypodontia in 5 Palestinian families. Mutational analysis in one family identified two heterozygous mutations in the first exon of Msx1 gene to co-segregate with non-syndromic autosomal dominant oligodontia. The first is a missense mutation that is predicted to result into 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. Both mutations screened negative in 200 Palestinian control subjects. The 4 other families were clinically examined and found to share common signs of ectodermal dysplasia including oligodontia of the primary and permanent dentition. Whole exome sequencing of family2 members identified a causative mutation in Kremen1 gene that is predicted to result in an amino acid change p.Phe209Ser. Sanger sequencing confirmed that Kremen1 is the causative gene in families 2, 3, 4 and 5. The syndromic disorder of ectodermal dysplasia is transmitted in an autosomal recessive manner. Homozygous mutant alleles are a common finding due to consanguinity and are responsible for symptoms that collectively constitute a special form of ectodermal dysplasia. Heterozygosity of this mutation in the Palestinian population is 0.5%. This is the first finding that implicates Kremen1 in a genetic disorder.

MSX1 homeoprotein is a transcriptional repressor of differentiation that is expressed at sites where cellular proliferation and apoptosis occur during pattern formation in embryogenesis. It demarcates the area from which neural crest cells emigrate. It also participates in specifying their differential fates as they populate the branchial arches, shape the skull and its sensory structures and confer positional identity to the dental field by taking part in the odontogeic homeobox code. (Bendall and Abate-Shen, 2000). Mutations in Msx1 gene have been associated with autosomal dominant selective tooth agenesis that affects mainly premolars and third molars (Kim et al., 2006).

Similarly in our study, third molars and first premolars are the most frequently absent teeth in family1. In addition to that, there is variability in the number and location of missing teeth among the affected individuals. Individual III-7 is the least affected with only 5 missing teeth. When compared to his siblings, his teeth show a more acceptable morphological appearance with a better alignment and fewer gaps and alveolar defects (Fig. 4.1). The phenotypic

differences among affected individuals could be attributed to modifying factors including the molecular environment, genetic background, environmental factors and antisense transcriptional regulation.

In order to investigate the influence of the splicing mutation on Msx1 expression, total RNA was harvested from the intraoral mucoperiosteal tissues of the affected member III-8. Even though our findings reflect Msx1 expression in postnatal adult tissues, similar consequences are expected to have occurred during the development of teeth and alveolar bone. Functional mRNA analysis of Msx1 gene in individual III-8 shows a truncated Msx1 transcript that is the result of paternal allele expression and an absence of the maternal allele (Fig. 4.4). There are several possible interpretations to this observation: (i) presence of a higher dosage of the paternal allele transcript (ii) there might be a silencing process of the maternal allele through an epigenetic mechanism. The offspring of affected mothers III-3 and III-5 however are uninformative since its members are wild-type. Epigenetic control of Msx1 by imprinting has been suggested to play a role in non-syndromic cleft lip and palate reflecting a higher risk of paternally transmitted alleles to be disease-causing (Suazo et al., 2010). Furthermore, the disrupted balance between sense-antisense Msx1 transcripts may be somehow involved in the phenotypic presentation. Long *cis* AS transcripts have been shown to exert regulatory functions on protein expression on the level of epigenetic imprinting, RNA maturation, edition and translation inhibition (Lavorgna et al., 2004). Aside from that, improvements in technology have recently unveiled the widespread nature of random monoallelic expression across the genome (Chess, 2012). This could account for the silencing of the wild-type allele in a time and tissue-specific manner and generating the severe phenotype that is seen in this individual.

The dominant phenotype of hypodontia in our study is most probably due to MSX1 haploinsufficiency rather than a dominant negative effect. The splicing mutation produces a loss-of-function effect secondary to aberrant mRNA splicing and a shift in the reading frame, introducing 54 foreign amino acids in the predicted protein after residue 106 and a premature stop codon, yielding a mutant and truncated protein composed of 160 amino acids. It is unlikely that this transcript is subject to nonsense mediated decay because the termination occurs in the last exon and there is no downstream exon-exon junction (Maquat, 2005). However, since the mutant allele produces very little of the native MSX1 protein sequence (106 of 303 amino acids), lacking the homeodomain, it is presumed inactive. Msx1 mutant proteins lacking their homeodomains are localized in the cytoplasm according to earlier findings (Wang et al., 2011). Therefore our truncated MSX1 protein is unlikely to interfere with the DNA- and protein-protein

interactions of MSX1 expressed from the wild-type allele when present. Previous studies encountered nonsense mutations in Msx1 gene. An Msx1 nonsense mutation (p.Ser105stop) produced oligodontia along with cleft lip and palate (CL+P) (van den Boogaard et al., 2000; De Muynck et al., 2004). Another Msx1 nonsense mutation, p.Ser202stop, produced oligodontia with nail dysplasia (Jumlongras et al., 2001).

