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Linkage Exclusion of FBXO10 Locus as a Cause of Hearing Impairment in Selected Palestinian Families with the Disease

By

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In Partial Fulfillment of the Requirements for the Degree

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Linkage Exclusion of FXB10 Locus as a Cause of Hearing Impairment in Selected Palestinian Families with the Disease

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ABSTRACT

Hearing loss is a common sensory disorder that typically illustrates genetic heterogeneity in human populations. About 1 in 1000 new born suffer a form of hearing loss where approximately half of all cases have genetic etiology. Genetic hearing impairment can be classified as either syndromic or non-syndromic. In about 30% of cases, genetically controlled hearing loss, is syndromically appearing as a pleotropic effect associated with other clinical features. However, a vast majority (70%) of inherited hearing impairment have been designated as non-syndromic. Indeed, this is the most heterogeneous trait known and thus far there are more than 114 mapped and 55 identified genes. Generally speaking, 75% of these genes have an autosomal recessive, 10-15% have an autosomal dominant, low portion are sex linked, and <1% are caused by mitochondrial DNA mutation in children with deafness.

The study presented here includes linkage exclusion studies of hereditary hearing impairment in 72 Palestinian families showing congenital non-syndromic autosomal recessive hearing loss. The fact that most of these cases resulted from consanguineous marriages; suggests that they are likely to be homozygous for the same gene defect. The high degree of inbreeding in these families facilitated the search for linkage exclusion. We started our analysis by running some basic computational biology techniques in order to define some novel candidates that might have a role in hearing impairment. FBXO10 which is substrate-recognition component of SCF type E3 ubiquitin ligase complex, has been predicted as being a good candidate for our analysis. Furthermore, we were motivated by the findings of Dr. Hashim Shahin who has identified a novel homozygous missense mutation in this gene in family DE. Therefore, we utilized many molecular biology techniques to check whether this missense mutation or other FBXO10 mutations are related to hearing loss in the tested families. Our findings suggest that hearing loss related FBXO10's mutations in family DE are family specific and no mutations in this FBXO10 gene have been in any of the 72 tested families with hearing loss.

إقصاء احتمالية الارتباط لجين FBXO10 كمسبب لمرض فقدان السمع بعد فحص عينية جينية لعائلات فلسطينية مصابة بالمرض.

بيان محمد قباجه

ملخص الدراسة

فقدان السمع الوراثي يعتبر واحد من أكثر الأمراض الوراثية المنتشرة في المجتمعات البشرية. حوالي 1 من 1000 من المواليد الجدد يعانون من فقدان السمع, لقد أثبتت الدراسات أن نصف حالات فقدان السمع جاءت نتيجة خلل في الجينات. يمكن تصنيف ضعف السمع الوراثي إلى متلازمات و غير متلازمات.

حوالي 30% من حالات فقدان السمع الوراثي هي متلازمات حيث تكون مرتبطة بأعراض أخرى. ومع ذلك فان الغالبية العظمى وتشكل 70% لمرض فقدان السمع الوراثي تأتي منفردة بدون أي أعراض أخرى. حتى الآن هناك أكثر من 114 طفرة تم تحديدها مسببة لفقدان السمع في 55 من الجينات التي تم تحديدها. وبشكل عام هناك 75% من هذه الجينات عبارة عن جينات متنحية, 10-15% سائدة, ونسبة قليلة جدا مرتبطة بالجنس, وحوالي 1% من فقدان السمع الوراثي ناتج عن طفرات في المادة الوراثية للمايتوكندريا.

في هذه الدراسة تم دراسة 72 عائلة فلسطينية مصابة بمرض فقدان السمع الوراثي المتنحي غير المتلازم. جميع هذه الحالات نتيجة لزواج الأقارب. حيث بدأنا هذه الدراسة باستخدام بعض تقنيات bioinformatics وذلك لتحديد أهم الجينات الأساسية والمرشحة لأن تكون لها علاقة في فقدان السمع في هذه العائلات.

وقد تبين لنا أن جين FBXO10 يعتبر مرشحا جيدا للدراسة و علاوة على ذلك فقد أخذ بعين الاعتبار النتائج التي توصل إليها الدكتور هاشم شاهين حيث تم تحديد طفرة متنحية في هذا الجين في العائلة DE وهي احدى العائلات التي تعاني من فقدان السمع و قد تم هذا الاكتشاف في مختبرات الأبحاث الوراثية في جامعة بيت لحم.

ولإثبات فيما إذا كانت هذه الطفرة في الجين FBXO10 منتشرة في المجتمع الفلسطيني. فقد استخدمنا تقنية linkage study و من خلال هذه الدراسة توصلنا أن الطفرة في الجين FBXO10 التي تم تحديدها من قبل الدكتور هاشم شاهين هي خاصة بالعائلة DE و ليس لها أي علاقة بفقدان السمع الوراثي في 72 عائلة التي تم دراستها.

DECLARATION

I declare that the Master Thesis entitled "dissertation title" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: _____Bayan Mohammad Qabaja___

Date_____21.8.2014_____

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Dedication

This thesis is dedicated to my parents who have supported me all the way along my studies. To Dr. Mahmoud, my supportive and proud husband. I dedicate this project to my sisters and my brothers especially to the big brother (Ala' Qabaja). Also, I dedicate this project to those who sacrificed their lives and their freedom, redemption of the beloved country Palestine.

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Abbreviations:

HI	Hearing Impairment
NSHL	Non-syndromic Sensorineural Hearing Loss
HL	Hearing Loss
WHO	World Health Organization
OAE	Anotoacoustic Emissions
SOAEs	Spontaneous Otoacoustic Emissions
EOAEs	Evoked Otoacoustic Emissions
ABR	Auditory Brainstem Response
BOA	Behavioral Observation Audiometry
VRA	Visual Reinforcement Auudiometry
PTA	Pure-Tune Audiometry
SHL	Syndromic Hearing Loss
USH2	Usher Syndrome Type II
ADNSHL	Autosomal Dominant Non-Syndromic Sensorineural Hearing Loss
TF	Transcription Factor
TFCP2L3	Transcription Factor Cellular Promoter2-Like3
HRL	Hereditary Research Lab's
SLC26A5	Solute Crrier Anion Transporter Family26, Member5
APC	Anaphase-Promoting Complex
SCF Complex	Skp1-Cullin-Fbox protein complex
PCR	Polymerase Chain Reaction

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CHAPTER 1

Introduction

In our community, the ability to hear is taken for granted as hearing and it is considered as an essential part of the oral communication. However, it is noteworthy that millions of people from all over the world are affected by very common sensory disability known as hearing impairment (HI). In fact, the non-life threatening nature of this has led to underestimating its consequences. Indeed HI may affect the nature of life and drastically, limit the quality of life of those affected, often reduced their communication, delay in language acquisition economic in addition to educational disadvantage. In case of more progressive states of this disease, many other medical abnormalities might evolve namely; hypothyroidism, diabetes, and possibly hyperlipidemia (Colin Mathers 2000).

In this chapter I am going first to discuss the structure of the ear in the first section. In the second section I will be discussing genes of the auditory system. In the third and the fourth section, I will be discussing the prevalence of hereditary impairment and high occurrence of consanguinity in the population respectively. In the fifth section, I will be providing a detailed description of different classes of hearing impairment.

1. Human Ear And Hearing

1.1. Ear Structure

The auditory system in humans consists of the ear which is a highly intricate organ and associated parts of the central and cranial nervous system. The ear is developed from the ectodermal embryonic thickenings of the early hind brain or rombomerecephalon at about the third week of embryonic life, and the morphologically differentiated ear appears at the sixth embryonic month. Ear is anatomically made up of three distinct parts, the outer, the middle, the inner ears (Figure 1.1), which function as one unit.

1.2. Sound Movement

The outer ear comprises the auricle or pina and auditory canal (acoustic duct). The sound waves captured by auricle, then the external auditory canal transferred the sound waves to the tympanic membrane (ear drum). The vibration of ear drum caused by the airborne sound wave, are transmitted through middle ear or tympanic cavity to inner ear by a chain of three movable ossicles (auditory bones), malleus, incus, and stapes. The sound vibration of the tympanic membrane is propagated through a piston-like mechanical movement of the three ossicles to the base of stapes, which moves in and out of the oval window of the vestibule, the central cavity of the inner ear (Figure 1.1).

1.3. Inner ear physiology

The inner ear or the labyrinth composed of two fluid-filled labyrinth, a bony and membranous labyrinth. The membranous labyrinth is filled with fluid, called endolymph which moves in response to sound or movement of head. Bony labyrinth contains three major cavities: the vestibule, the cochlea and the semicircular canal, with the sacculus and the utriculus (Figure 1.1). These channels are lined by membranes. The parts of the inner ear involved in balance are semicircular canals and vestibule. The semicircular canals respond to the rotatry acceleration, while the base of semicircular canals, called ampulla and vestibule (contain two small membranous sacs. Called sacculus and utriculus), contain little hair cells and respond to the linear acceleration.

The part of inner ear involved in hearing is a complex organ, cochlea, three canals namely; Vestibular canal, the cochlear canal and the tympanic canal. These canals are located within the tubular cochlea. The cochlea canal contains hair cells called organ of Corti. The organ of Corti form the transduction system of the ear and convert the sound waves, generated in the endolymph of the cochlear duct which comprises 2.5 turns and can process 20Hz-20kHz sound into the electric impulses(Petit 1998; Bevan Yueh 2003), these electrical impulses are passed through the cochlear VIIIth cranial nerve to multiple nuclei that found in the central auditory system, then to the auditory cortex of the temporal lobe of cerebrum(Willems 2000). Tunnel of Corti, tectorial membrane is a highly organized array of supporting cells and hair cells that are considered as the component of the organ of corti. There are two types of the sensory hair cells (about 15 000 cells in the human cochlea), which differ in their function (Renato Nobili 1998). One row of inner hair cells act as the

primary receptor cells and transmits their signals to the cochlear nerve and the auditory cortex. Three rows of outer hair cells have both sensory and motor elements that respond to variation in potential. Outer hair cells generate the forces for altering the delicate mechanics of the cochlear partition, that contribute to hearing sensitivity and frequency selectively (Renato Nobili 1998).

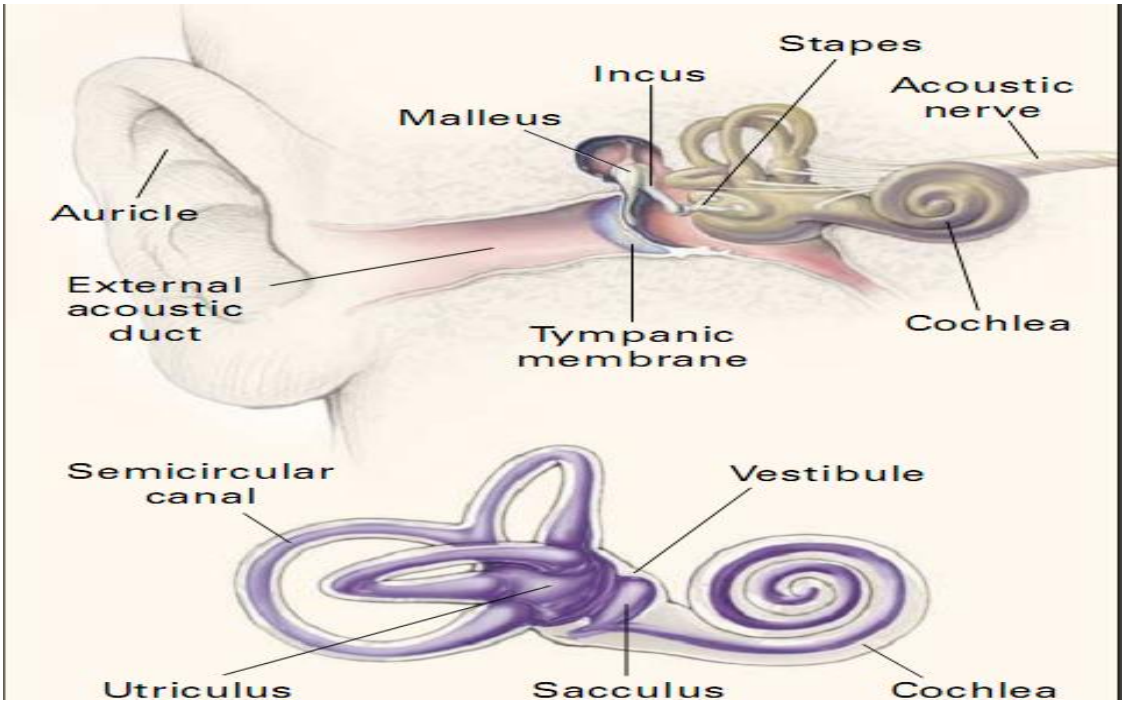


Figure 1.1: Drawing of the Outer, Middle, and Inner Ear. Auricle capture the sound waves and conveyed through the external acoustic duct then to the tympanic membrane, which lead to vibrate this membrane. These vibrations are transmitted through the auditory bones of the middle ear to the footplate of the stapes, which is anchored in the oval window of the vestibule of the cochlea.
(adapted from (Willems 2000)

2. Genes and Hearing Loss

A genetic component is involved in development of hearing impairment, and it accounts for approximately 60% of hearing impairment cases (Klemens Frei 2002; Faiqa Imtiaz 2011). Because there are more than 100 different genes which are estimated to be involved in the inner ear and in the hearing process in general (Klemens Frei 2002; Kramer 2010), and only single- gene mutations can lead to damage the cochlea and cause hearing loss (Willems 2000), genetic effects can be rarely detected and more investigations are needed to uncover the actual genes that are involved in the processes of hearing. Studying of genes that control hearing process can give us more information about the structure and the function of the proteins in various types of cells and cochlea where any mutations in their genes may cause deafness.