On the other side, there is also a possibility that the splicing mutation is 'leaky' in some of the affected individuals, holding the missense mutation accountable for the lost function as a consequence to the greater hydrophobicity of Leucine and the resultant three dimensional change at the position of Proline. Cases exist where splicing mutations are partially penetrant generating both mutant and wild-type transcripts (Svenson et al., 2001).

Ectodermal dysplasia comprises a large group of syndromes deriving from abnormalities of the ectodermal structures. Despite the myriad of genetic causes that lead to the development of this disorder, there is a great similarity in the symptoms (Visinoni et al., 2009). KREMEN1 has been shown to have a dual role in Wnt signaling by acting as a context-dependent endocytosis regulator of LRP6. In the presence of DKK1, it acts as a transmembrane receptor for the Wnt antagonist DKK1and an inhibitor of the Wnt co-receptor LRP6, causing its rapid internalization and inhibition of the Wnt signal. In the absence of DKK1, it may attenuate LRP6 endocytosis and degradation, thus promoting cell surface localization of LRP6 and Wnt signaling (Hassler et al., 2007). Still the nature of its involvement in odontogenesis and/or ectodermal dysplasia hasn't been addressed.

In this study, members from 4 Palestinian families displayed similar syndromic signs of ectodermal dysplasia and severe tooth agenesis. The disorder is inherited in an autosomal recessive manner. Homozygous Kremen1 F209S individuals display characteristic ectodermal abnormalities such as severe oligodontia of primary and permanent teeth, fragile and wiry scalp hair with abnormal distribution, forehead fuzziness with a prominent widow's peak and characteristic nose broadening with a lower nose bridge and thick lips. Hypertelorism and downward slanting of palpebral fissures is also a common finding. The spatial arrangement of hair follicles is thought to be the result of competing activities of diffusible signaling molecules and their antagonists, and a Wnt/Wnt inhibitor–based reaction–diffusion mechanism (Sick et al., 2006). This explains the characteristic hair pattern in those individuals. Moreover, they have a great tendency towards agenesis of lower anterior primary and permanent teeth and upper primary and permanent lateral incisors. They are usually born to Kremen1 heterozygous parents

who appear phenotypically normal. Conversely, a few heterozygous parents/individuals present with isolated tooth agenesis (up to 6 missing teeth) as the only evident abnormality. They usually lack permanent upper lateral incisors and lower incisors. Primary predecessors to those teeth, however seem to develop normally as can be seen in II-6, II-7 and III-1 in family3. A different genetic determinant is expected to be responsible for the non-syndromic phenotype (data require validation). Previous reports exist that describe offspring with ectodermal symptoms born to parents presenting with isolated hypodontia in a Norwegian family (Lyngstadaas et al., 1996). Digenic mutations in Eda and Wnt10A were found in cases of isolated oligodontia and syndromic tooth agenesis (He et al., 2013).

Kremen1 expression has been observed in the adult blood and mucoperiosteal tissues to harbor the mutation in the affected individuals with no significant differences between selected homozygotes and heterozygotes. As a result, the phenotypic variation between heterozygotes does not depend on Kremen1 dosage.

On the molecular level, the observed syndromic phenotype is a consequence of loss of function of KREMEN1 mutant protein. The substitution of a Phenylalanine residue for Serine in the WSC extracellular domain seems to attenuate the role that KREMEN1 plays in the activation of Wnt signaling. More specifically, it seems to interfere with KREMEN1-dependant cell-surface localization of LRP6 in the absence of DKK1. The importance of WSC domain has been established previously. Its deletion in KRM2, which is a KRM1 homolog, abolishes its binding with LRP6 receptor and the formation of a ternary complex after DKK1 addition in 293T cells (Mao et al., 2002). The persistent inhibition of Wnt/β-catenin signaling and the resultant ED phenotype suggest an abolished downstream Eda signaling is responsible for the observed phenotype. TNF signaling has been shown to be regulated by Wnt and to control the function of epithelial signaling centers (Laurikkala et al., 2001). Conversely, the role of KREMEN1 as an inhibitor may be lost and cause abnormally increased Wnt signaling. Loss-of-function mutations in AXIN2, which is a negative Wnt regulator, were reported to be responsible for non-syndromic autosomal dominant hypodontia (Lammi et al., 2004). The mechanism by which the missense mutation in this work interferes with Wnt signaling is still unclear. Homozygous mutants are severely affected and demonstrate similar dental findings to X-linked ectodermal dysplasia arising from Eda mutations (Song et al., 2009).

Highly consanguineous marital relationships within each family encouraged the appearance of homozygous Kremen1 mutant alleles and the autosomal recessive inheritance of a novel form of ectodermal dysplasia.

CHAPTER 6

Conclusions

In this study we report novel mutations that are responsible for the inheritance of tooth agenesis in the Palestinian population.