Add to that the hereditary hearing loss is genetically and clinically heterogeneous (Bitner-Glindzicz 2002; Nikolay A Barashkov 2011). Hetrogenetic nature of this disease lead to different mode of inheritance. With the present information in hand, a minimum of 114 loci and 55 genes are known to control the inherited non-syndromic hearing loss (Nikolay A Barashkov 2011). It is generally estimated that 75% of these genes have an autosomal recessive, 10-15% autosomal dominant, low portion as X- linked, and <1% are caused by mitochondrial DNA mutation in the children with prelingual deafness (Haris Kokotas 2010; Nikolay A Barashkov 2011).

3. Prevalence of Hereditary Hearing Impairment

Hearing impairment like the loss of hearing to different degrees, is one of the most prevalent sensory defect, worldwide (Willems 2000; Jiann-Jou Yang 2010; Nikolay A Barashkov 2011). Hearing impairment with severe degree is the highest prevalence (D. P. Kelsell 1997), affecting more than 250 million people in the world (Colin Mathers 2000). About 1 in 1000 new born suffer a form of hearing loss (Willems 2000; Hong Joon Park 2001; Hashem Shahin 2002; Borck G 2011), approximately half of all cases have genetic etiology (Hong Joon Park 2001; Nikolay A Barashkov 2011). Hearing loss can affect people of all ages with a variable degree of severity. Furthermore, the prevalence of hearing loss increases dramatically with age (Petit 1998; Bevan Yueh 2003; Demers 2007): affect 5% of people less than 45 years old, 14% of people between 45-65 years old and half the population by the age 80 (Keats BJ 1999; Willems 2000; Jiann-Jou Yang 2010). Hearing loss present at birth is known as congenital deafness, while one that occurs after birth is called adventitious deafness. Congenital hearing impairment affect live-born infant with a moderate to profound degree of hearing loss (Borck G 2011).

4. Consanguinity in Palestinian Population

In some communities, like Palestine, the rate of consanguinity is very high, and about 44.3% of the marriage are between relatives (22.6% of them between first cousin) (J. 1997). The prevalence of inherited prelingual autosomal recessive hearing loss is among the highest in the world (Hashem Shahin 2002). It is well documented that children of parent who are related have a higher percentage of homogenous alleles than children of unrelated parents. Theoretical calculations

predict a level of 6.25% homozygosity in the genome of children from first cousin marriages(Maria H. Chahrour 2012), but empirical calculations suggest a higher level of homozygosity.

Increasing the level of homogeneity increases the probability of inheriting two homozygous mutations resulting in a recessive disease and prelingual hereditary hearing impairment is with no exception. Prelingual hereditary hearing impairment occur in the Palestinian population at a frequency of approximately 1.7 per 1000 (Hashem Shahin 2002) and may exceed in isolated communities. Furthermore, in Palestinian population until now, twenty nine published mutated alleles are known to cause NSHL. The emergence of hearing loss in early stages of childhood, highly affect their language acquisition and educational process. However, more effects on social and working status may result in when emergence appears in adults or even late childhood stages(Petit 1998).

5. Hearing Impairment Classification

Hearing impairment are categorized depend on their type, their severity, and the age of onset (before or after language is acquired)

5.1. Degree of Severity

The normal hearing individual could have 250-8000 cycles of frequency of sound wave per second (250-8000 HZ) (Jiann-Jou Yang 2010). This measure is used to categorize the hearing capabilities of people. In details, people with <500Hz known to have low hearing loss frequency, people with 501-2000Hz known to have medium hearing loss frequency, and people with >2000Hz known to have high hearing loss frequency (Richard JH Smith 2012).

According to The World Health Organization (WHO) classification, the threshold level hearing impairment ranges from "no impairment" to "profound impairment" (Colin Mathers 2000) . In details normal hearing ranges from (0-25dB). While the threshold of affected people with hearing loss is graded as mild impairment when hearing threshold (26-40dB), moderate impairment when hearing threshold (41-55dB), moderately severe impairment when hearing threshold (56-70dB), severe (71-90dB) or profound (>90dB), the individual has no hearing at all (Petit 1998; Colin Mathers 2000).

5.2. Causes of HL

The cause of hearing loss are categorized as genetic, and environment factors (Wylie Burke 2003). Some of these environmental causes are; exposure to sustained high sound pressure levels (>90 dB), head trauma, prenatal infection (eg. prenatal toxoplasmosis, rubella), postnatal infection (eg. Cytomegalovirus, meningitis and immunization), provision of neonatal (neonatal hypoxia, low birth weight, severe neonatal jaundice) and postnatal oxotoxic medication (eg.Aminoglyside antibiotics) (Willems 2000). Some of hearing impairment is of still unknown causes.

5.3. Types of HL

On the basis of ear defect types, hearing impairment can be categorized into four types:

5.3.1. Conductive HL

This type of hearing impairment occurs when the sound is not reaching the inner ear, the cochlea due to a problem somewhere within the outer and middle ears, including ear canal, eardrum, and the tiny bones or ossicles. Conductive hearing losses are often treated via medication or surgical intervention e.g. repair of perforated ear drum, draining of fluid-filled middle ear, reconstruction of ossicular chain (Vora. 2003).

5.3.2. Sensorineural HL (NSHL)

It has been found that the genetic etiology is associated with the majority (70%) of NSHL cases in developed countries (David P. Kelsell 2001).This kind of hearing impairment results due to malfunctioning of the inner ear structure (cochlea), on the account of damaged or missing tiny hair cells in the cochlea. This malfunctioning will result in dispersion in the transmission of the sound of signal from the inner ear to the cortical auditory centers of the brain. Noting that, sensorineural hearing loss is a permanent loss.

5.3.3. Mixed HL

It is a combination of both conductive and SNHL. The surgery is often attempted but not always.

5.3.4. Central auditory dysfunction

this type of hearing impairment results from damage or dysfunction at the level of the VIIIth cranial nerve, auditory brain stem, or cerebral cortex due to tumor, disease, heredity, injury or other unknown causes (Richard JH Smith 2012).

5.4. Onset

Before language: which referred as Prelingual HL; is sustained before the acquisition of language, this type of HL occur due to a congenital condition or through hearing loss in early infancy.

After language: opposed to the prelingual and referred as Postlingual HL; is occur after the development of normal speech.

5.5. Association with other symptoms

In about 30% of inherited hearing impairment cases it is syndromic HL. More than 400 syndromes associated with hearing loss have been identified (Eggermont 2012). These syndromes have malformations of the external ear or with medical problems involving other organs system. The remaining (70%) of inherited hearing impairment is non-syndromic HL (Jiann-Jou Yang 2010). These non-syndromic impairments have no associated visible abnormalities of the external ear, or other related medical problems. However it can be associated with abnormalities of the middle ear and/or inner ear.

6. Hearing Tests

Early clinical diagnosis in all newborn children can help to start hearing rehabilitation within the first months of life. Hearing can be measured by using two main tests; physiological tests and behavioral tests. These are described below in details.

6.1. Physiologic tests

6.1.1. Otoacoustic Emissions (OAE)

An otoacoustic emissions (OAE) is a sound which generated from within the inner ear (cochlea) (Christopher A. Shera 2004). OAEs are often used in clinic when inner ear is healthy because OAEs disappear when inner ear has been damaged. There are two types of otoaoustic emissions, spontaneous otoacoustic emissions (SOAEs), which can occur without external stimulation. The other type is evoked otoacoustic emission (EOAES), which reflect primarily the activity of the outer hair cells of the cochlea in the response to either spontaneous or sound stimuli (Renato Nobili 1998; Richard JH Smith 1999; Richard JH Smith 2012), which are partially passed on to the external auditory canal.

6.1.2. Auditory Brainstem Response (ABR)

ABR (also known as BAER, BSER) is a neurological test of auditory brainstem function in response to auditory click stimuli, that is transmitted from an acoustic transducer in the form of an insert earphone or headphone (Neil Bhattacharyya 2011). Although ABR test measures hearing sensitivity in the range of 15,000-4,000 Hz (Richard JH Smith 1999; Anne M Delaney 2012; Richard JH Smith 2012), ARB does not assess low frequency (less than 1500HZ) sensitivity (Richard JH Smith 2012).

6.2. Behavioral Testing

This test includes the behavioral observation audiometry (BOA) and the visual reinforcement audiometry (VRA). BOA is used in child from birth to six months of age (Ruth RA 1983; Richard

JH Smith 1999; Richard JH Smith 2012). Child's responses may consist of quieting, eye widening, startle, etc.

6.3. Pure-Tone Audiometry

The Pure-tone audiometry (PTA) is the key of hearing test; this type of hearing test used to identify hearing levels of a person, helping in determination of the degree of hearing, type and configuration of a hearing loss. In this procedure the sensitivity threshold for each ear are measured for pure tone stimuli of different frequencies (250, 500, 1,000, 2,000, 4,000, and 8,000 Hz) (Joe Walter Kutz Jr 2012). PTA uses both air and bone conduction audiometry, and the type of loss can be identified via the air-bone gap.

CHAPTER2

Background

In this chapter, I dig deeper into the heritable causes of hearing loss and the genes that control auditory function, indicating different genes associated with etiological hearing impairments.

1. Heritable causes

Hearing loss is a heterogeneous trait, and may be caused from genetic or environmental factors. Genetic causes account for half of the hearing impairments (Hong Joon Park 2001), and can be divided into two basic categories: syndromic hearing loss and non-syndromic hearing loss.

1.1. Syndromic Form of Hereditary Hearing loss

About 30% of genetically inherited hearing loss is associated with other symptoms and is termed syndromic hearing loss (SHL) (Petit 1998), it is associated in malformations of outer ear or other organs or with other medical problems involving different organ systems. Hearing loss in most such cases is conductive or mixed. Over 400 genetic syndromes that include hearing loss have been described (Saima Riazuddin 2000). Syndromic hearing loss can be either dominant (Waardenburg syndrome, Stickler syndrome, Branchial-oto-renal syndrome), recessive (Usher syndrome), X-linked (Nance syndrome, Alport syndrome) or mitochondrial. It is worth mentioning that Usher syndrome type II (USH2) is regarded as the most common type, it is accounts up to 50% of USH cases and characterized by moderate to severe congenital hearing loss (Xiaowen Liu 2010).

1.2. Non-syndromic Form of Hereditary Hearing loss

Non-syndromic hearing loss has no other recognizable abnormal phenotype beside the hearing loss, it can be associated with abnormalities of the middle ear or inner ear. It is a more common cause of hearing loss than syndromic hearing loss. Non-syndromic hearing loss is considered one of the most genetically heterogeneous traits, with up to 100 genes being involved (Grove 1998). Mutations within the same gene may lead to recognizable variety of clinical phenotypes with different modes of inheritance. Examples DFNB1 and DFNA3; both map to 13q12 and both are

caused by mutations in the genes GJB2 and GJB6 (Grove 1998) . Similary DFNB2 and DFNA11both caused by mutation in MYO7A gene at region 11q13.5. also DFNB21 and DFNA8/12; both are caused by defect in TECTA gene. (Grove 1998; Saima Riazuddin 2000). Similary NSHL and SHL can caused by mutation in one gene. Example DFNB18 and Usher syndrome type type 1c, caused by mutation in USHIC gene. Similary DFNA6/14/38 and Wolfram syndrome, caused by mutation in the same gene WFS1 (Http://www.ncbi.nlm,nib.gov/books/NBK1434/).

The different gene loci for NSHL are designated DFN. Loci are named based on modes of inheritance, and referred to as DFN for the X-linked forms, DFNA for the autosomal dominant forms and DFNB for autosomal recessive forms. The numbers following these designations reflect the order of identification of the loci in chronological time.

1.2.1. Autosomal Recessive Non-Syndromic Hearing Loss

The most frequent form of non-syndromic hearing loss is caused by autosomal recessive alleles. Autosomal recessive non-syndromic hearing loss is the most common form of severe inherited childhood deafness. Till now, 70 autosomal recessive non-syndromic hearing impairment loci have been mapped and ~40 causative genes have been cloned(Borck G 2011), which express different proteins. Numerous loci have been identified on several chromosomes, but there are also chromosomes to which no loci have been mapped (chromosome 8 and 20). Table 2.1 shows the autosomal recessive hearing impairment loci mapped so far and their chromosomal locations.

Table 2.1: Loci and genes for autosomal recessive non-syndromic hearing loss

S#	Locus	Localization	Gene
1	DFNB1A	13q12	*GJB2*
2	DFNB1B	13q12	*GJB6*
3	DFNB2	11q13.5	*MYO7A*
4	DFNB3	17p11.2	*MYO15A*
5	DFNB4	7q31	*SLC26A4*

6	DFNB6	3p21.31	*TMIE*
7	DFNB7/11	9q21.13	*TMC1*
8	DFNB8/ 10	21q22	*TMPRSS3*
9	DFNB9	2p23.3	*OTOF*
10	DFNB12	10q22.1	*CDH23*
11	DFNB15/72/95	19p13.3	*GIPC3*
12	DFNB16	15q15.3	*STRC*
13	DFNB18	11p15.1	*USH1C*
14	DFNB21	11q	*TECTA*
15	DFNB22	16p12.2	*OTOA*
16	DFNB23	10q21.1	*PCDH15*
17	DFNB24	11q23	*RDX*
18	DFNB25	4p15.3	*GRXCR1*
19	DFNB28	22q13	*TRIOBP*
20	DFNB29	21q22	*CLDN14*
21	DFNB30	10p12.1	*MYO3A*
22	DFNB31	9q32	*WHRN*
23	DFNB35	14q24.1	*ESRRB*
24	DFNB36	1p36.3	*ESPN*
25	DFNB37	6q13	*MYO6*
26	DFNB39	7q21.11	*HGF*
27	DFNB42	3q13.33	*ILDR1*
28	DFNB49	5q12.2	*MARVELD2*
29	DFNB53	6p21.3	*COL11A2*
30	DFNB59	2q31.2	*PJVK*

31	DFNB61	7q22.1	*SLC26A5*
32	DFNB63	11q13.4	*LRTOMT/COMT2*
33	DFNB66/67	6p21.31	*LHFPL5*
34	DFNB74	12q14.3	*MSRB3*
35	DFNB77	18q21.1	*LOXHD1*
36	DFNB79	9q43.3	*TPRN*
37	DFNB82	1p13.3	*GPSM2*
38	DFNB84	12q21.31	*PTPRQ*
39	DFNB91	1p34.3	*GJB3*

1.2.2. Autosomal Dominant Non-Syndromic Hearing Loss

The heterogeneity in autosomal dominant non-syndromic hearing loss is high (http://www.ncbi.nlm.nih.gov/books/nbk1434/). Currently, 64 autosomal dominant non-syndromic sensorineural hearing loss (ADNSHL) loci have been mapped and 27 genes have been identified (Hereditary hearing home page). Characteristic phenotypic features can be used to distinguish between the different forms of autosomal dominant non-syndromic hearing loss. The typical phenotype of individuals with autosomal dominant non-syndromic hearing loss is late onset. Table 2.2 shows the autosomal dominant hearing impairment loci mapped so for and their chromosomal locations.

Table 2.2: Loci and genes for the autosomal dominant non-syndromic hearing loss

S#	Locus	Localization	Gene
1	DFNA1	5q31	*DIAPH1*
2	DFNA2A	1p34	*KCNQ4*
3	DFNA2B	1p35.1	*GJB3*
4	DFNA3A	13q11	*GJB2*
5	DFNA3B	13q12	*GJB6*

6	DFNA4	19q13	*MYH14*
7	DFNA5	7p15	*DFNA5*
8	DFNA6/14/38	4p16.3	*WFS1*
9	DFNA8/12	11q23.3	*TECTA*
10	DFNA9	14q12	*COCH*
11	DFNA10	6q23.2	*EYA4*
12	DFNA11	11q13.5	*MYO7A*
13	DFNA13	6p21	*COL11A2*
14	DFNA15	5q31	*POU4F3*
15	DFNA17	22q	*MYH9*
16	DFNA20/26	17q25	*ACTG1*
17	DFNA22	6q13	*MYO6*
18	DFNA25	12q23.1	*SLC17A8*
19	DFNA28	8q22	*GRHL2*
20	DFNA36	9q13-q21	*TMC1*
21	DFNA44	3q28-29	*CCDC50*
22	DFNA48	12q13.3	*MYO1A*
23	DFNA50	7q32.2	*MIRN96*
24	DFNA51	9q21	*TJP2*
25	DFNA64	12q24.31	*SMAC/DIABLO*

1.2.3. X-linked Dominant Non-Syndromic Hearing Loss

Although X-linked form of non-syndromic hearing loss is much less common than autosomal hearing loss, advances in genome research have facilitated the identification of defective X-linked genes in this form of hearing impairment. For non-syndromic hearing impairment locus mapped showed an X-linked inheritance. DFNX3 (Xq21.1) is characterized by a mixed conductive-

sensorineural hearing loss. The defective gene (DFNX3) encodes the transcription factors POU3F4 (Keats BJ 1999), which is the most common in DFN (Willems 2000). Other X-linked non-syndromic hearing loss phenotypes include profound prelingual hearing loss characteristic of both DFNX2 and DFNX4. Later on, bilateral high-frequency impairment beginning at five to seven years of age and progressing by adulthood to severe-profound hearing impairment, over all frequencies, characteristic of DFNX6 (Richard JH Smith 2012).

2. Genes That Have Regulatory Functions

The Function of genes is to provide information needed to make molecules called proteins. The auditory system is complex, because there are more than 100 different genes that control the auditory system, and any mutation in these genes lead to deafness especially mutations in the proteins that make up the cochlea, which considered as the most intricate organ in the auditory system. Grouping of the known genes associated with the etiology of deafness disorder functional categories which can help us in elucidating their functional roles in the process of hearing. Another important aspect of gene discovery for deafness disorders is that it allows the possibility for development of diagnostic tests and accurate genetic counseling. The identified genes that are associated with hearing loss encode a wide variety of proteins, including transcription factors, gap junctions, extracellular matrix component ubiquititins and ion channels.

2.1 Transcription Factors

Transcription factors (TF) are proteins that bind to specific sites on DNA to activate transcription. TF as any other proteins are essentials in hearing, and mutations in the genes that code for these proteins will help to explain critical components in the process.

TFCP2L3 (transcription factor cellular promoter 2-like 3) is one of the family TF genes, this genes is highly expressed in epithelial cells lining the cochlear duct. A Frame shift mutation in this gene resulting in a premature translation stop codon in exon 14 was identified (Linda M. Peters 2002).

The *EYA4* proteins is called a transcription factor or transcription coactivator, this gene is important in inner ear (http://ghr.nlm.nih.gov/gene/EYA4). It has been discovered that the postlingual and the progressive DFNA10 is due to mutation in *EYA4* gene. These mutations create a premature stop signal in the instruction for making the EYA4 protein. *EYA4* gene is a ortholog to the *Drosophila* gene *eya* (Pfister M 2002).

The POU proteins are another family of the TFs that bind to DNA through their POU-domain regions. Different members of POU family are expressed in auditory system, especially in inner ear. The gene POU4F3 is expressed in hair-cells and mutation in this gene cause DFNA15 (Alex Robert 2010). Another member of the POU family is POU3F4 which located in the X chromosome, and mutations in this gene cause X-linked mixed (DFN3) (Willems 2000; Alex Robert 2010). Targeted deletion of both alleles POU4F3 in mice have been shown to cause deafness (Ronna Hertzano 2004).

2.2. Genes involved in structure of inner ear.

There are several genes have been identified and play important role in hearing process by forming the structure components of the cochlea, and mutation in any of these genes lead to the abnormal structure in outer and inner ear especially in the cochlea and cause hearing loss.

Myosins is one member of the genes that involved in the structure of inner ear. Myosins are molecular motor proteins that bind to actin filament and that hydrolyze ATP to exert mechanical force to move along (Willems 2000). The myosin proteins are organized into head, neck and tail domains, which involved in several cellular functions, including phagocytosis, secretion, muscular contraction, transport of intracellular organelles and cellular movement (Willems 2000). Mutations within genes that encode some myosins may lead to non-syndromic hearing loss. In Palestinian Arab families and Israel Jewish nine novel mutation was identified in genes encoding *myosinVI*, *myosinVIIA* and *myosinXVA*, doubling the number of myosin mutations in the Middle East (Zippora Brownstein 2013). Mice with mutations in any myosins have abnormal stereocilia and are deaf (Willems 2000).

The *TECTA* gene encodes the protein α-tectorin which is an important structural component of the tectorial membrane (Spencer 2011). Tectorial membrane is an extra cellular matrix of the inner ear, it is very important for mechanoelectrical transduction by the organ of Corti. Different mutations in this gene lead to disruption in the tectorial membrane structure and result in non-syndromic hearing loss (Spencer 2011). Other structural proteins which important in inner ear are collagens, collagen molecules combine to form the tectorial membrane. Approximately 30 genes codes for collagen proteins (Spencer 2011), mutations in different collagen genes can cause hearing loss.

SLC26A5 (solute carrier anion transporter family 26, member 5) gene that encode a motor protein called prestin which is essential in auditory processing and expressed in cochlea to form the lateral membrane of the outer hair cells (http://ghr.nlm.nih.gov/gene/SLC26A5). Mutations in this gene have been shown to cause autosomal recessive hearing loss (DFNB61) (http://www.phosphosite.org/proteinAction.do?id=5133775&showAllSites=true).

Diaphanous is another protein which important in structural proteins of inner ear. It may also be involved in establishing the rigid structure of the actin core of steriocilia, which is the major component of the cytoskeleton of hair cells of the inner ear, by regulating the polymerization of actin in hair cells of inner ear. Diaphanous is encoded by the gene (DIAPH1) located on 5q31 and cause progressive DFNA1. Abnormal formation of either the hair cells or tectorial membrane would likely to disrupt the auditory pathway.

2.3 Connexins

Connexins (or gap junction proteins), which are essential for many physiological process like hearing. Mutation in connexin-encoding genes lead to disruption of ion channel that regulates ion's movement in and out hair cells during hearing process will result in homeostasis disruption. Consequently, sound waves cannot be converted into electrical stimuli, and then cause hearing loss.