Genetic evidence has revealed two loss-of-function mutations in the transcriptional repressor Msx1 to be responsible for autosomal dominant selective tooth agenesis in a single family. The proposed mechanism for the phenotypic manifestations is haploinsufficiency secondary to a truncated nonfunctional product. A splicing heterozygous mutation (4862095 A>T) is expected to cause a shift in the reading frame generating an early termination codon which leads to loss of the conserved homeodomain sequences. These conclusions are based on functional mRNA analysis of Msx1 in adult oral periosteal tissue suggesting similar consequences in the context of the developing tooth and surrounding bony environment.

An upstream heterozygous missense mutation is expected to lead to p.Pro24Leu and is found to co-segregate with the splicing mutation in all affected individuals. Its pathogenic contribution cannot be therefore confirmed with certainty.

As reported with previous Msx1 mutations, the teeth most commonly absent in affected individuals are third molars and first premolars. Variable expression is encountered among affected individuals. Modifying, yet unknown genetic and epigenetic factors are held accountable for these observations. Our results suggest an imprinting mechanism resulting into silencing of the maternal allele. We also expect that an imbalance of sense/antisense Msx1 transcripts is involved in the variation of phenotypic presentation among affected individuals. The proper genetic ‘balance’ generated by these factors in mildly affected individuals rescues tooth development and the alveolar bone environment that harbors the teeth.

Whole exome sequencing identified a homozygous missense mutation in Kremen1 gene (F209S) to be responsible for a number of syndromic ectodermal symptoms including severe oligodontia of the primary and permanent dentition along with wiry and abnormally distributed scalp hair, lower nose bridge, broad nose, thick lips, slight hypertelorism and downward slanted palpebral fissures. This is the first report of this syndrome and the first evidence of the involvement of Kremen1 gene mutation in a human disorder, giving rise to a new form of ectodermal dysplasia and placing this gene on the mutational spectrum of ectodermal dysplasias.

The homozygous mutation most probably exerts a loss of function of KREMEN1 transmembrane receptor, affecting the role of KREMEN1 in Wnt signaling and the proper formation of epithelial appendages such as hair and teeth. The loss of Wnt-gradient tends to hinder the development of lower anterior primary and permanent teeth and the upper primary and permanent lateral incisors which is an indicator that the development of these teeth requires a balanced level of Wnt/β-catenin signaling.

Mutation carriers of different related families reside in the same region and should be aware of their genetic status before stepping into marriages especially when consanguineous marriages are opted for.

Msx1 and Kremen1 mutations were investigated in 200 healthy Palestinian controls. Msx1 mutations screened negative, whereas Kremen1 reported a heterozygosity of 1/200.

CHAPTER 7

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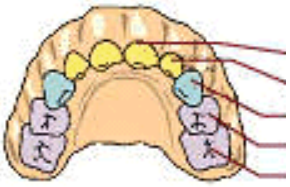
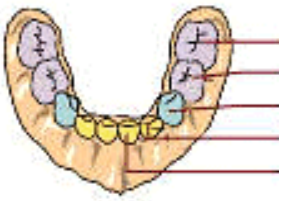
CHAPTER 8

Appendix 1

Temporary Teeth

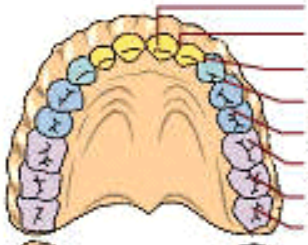


	Upper	Time of eruption
1.	Central incisor	**7.5 mo**
2.	Lateral incisor	**9 mo**
3.	Canine	**18 mo**
4.	First molar	**14 mo**
5.	Second molar	**24 mo**

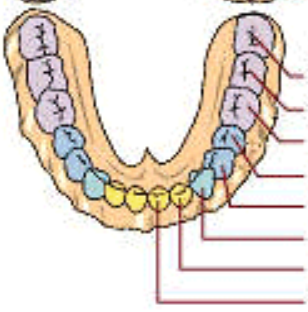


	Lower	
5.	Second molar	**20 mo**
4.	First molar	**12 mo**
3.	Canine	**16 mo**
2.	Lateral incisor	**7 mo**
1.	Central incisor	**6 mo**

Permanent Teeth



	Upper	
1.	Central incisor	**7-8 yr**
2.	Lateral incisor	**8-9 yr**
3.	Canine	**11-12 yr**
4.	First premolar	**10-11 yr**
5.	Second premolar	**10-12 yr**
6.	First molar	**6-7 yr**
7.	Second molar	**12-13 yr**
8.	Third molar (wisdom tooth)	**17-21 yr**



	Lower	
8.	Third molar (wisdom tooth)	**17-21 yr**
7.	Second molar	**11-13 yr**
6.	First molar	**6-7 yr**
5.	Second premolar	**11-12 yr**
4.	First premolar	**10-12 yr**
3.	Canine	**9-10 yr**
2.	Lateral incisor	**7-8 yr**
1.	Central incisor	**6-7 yr**