The prevalence of mutations in a single gene (*GJB2*) that encoding the gap junction protein connexin 26, accounts for up to 50% of all cases of recessive NSHL in some populations (Hong Joon Park 2001; Hashem Shahin 2002), in some Palestinian communities, five different mutations were identified: ivs1(+1) G→A, 35delG, 167delT, T229C, 235delC (Hashem Shahin 2002). Connexin 26 interact with other connexin 26 protein or other connexin proteins, such as

connexin 32, connexin 46, connexin 50 to form a six identical connexin subunit (hexameric) of homotypic in the endoplasmic reticulum, which are then translocated into the plasma membrane (David P. Kelsell 2001) or composed of more than one species of connexins (heterotypic) to form half-channel named connexon of gap junctions and two connexons align to form a complete inter-cellular channel (Grove 1998). Such gap junctions are very important for the recycling of endolymphatic potassium ions (David P. Kelsell 2001; Klemens Frei 2002; Jiann-Jou Yang 2010) by local circulation of potassium ions in the inner ear, where other potassium channels pump potassium back into endolymph of cochlear duct (D. P. Kelsell 1997). Besides *GJB2*, the genes *GJB3*, GJB4, *GJB6* and *GJA*, that encode gap junction proteins connexins 31, 30.3, 30 and 43, known to be associated with hearing loss (David P. Kelsell 2001; Jiann-Jou Yang 2010; Nikolay A Barashkov 2011).

2.4 Ubiquitin ligase proteins

Ubiquitin ligase is another important protein involved in cell cycle progression, signal transduction and transcription (Ning Zheng 2002), which also called an E3 ubiquitin ligase. It plays the major role in providing the specificity of substrate recognition for ubiquitin-dependent proteolysis (Howley 1998). Ubiquitin-protein ligase acts at least the last step of a three-enzyme cascade involving the ubiquitin-activating (E1) and ubiquitin-conjugating (E2) enzymes. The E3 is a protein that in combination with an E2 ubiquitin-conjugating enzyme causes the attachment of ubiquitin from the Ubcaroxy-terminus to a lysine on a target protein via an isopeptide bond (Ning Zheng 2002). Most of ubiquitin ligases are involved in polyubiquitination: A second ubiquitin binds to the first, a third binds to the second, and so forth. Polyubiquitination marks proteins for degradation by the proteasome.

There are various lysines that can be targeted by E3 to make chains. The most common lysine is lys48 on the ubiquitin chain. This lysine is used to make polyubiquitin, which is recognized by the proteasome. There are two examples of ubiquitin ligase protein scaffold: the anaphase-promoting complex (APC) and the SCF complex (Skp1-Cullin-F-box protein complex) which considered as the largest family of ubiquitin-protein ligase (Ning Zheng 2002), they involved in recognition and ubiquitination of specific target proteins for degradation by the proteasome. SCF complex are RING-type E3s, the component cullin form a catalytic core complex that recruit a

cognate E2, while the variable F-box protein subunit binds the substrate, Rbx1 is another component of SCF complex, which contains the RING domain (Ning Zheng 2002). The human SCF complexes include the SCF^{Skp2} complex (the superscript denotes the F-box protein (Figure 2.1).

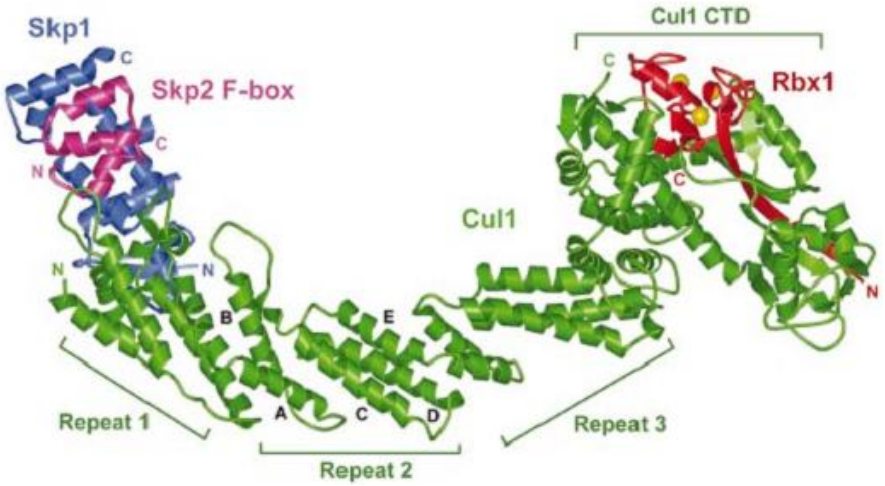


Figure 2.1.Overall structure of the Cul1-Rbx1-Skp1-Fbox quaternary complex. Cul1, Rbx1, Skp1 and the F-box of Skp2 are colored in green, red, blue and magenta, respectively. The five helicles that make up the culin-repeat motif are labeled for second repeat. (Ning Zheng 2002).

FBXO10 is substrate-recognition component of the SCF (SKP1-CUL1-F-box protein)-type E3 ubiquitin ligase complex. *FBXO10* gene was mapped on chromosome 9p13.2 with size 77,983 bases (105195 Da) and is composed of 11 exons and encodes 956 amino acid protein. Mutation in this gene may lead to deafness, as in family DE which is a deafness family studied at Hereditary Research Lab HRL at Bethlehem University. The affected individuals in this family were diagnosed to have bilateral severe to profound SNHL. Deafness phenotype in this family was proposed to be caused by a novel homozygous missense mutation in exon 2 of *FBXO10* gene as shown in table 2.3. This mutation was identified by Dr. Hashem Shahin (not published) ; further investigation is needed to support this finding.

Table 2.3.EP34-sequencing for *FBXO10* gene

Exon No	Sequence Results	Note
Exon1	**rs4077401**	Heterozygous
Exon2	E54K	Heterozygous

Exon3	A 323A rs7044153	
Exon4	---	
Exon5	----	
Exon6	----	
Exon7	----	
Exon8	----	
Exon9	----	
Exon10	---	
Exon11	rs10973387 rs12237351	Heterozygous Heterozygous

FBXO11 is another example of F-box protein family, *FBXO11* is expressed in epithelial cells of the middle ear from late embryonic stages through day 13 of postnatal life (Rachel E. Hardisty-Hughes 2006). In mouse the semi-dominant mutation in *FBXO11* gene lead to Jeff mouse. The Jeff mouse mutant is a model of chronic otitis media in the human population. Additional mutant alleles at the *FBXO11* locus, the Mutt mutation. Both Jeff and Mutt homozygotes demonstrate cleft palate defects. This makes *FBXO11* an important candidate gene for the study of the genetics pathway involved in hearing loss in human. as shown in figure 2.2

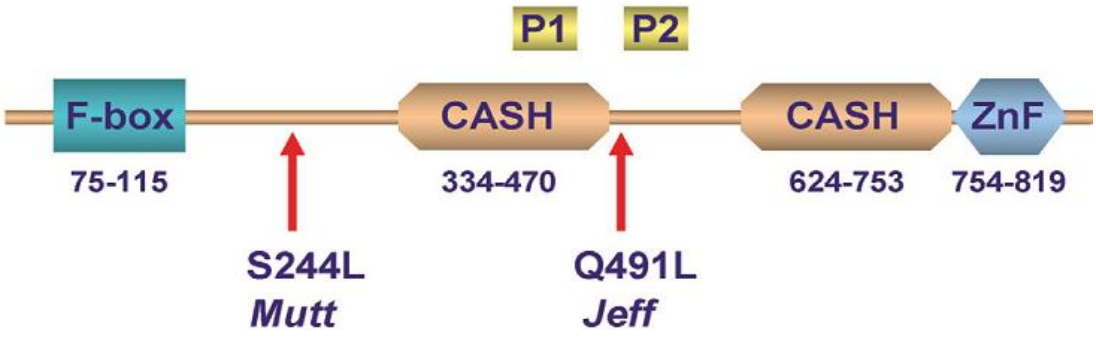


Figure 2.2. Protein structure of Fbox from sequence predictions. The molecules consists of an F-box motif, two carbohydrate-binding (CASH) domains and a zinc finger domain (ZnF). The amino acid numbers consisting these domains are shown below the appropriate domain. The positions of the Jeff and Mutt mutation are also indicated (arrows).the position of peptides, flanking the Jeff mutation, used to raise a polyclonal antibody CIYVHEKGRGQFIEN (P1) and CPIVRHNKIHDGQHG (P2) is indicated.

3. Mouse Model for Human Hereditary Hearing Loss

Hereditary hearing loss is a frequent condition in humans. The identification of genes that are related to deafness is one of the things that should be studied . Because of its high similarity to human cochlea, mouse cochlea has been identified as being an excellent model to study hearing loss. Consequently, mouse deaf mutants have played a vital role in determining genes that can be involved in causing hearing impairment and in elucidating the functional role of critical molecules involved in the process of auditory transduction.

Adding to its human similarity, mice have the ability to breed large number of offspring in short time to narrow the period of study. This gives mouse the advantage of being an experimental model for genetic studies. In addition, it has been found that under the special crosses all offspring, carrying the same mutant gene, can be produced. This can help in the localization of causative mutations to a specific location on chromosomes by linkage analysis. The determination of the mouse mutations has facilitated the process of identifying human genetic disorders.

4. Contribution

The study presented here includes linkage exclusion studies of hereditary hearing impairment in 72 Palestinian families showing congenital non-syndromic autosomal recessive hearing loss. Most of the affected members of these families resulted from consanguineous marriages, suggesting that they are likely to be homozygous for the same gene defect. The high degree of inbreeding in these families facilitated the search for linkage.

We started our analysis by running some basic bioinformatics and computational biology techniques in order to define some novel candidates that might have a rule in hearing impairment. After defining *FBXO10* as being a candidate, we used many biological techniques to validate the results. *FBXO10* is paralogue to *FBXO11* and in in mice, mutation in *FBXO11* lead to hearing impairment and this hinted to us that mutation in *FBXO10* in human also may lead to hearing impairment. And this gene recently has been shown to be harbor a novel homozygous missense mutation that might explain the hearing loss in family DE by Dr. Hashem Shahin (personal communication). We studied whether this missense mutation or other mutations might exist in *FBXO10* related to hearing loss in these families or not . So we used DNA samples that were

collected from 72 families affected with hearing loss, and then carried out genotyping using 5 microsatellite markers which we selected on the basis of their map position (UCSC Genome Browser) and heterozygosity coefficient.

CHAPTER 3

Materials and Methods

1. Computational Analysis:

1.1. Building the network related to hearing impairment

In this analysis we were trying to predict whether there would be any candidate gene to be involved in hearing impairment using already known genes. To do so, we started by building the gene-gene interaction network using Databases, Text-mining, co-expression, gene fusion or gene neighborhood. We started by a set of genes that represents seed genes. This set of genes represents number of genes that have been biologically validated to be involved in defenses. These genes are: CDH23, CX26, GPSM2, MYO15A, MYO7A, OTOA, OTOF, OTOTARA, PTPRQ, PVJK, Pendrin, TECTA, TMHS and TMPRSS3. We dropped these genes in STRING database that stores huge amount of gene-gene interactions curated from databases, text-mining, co-expression, gene fusion and gene neighborhood. We extracted the network for these genes' interactions in addition to other genes that might interact with those genes. Figure 3.1 shows the network where each node (circle) represents a gene and each edge (line) represents an-interaction between these two nodes according to one of the criteria mentioned above.

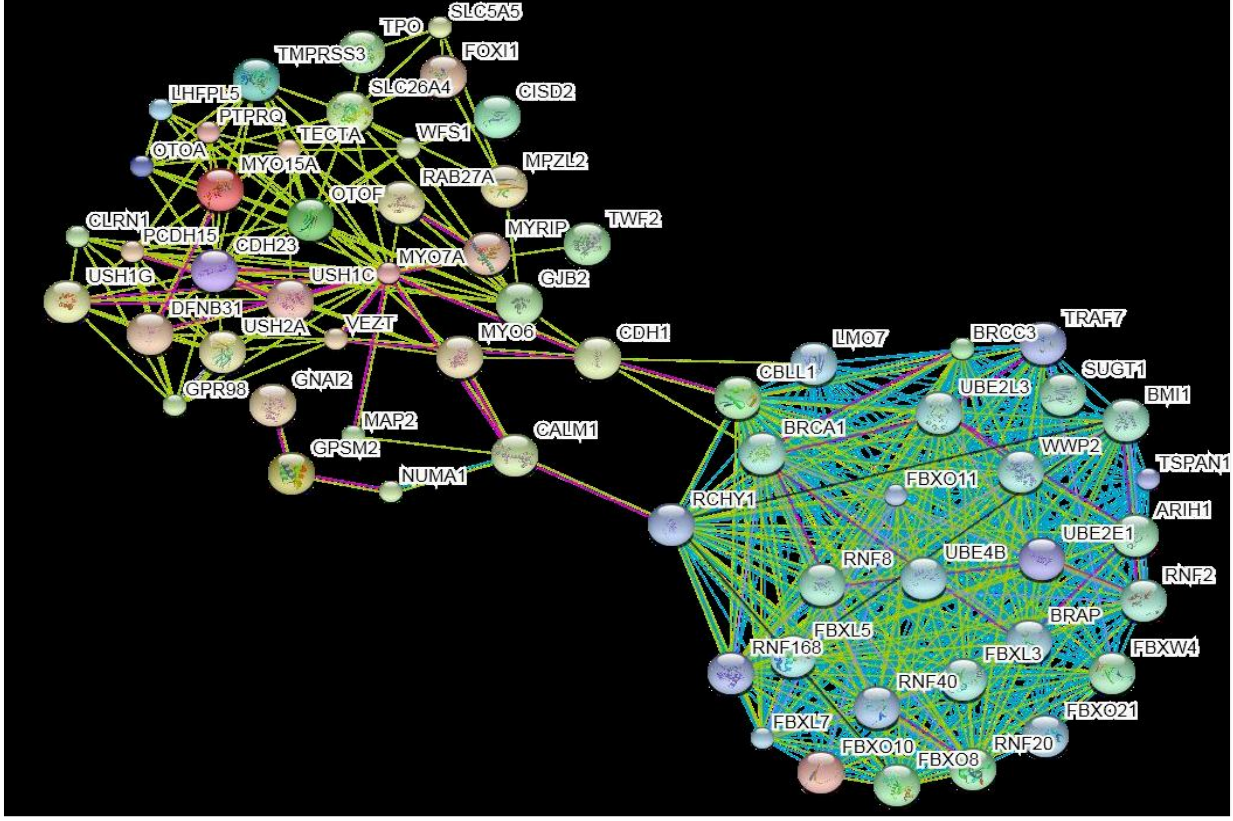


Figure 3.1: shows the gene-gene network using a set of seed genes involved in hearing impairment. An edge is built between two nodes if they co-appear in database to share a function, if they co-appear in the same abstract and many other criteria

As shown in figure 3.1 there are many interactions between these genes, indicating that there is a big chance that those genes are sharing biological processes or functioning together. In addition although FBXO10 is not biologically validated that it has a relationship with defenses but it appears as a node in the built network indicating its importance in this process. On the other hand, it's obvious from this figure that the network is almost divided into two major clusters that are connected by two bridges (CDH1, CALM1). That's to say the genes on the right hand side of the figure might share a biological process with genes on the left hand side through these two genes. There for, CALM1 and CDH1 are considered to have the highest Betweenness Centrality measure indicating its high importance in keeping this network connected. Betweenness centrality is a property of biological networks that describes how strong a particular node can connect al other node in a network. Another thing we might learn from this network that all the genes in one cluster are more strongly connected than the genes with the other cluster.

1.2. Building Clusters

To further analyze and understand the Network topology (structural feature) we included 10 more genes in the network to check whether there are any other connections that can be built between these clusters. Noting that the addition of these genes is completely unsupervised (we didn't control the names of genes to be included nor we control the location, it's all based on their similarity with the genes already existed in the network where the similarity stands for the previously mentioned criteria to build the network; text-mining, databases, co-expression ….etc.). Finally we run k-mean clustering algorithm (an algorithm that groups the genes related to each other together and separate them from all other genes) to check the behavioral characteristic of the network. **Figure 3.2** shows the new network after inclusion of genes and running clustering. A small analysis of the biological process dominate in each cluster is shown below.

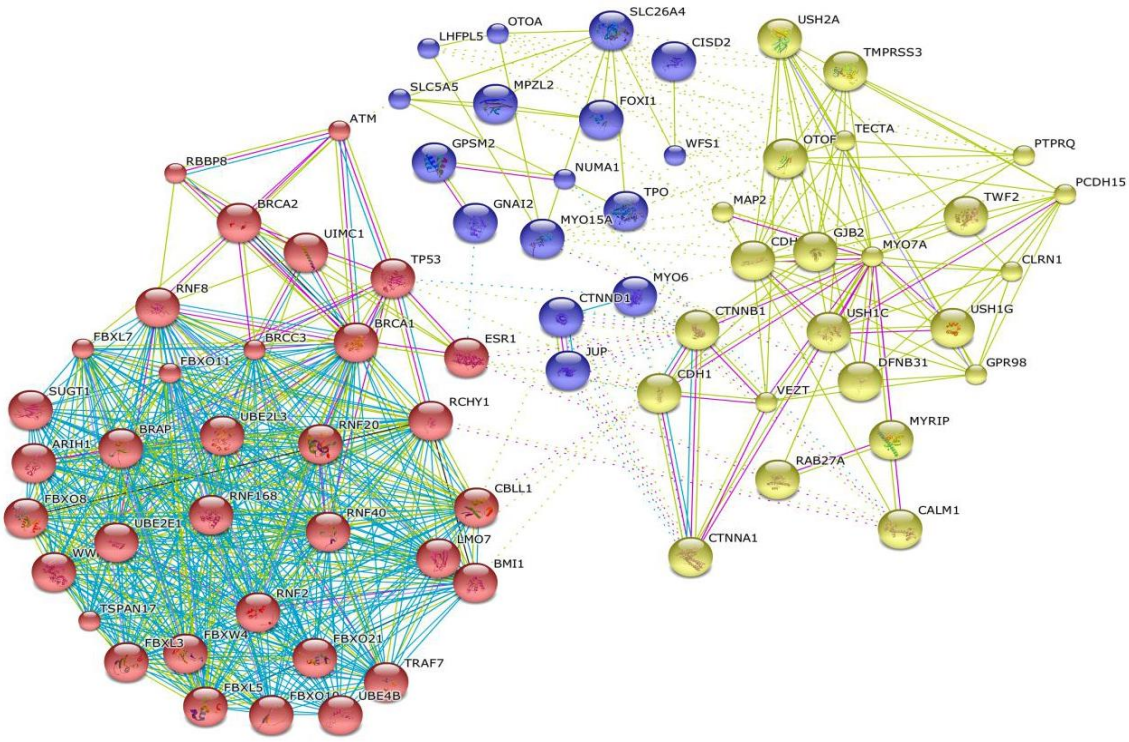


Figure 2: Shows three gene clusters (groups) based on their similarity with each other using k-mean clustering algorithm.

1.3. Functional Characterization

To better understand the molecular function for these genes, we used Gene-Ontology tools. These tools take as input a set of genes and output the most important functions these genes share together. Noteworthy that the first biological process indicates the most important function to represent these genes and the last one is the least important function to represent them. This is very helpful in the field of biomedical sciences to better understand the pathway or the biological process dominates for a set of genes. To do this, I used an online tool called DAVID. The result was really interesting where functions related to deafness has got a very high score to represent these genes. This indicates that our sets of genes are really involved in hearing loss. This, in other words, means that there is a great chance that the newly added genes to the network might also play a role in hearing loss and thus making them a tempting target for our analysis. The results from DAVID gene ontology tool are listed in **Figure 3.3**.

Functional Related Terms

Options

Rerun using options

1620 term(s) were searched. 27 term(s) passed the filter. Download File

Similarity Score: Very High (0.75-1) High (0.5-0.75) Moderate (0.25-0.5) Low (<0.25)

#	Category	Term	Kappa
1	SP_PIR_KEYWORDS	Hearing	1.00
2	SP_PIR_KEYWORDS	deafness	0.80
3	SP_PIR_KEYWORDS	vision	0.75
4	SP_PIR_KEYWORDS	usher syndrome	0.75
5	SP_PIR_KEYWORDS	sensory transduction	0.75
6	GOTERM_BP_FAT	sensory perception of mechanical stimulus	0.75
7	GOTERM_BP_FAT	cognition	0.75
8	GOTERM_BP_FAT	neurological system process	0.75
9	GOTERM_BP_FAT	sensory perception of sound	0.75
10	GOTERM_BP_FAT	sensory perception	0.75
11	GOTERM_BP_FAT	sensory perception of light stimulus	0.70
12	GOTERM_BP_FAT	visual perception	0.70
13	SP_PIR_KEYWORDS	retinitis pigmentosa	0.68
14	GOTERM_CC_FAT	stereocilium bundle	0.65
15	GOTERM_CC_FAT	stereocilium	0.65
16	GOTERM_BP_FAT	equilibrioception	0.65
17	GOTERM_BP_FAT	neuromuscular process	0.65
18	GOTERM_BP_FAT	neuromuscular process controlling balance	0.65
19	GOTERM_CC_FAT	microvillus	0.65
20	GOTERM_BP_FAT	photoreceptor cell maintenance	0.60
21	GOTERM_BP_FAT	cellular component maintenance	0.60
22	SP_PIR_KEYWORDS	non-syndromic deafness	0.58
23	SP_PIR_KEYWORDS	disease mutation	0.56
24	GOTERM_CC_FAT	cell projection	0.54
25	GOTERM_BP_FAT	inner ear development	0.52
26	GOTERM_BP_FAT	sensory organ development	0.52
27	GOTERM_BP_FAT	ear development	0.52

Figure 3.3: Shows the Most relevant biological processes to represent the prevailing function of our set of genes.

1.4.Choosing FBXO10 as a candidate gene

Finally I sought to check the molecular functions for different clusters detected in figure3.2.
For the red cluster, protein ubiquitins and protein modification was the most important function thus indicating their importance in being a tempting previously undescribed candidate for hearing impairment. For blue cluster, sensory perception of sounds and more generally development process was the most important function. On the other hand, sensory perception of sounds and inner ear morphogenesis was the most dominant function for genes in the yellow cluster.

Having these computational results in hand, in addition to that mutations in *FBXO11* in mice cause hearing impairment, this gene is paralogue to *FBXO10* in human, and this gene recently has been shown to harbor a novel homozygous missense mutation in family DE by Dr. Hashem Shahin (not published) . We decided to choose *FBXO10* for further biological validations. Thus we decided to study the possibility of validating *FBXO10* as being a novel gene involved in hearing impairment process.

2. Genotyping Analysis

2.1. Materials

2.1.1.Buffers, Gels and Solutions

- **Ethidium Bromine**

  Ethidium bromide was dissolved in the double distilled sterile water to a final concentration of 1mg/ml. (or bought premade to this concentration)

- **Agarose gel**

  1.5%agarose

  1X TBE buffer

  Final concentration of 0.01% ethidium bromide

- **Red blood cell lysis buffer**

155 mM NH4Cl

10m NH4HCO3

0.1 mM EDTA with (pH=7.4)

- **1X lysis buffer**

50 M Tris HCL with (pH=7.5)

100 mMNaCl

mM EDTA with (pH=8)

- **5X loading buffer**

0.25% bromophenol blue

0.25% Xylene cyanol FF

30% Glyserol in water

- **50X TAE Buffer**

  2M Trisph 8.0

  1M Acetic acid

  0.05M EDTA

  Ajust to PH=8.0

- **Proteinase K**

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

2.1.2. Reagents, Instruments and Kits

Reagents

Reagent	**Supplier**	**Product specifications**
Agarose	ORNAT	SeaKem® LE Agarose
Oligonucleotide primers	Hylabs	
Super therm polymerase	Eisenberg Bros	CAT# JMR-80
10x polymerase Buffer	Eisenberg Bros	CAT# JMR-420
dNTPs 2.5mM	TAMAR	CAT# R0181,4X0.25mM
100bp plus DNA ladder	TAMAR	ThermoScientific, GeneRuler™
Q solution	Qiagen	
Proteinas K	aMReSCO®	LOT# 1311C384
Hi Di Formamide	Applied Biosystems	CAT#4311320,
20% SDS	aMReSCO®	CAT # 083754-500ml

Kits

Kit	Supplier	Product specification
GENESCAN®400HD {ROKit	Applied Biosystems	CAT# 402985

Instruments

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

2.2. Methods

2.2.1. Pedigree collection and analysis:

Seventy two families were there DNA samples kindly provided by Prof. Moein Kanaan, director of the Hereditary Research Lab (HRL) at Bethlehem University where each family is referred to with a different symbol. These families show hereditary recessive hearing loss in some of their members. These families come from different parts of the West bank, but mainly from Hebron and Bethlehem. All of these families were studied by HRL at Bethlehem University for all Palestinian known deafness mutations, and till now they did not know the main reason that caused the deafness. HRL collected all possible information about these families, and they took one of the members as a proband. The information on the degree of hearing loss and the age of onset were carefully recorded. The pedigree was constructed from available information, for each family using the

standard methods, described by Bennett (1995). Males were symbolized by squares and females by circles. The normal individuals were designated with unfilled symbols while the affected one's by filled symbols.

2.2.2. Blood Sampling

The samples from all families already found as extracted DNA from the HRL at Bethlehem University. Only in family BN, Venous blood samples were drawn from the affected and normal members, using 10ml clean and sterilized syringes. Then the blood was immediately transferred into the blood vacutainer sets containing EDTA. These samples were kept at 4^{o}C, before being processed for genomic DNA extraction.

2.2.3.Isolation of the DNA by Salting-Out technique

Approximately 10 ml of the blood was collected in asterile EDTA vacutainer. 20ml of the red blood cell lysis buffer (section III.1.1) was added, and after a gentle mix, tubes were kept on ice for 10-20 minutes (min), being shaken by hand from time to time, until blood becomes transparent. Centrifugation was done at 2000 round per minute (rpm) for 10min. at 4 ^{o}C. The supernatant was carefully removed and the pellet was resuspended in 3 ml red blood cell lysis buffer and centrifugation was repeated. The pellet was then suspended in a mix of 1x lysis buffer, 100µl of 20%SDS and proteinase K. Incubation was done at 55 ^{o}C for 3 hours or 37 ^{o}C overnight. One ml of 6M NaCl was added to lysate and vigorously mixed until the solution appeared foamy. Centrifugation was done at 3000 rpm for 20 min. at room temperature, followed by gently transferring the upper phase into a 15 ml tube, avoiding the salt protein deposit. Two volumes of 100% ethanol (E-OH) were added to the upper phase followed by its gentle inversion. DNA was removed with a glass Pasteur pipette, followed by washing in 70% E-OH (in Eppendrof tube) and then air drying for a few minutes on Pasteur pipette. DNA was then dissolved in DDW (200-600µl depending on the amount of DNA) and left at room temperature overnight.

2.2.4. DNA Dilution:

The stock DNA was diluted to 100 ng/ml for PCR amplification

2.2.5. Polymerase Chain Reaction (PCR)

Table 3.1. Standard PCR reaction mix per 25µl of total volume

Reagent	**Volume in µl**
10X Buffer	2.50
Polymerase (super therm polymerase)	0.25
Q-Solution	5.00
dNTPs (2.5Mm)	2.00
Forward primer	0.50
Reverse primer	0.50
Nuclease free H2O	13.25
100ng/ µl DNA Template	1

PCR Programm

The Amplification process was performed using our PCR machine GeneAmp-PCR system 9700 from Applied Biosystem.

94 □ C 3min

94 □ C 30sec
63 □ C 30sec* X2
72 □ C 30sec**

94 □ C 30sec
61 □ C 30sec X2
72 □ C 30sec

94 □ C 30sec

57 □ C 30sec X2

72 □ C 30sec

94 □ C 30sec

55 □ C 30sec X35

72 □ C 30sec

72 □ C 5 min

4 □ C∞

*Annealing temperature depends on the TM of the primers used for PCR amplification.

**Extension temperature depends on the size of amplified product □1Kb = 1min.

2.2.6.Electrophoresis of PCR product using Agarose gels

The desired percentage of agarose was prepared and thenboiled until transparent , after ashort cooling period, Ethidium Bromide (section2.1.1) was added to a final concentration of 0.5µg/ml. the percentage of agarase dissolved in the TAE was 1.5% depend on the size of the PCR product. Three µl of the PCR product were mixed with loading dye and loaded intothe wells and run in 1X TAE running buffer at 120V for half an hour. DNA fragments were observed using ultraviolet light and photographed using the (Molecular Imager®, Gel DOC ™ Imaging System, BioRAD). For size estimation, 6µl of molecular weight marker (ThermoScientific, GeneRuler™, 100bp plus DNA ladder) was loaded in the first well.

2.2.7. Genotyping and Primers

2.2.7.1. Choose Primers

We searched one megabase around the *FBXO10* gene locus on chromosome 9 and we choose five microsatellite markers which are the most repeats and proximity to the *FBXO10* gene. The location of these five markers on chromosome 9 was obtained from UCSC genome browser (genome.ucsc.edu/) and Marshfield Madical Center (www.marshmed.org.gov/genetic/). Average heterozygosity of each marker was above 70%, implying that these markers are highly informative for genotyping in family members. The name, the locations and the size of the five microsatellite

markers on chromosome 9 are shown in table 3.2, and the primers for PCR amplification shown in table 3.3.

Primers, are short single strands from either DNA or RNA in order to bind to a complementry strand. During PCR the denaturing step will break the hydrogen bonds, separating the two strands apart, this allows the primers to anneal to the target region on the DNA during annealing step.

During designing primers for PCR its necessary to take into consideration things like: the length of primer the typical primers are 18-30 nucleotide in length, the 5´ and 3´ end , the primer melting /annealing temperature depending on the G/C content, the G-C content (50-60%), have a balanced distribution of G/C and A/T domains and the distance between the forward and reverse primers.

Table 3.2. Name, location and size of the five markers that surround FBXO10 gene.

Primer Name	Start	end	Size
D9S1794	36,216,218	36,216,465	150-182
D9S1791	36,393,375	36,393,713	168-190
D9S1874	37,222,265	37,222,559	167-203
AC(21)	37,741,348	37,741,589	160
GT(21)	38,477,910	38,478,147	223

Table 3.3. Primers for PCR amplification

	Primers name	**Primers sequence**
	Forward	**Reverse**
D9S1794	5’- GAATTGCTTGAACCTGGG-3´	5’- TCTGTGATCTTAGTTTGGGG-3´
D9S1791	5’- GTAATCTTGGGCAACCTATGTATG-3´	5’- TCAAAATAAGTCTGGGACAAAACC-3´
D9S1874	5’- GTATAGTATGGAGCAGAAATGTAAC-3´	5’- GGCCAAGGGATAAACAG-3´
AC(21)	5’- GGGATCTGGTGGCTGTGTTT-3´	5’- CCAATATGTTTCCTTCAGAATGC-3´
GT(21)	5’- GGGATTTGGACTTGATGGTTC-3´	5’- CCTAACTTTTCTCTCAATGCTC-3´

2.2.7.2 Genotyping

PCR amplification was performed for all 72 probands using the five different markers. Genotype analysis was first performed on all probands and later we considered the best nine probands, which have homozygote alleles for at least four markers, for further analysis. Genotyping reaction mixture is shown in table 3.4.

Table 3.4. Genotyping reaction mix per 12.5µl of total volume

Reaction	Volume in µl
Hi-dye	11.2
Rox	0.3
DNA (PCR)	1

Genotyping was carried out by adding all components in table 3.4. After pipetting and vortexing the reaction components, we denatured samples for 2 minutes at 95 ^{o}C using a 96 well hot plate. Samples were then snap cooled on ice for 5 minutes before transferring them into sequencer plate. Samples were finally mounted on the Applied Biosystems 3130XL sequencing machine for fragment analysis.

In a typical microsatellite analysis, microsatellite loci are amplified by PCR using fluorescently labeled forward and unlabeled reverse primers. The PCR amplicons are separated by size using electrophoresis; then the dye labeled products are identified by fluorescence detection.

Amplified fragments, along with appropriate size standards, migrate through a polymer-filled capillary and are detected using Data Collection Software (DCS). During an electrophoresis run, the DCS records the fluorescence intensity as a function of time and wavelength from regions on a CCD camera that correspond to different detection wavelength ranges. Data collected was represented in the form of peaks on the electropherogram.

These peaks were generated by GeneScan software version 4.0. The number of peaks and their intensities are proportional to the length of the repeat and the number of repeats in the PCR product.

Chapter4: Results

Our current study is based on 72 consanguineous families with non-syndromic hereditary hearing impairment. These families were taken from HRL at Bethlehem University. These families were screened by HRL for all mutation that have been found in the Palestinian population and till now and so far they didn't know the genetic cause for their phenotype. So these families are considered suitable for our study using linkage exclusion approach.

Figure 4.1 shows the results for genotyping using five microsatellite markers on the 72 probands that represent the families we studied. More specifically, Figure 4.1 shows the number of families having 1, 2, 3, 4, or 5 homozygote alleles out of the five markers we analyzed. To further investigate the presence or absence of a mutation in *FBXO10* we considered the probands that have homozygote alleles for at least 4 markers. We argue that studying these probands' families would enable us to check if genotyping results were generated by chance or are related to a mutation in *FBXO10*.

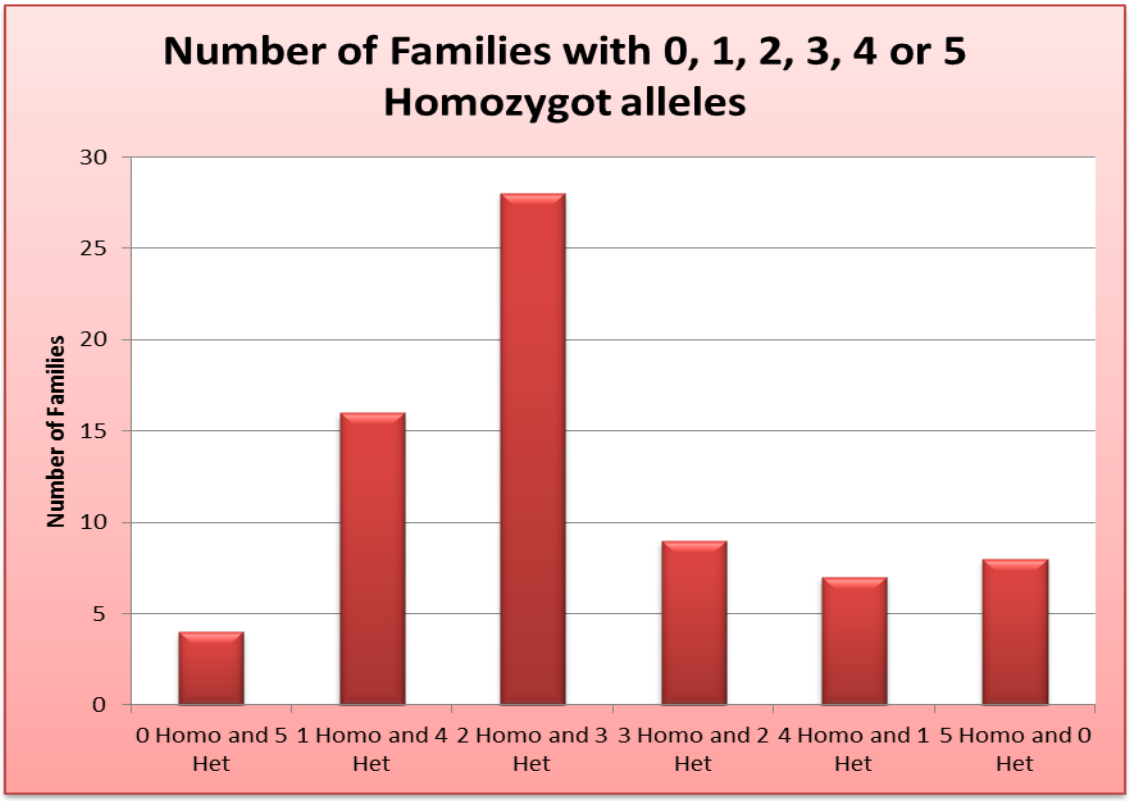


Figure 4.1: Genotyping result for 72 probands by using five microsatellite markers

Table 4.1. Genotyping result for the nine probands that each has at least homozygote alleles for at least four markers.

#	Name	GT(21)	AC(21)	1874	1791	1794
1	DE3	Homo	Homo	Homo	Homo	Homo
2	DN3	Homo	Homo	Homo	Homo	Homo
3	X1	Homo	Homo	Homo	Homo	Homo
4	CT3	Homo	Homo	Homo	Homo	Homo
5	AH2	Homo	Homo	Homo	Homo	Homo
6	BV3	Homo	Homo	Homo	Homo	Het
7	BN3	Homo	Homo	Homo	Homo	Homo
8	BA3	Homo	Homo	Homo	Homo	Homo
9	AR1	Homo	Homo	Homo	Homo	Homo

1. Description of families studied:

Family DE

Family DE is from Bethlehem and was ascertained by HRL, and was used as a positive control in this analysis. Traditionally, the family members prefer to marry within the community and consequently consanguineous marriages are very common. Pedigree of this family is shown in Figure 4.2. Three individuals including two females and one male are diagnosed to have bilateral severe to profound NSHL. The affected individuals present in generation II of the pedigree. Analysis of the pedigree is strongly suggestive of an autosomal recessive mode of inheritance. The DNA samples for all family members were already available for our use.

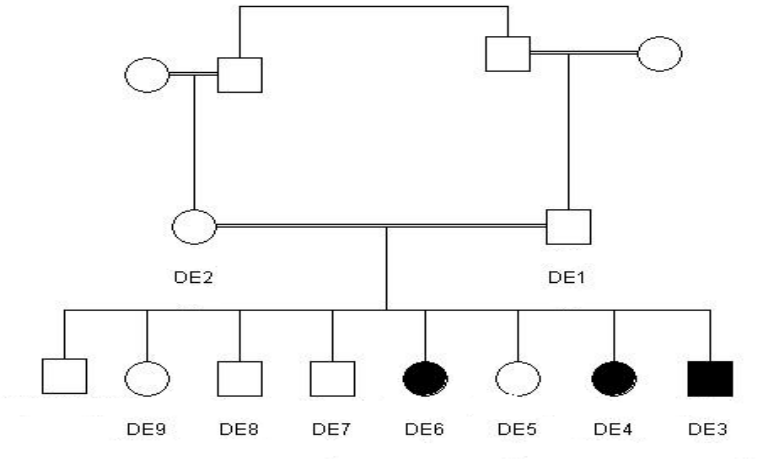


Figure4.2. Pedigree of family DE with NSHL. Circles represent females, and squares represent males. Filled circles and squares represent affected individuals.

Family DN

The family DN is a consanguineous family from Hebron with only one affected individual in generation II. Pedigree for this figure 4.3.The affected male individual suffers from prelingual non-syndromic hearing loss. Analysis of the pedigree is suggestive of an autosomal recessive mode of inheritance. DNA samples for the family members were already at the HRL.

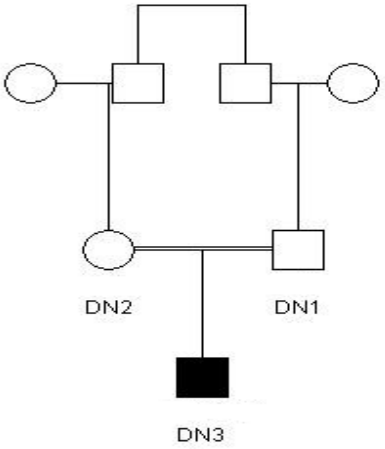


Figure 4.3. Pedigree of family DN with NSHL.

Family X

Family X is a large extended family from Bethlehem with six affected individuals both a male and female Pedigree of this family is shown in Figure 4.4. Affected individuals are diagnosed to have bilateral severe to profound NSHL. The affected persons are present in generation I of the pedigree. Analysis of the pedigree is strongly suggestive of an autosomal recessive mode of inheritance.

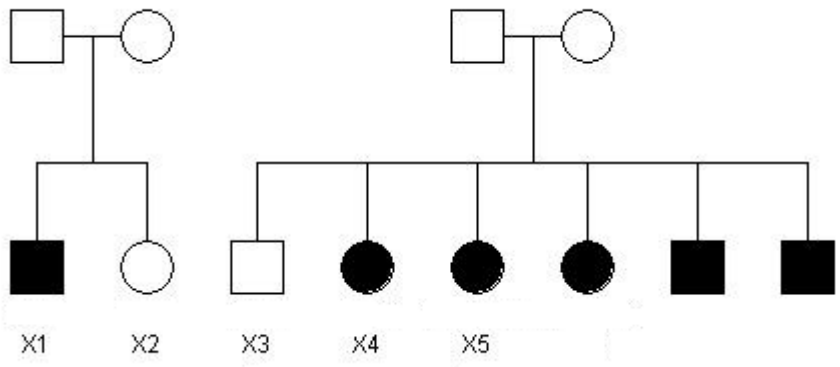


Figure 4.4. Pedigree of family X with NSHL.

Family CT

The family CT is originated from south of Bethlehem. Pedigree for this consanguineous family is shown in figure 4.5. Two individuals present in first generation and affected by prelingual non-syndromic hearing loss. As shown in figure 4.5, the affected persons are females. Analysis of the pedigree is suggestive of an autosomal recessive mode of inheritance.

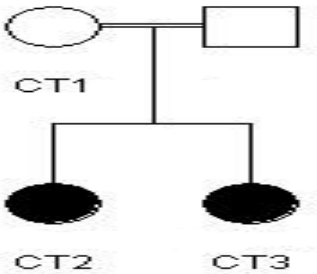


Figure 4.5. Pedigree of family CT with prelingual non-syndromic hearing loss.

Family AH

Family AH is another consanguineous family from Bethlehem with multiple affected members including three females and 2 males. Pedigree of this family is shown in Figure 4.6. showing the affected family members who suffer from bilateral severe to profound SNHL. The affected persons are present in generation II and III of the pedigree, two males and two females in generation II and one female in generation III. Analysis of the pedigree is strongly suggestive of an autosomal recessive mode of inheritance.

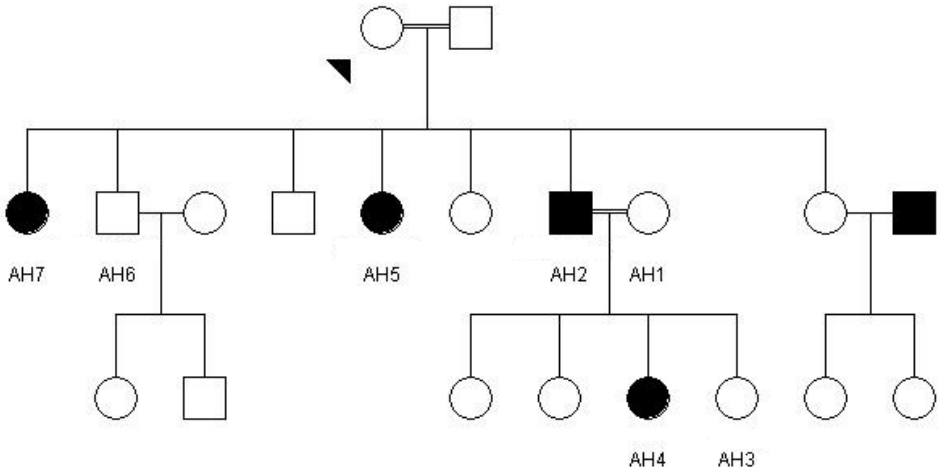


Figure 4.6. Pedigree of family AH with NSHL.

Family BV

Family BV is consanguineous extended family from Hebron. Pedigree of this family is shown in Figure 4.7. Six individuals including three males and three females are diagnosed to have bilateral severe to profound SNHL. The affected individuals present in generation IV and V of the pedigree, one female in generation IV, two females and three males in generation V. Analysis of the pedigree is strongly suggestive of an autosomal recessive mode of inheritance.

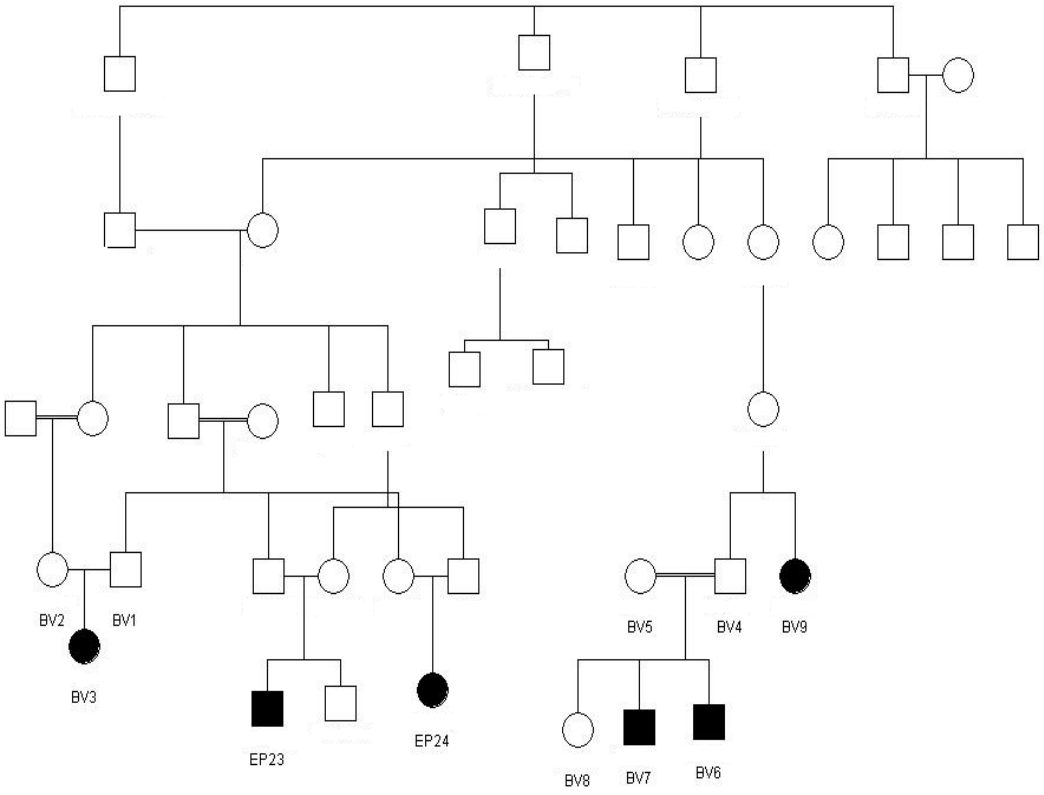


Figure 4.7. Pedigree of family BV with NSHL.

Family BA

Family BA is a large extended family from Bethlehem, with seven affected individuals both male and female. The parents of the affected individuals are second degree relatives. Pedigree of this family is shown in Figure 4.8 indicates four affected females and three males are diagnosed to have bilateral severe to profound SNHL. Analysis of the pedigree is strongly suggestive of an autosomal recessive mode of inheritance. The DNA samples for BA1, BA2, BA3 and BA4 were already found in HRL. DNA samples for the other family members were not available for the study.

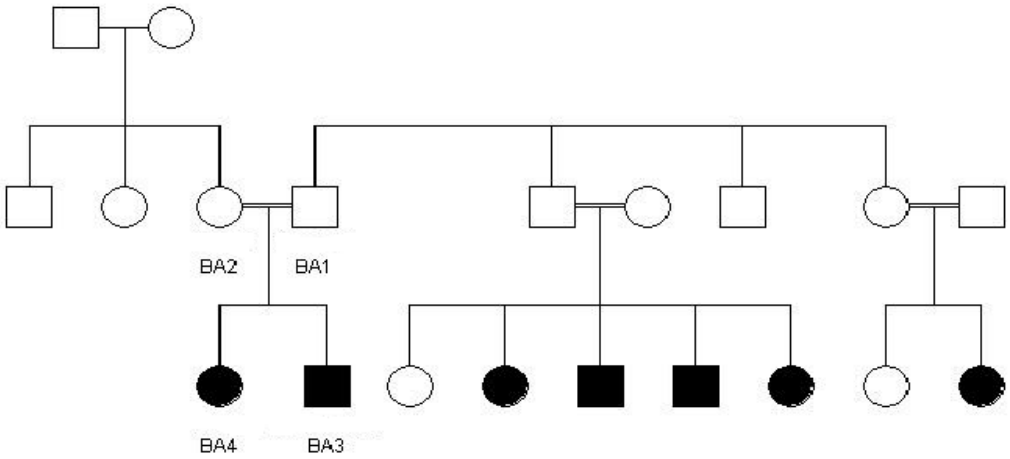


Figure 4.8. Pedigree of family BV with NSHL.

Family AR

The family AR comes from Hebron; pedigree for this family is shown in figure 4.9. Only one individual presents in first generation and is diagnosed to have bilateral severe to profound NSHL. Analysis of the pedigree is suggestive of an autosomal recessive mode of inheritance.

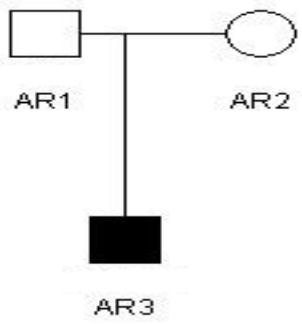


Figure 4.9. Pedigree of family AR with NSHL. Circles represent females, and squares represent males. Filled circles and squares represent affected individuals.

Family BN

The family BN is originated from Bethlehem with two affected and one unaffected family members marriages. Pedigree for this consanguineous family is shown in figure 4.10. The affected individuals are present in first generation and are diagnosed to have bilateral severe to profound SNHL. Analysis of the pedigree is suggestive of an autosomal recessive mode of inheritance. DNA samples for BN1, BN2, BN3 were already found in HRL. We collected blood samples from all family members, to study BN4 and BN5 members, and to make confirmation for our results.

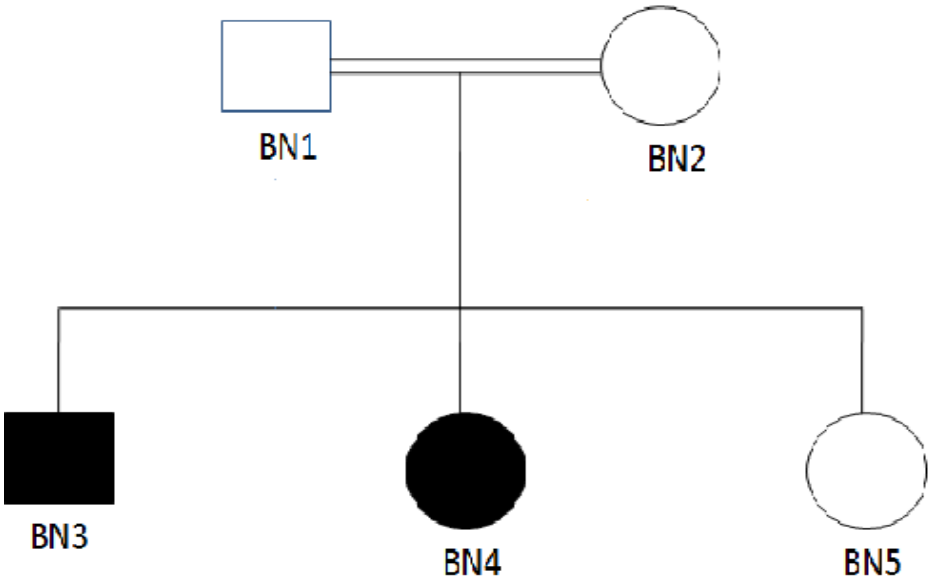


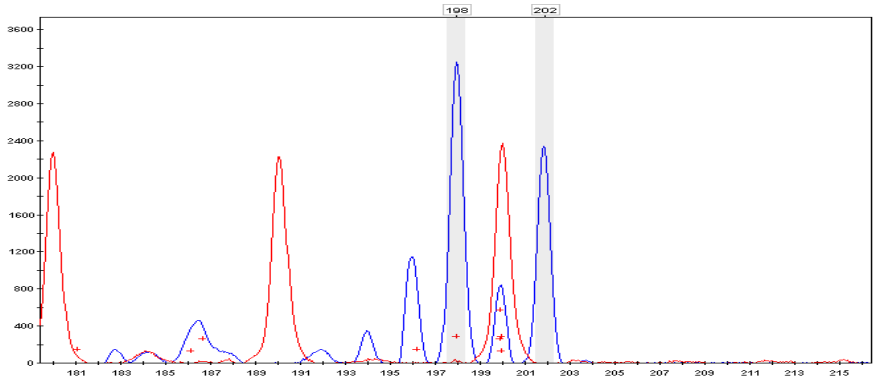
Figure 4.10. Pedigree of family BN with NSHL.

2. Genotyping Result:

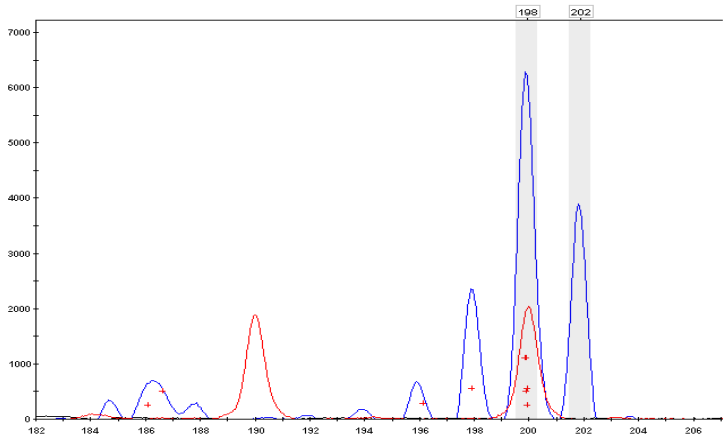
Based on genetic linkage studies on other forms of hereditary hearing impairment, it is clear that FBXO10, as a candidate gene interval, must be tested for linkage analysis. As being shown in missense mutation in FBXO10 in family DE, we performed linkage exclusion for both normal and affected individuals from all the nine families for FBXO10 gene on chromosome 9. This have been achieved using two markers D9S 1874 and AC (21) to check if FBXO10 is linked to for the hearing loss in these families or not.

Family DE

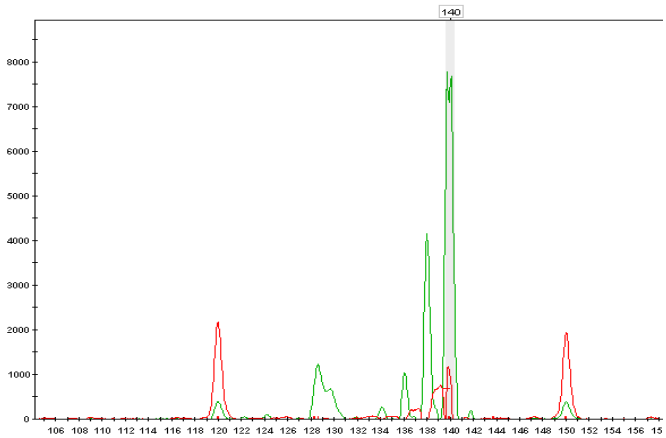
In this analysis family DE has been used as a control. That is because the first result for DE3 has homozygous allele with using the five markers as shown in table 4.1. For the normal individuals DE1, DE2, DE5, DE7, DE8 and DE9 the results indicated that they have heterozygous allele both for D9S 1874 and AC (21). The result for affected individuals DE3, DE4 and DE6 were homozygous for both markers as shown in table 4.2. The results confirm that *FBXO10* is related to hearing loss in this family.



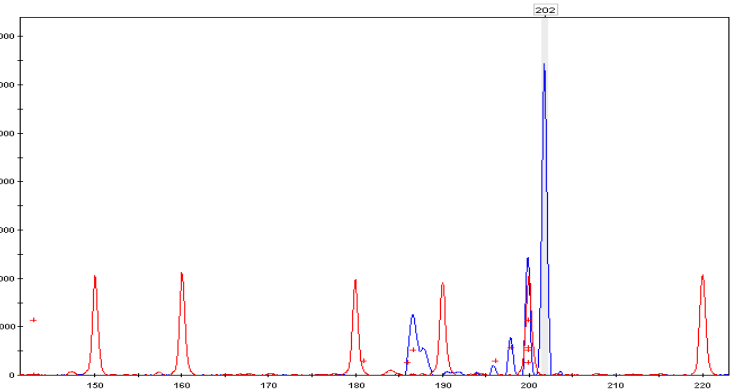
Hetrozygous alleles for DE1 by using D9S1874 marker



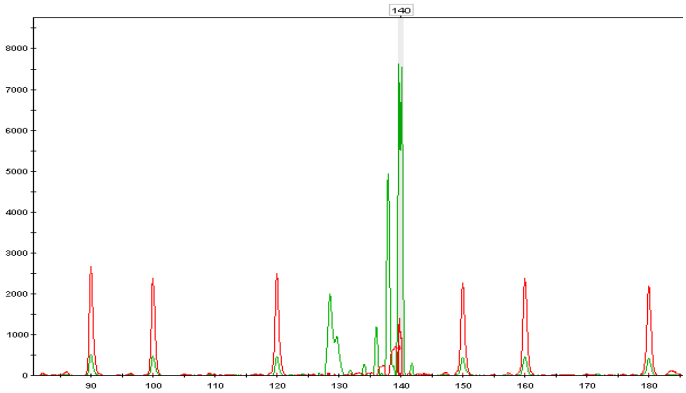
Heterozygous alleles for DE2 by using D9S1874 marker



Heterozygous alleles for DE2 by using(AC21) marker



Homozygous alleles for DE4 by using D9S1874 marker



Homozygous alleles for DE4 by using (AC21) marker

Table 4.2. shows the results of genotyping for family DE by using D9S 1874 and AC (21) markers.

Family members	D9S1874	AC (21)	Affected
DE1	Hetrozygous	Hetrozygous	Unaffected
DE2	Hetrozygous	Hetrozygous	Unaffected
DE3	Homozygous	Homozygous	Affected
DE4	Homozygous	Homozygous	Affected
DE5	Hetrozygous	Hetrozygous	Unaffected
DE6	Homozygous	Homozygous	Affected
DE7	Hetrozygous	Hetrozygous	Unaffected
DE8	Hetrozygous	Hetrozygous	Unaffected
DE9	Hetrozygous	Hetrozygous	Unaffected

Family DN

We carried out genotyping analysis for three individuals in family DN; DN1, DN2 and DN3 using two markers D9S 1874 and AC (21). The results for the affected individual (DN3) was homozygous for both markers. On the other hand, the results for the unaffected individuals DN1 were heterozygous for both markers. Finally the genotyping results for the unaffected individual, DN2, were homozygous for both markers. The genotyping results for this family are shown in table 4.3. This indicates that FBXO10 is not related to hearing loss in this family.

Table 4.3. shows the result of genotyping for family DN by using D9S 1874 and AC (21) markers.

Family members	D9S1874	AC (21)	Affected
DN1	Hetrozygous	Hetrozygous	Unaffected

DN2	Homozygous	Homozygous	Unaffected
DN3	Homozygous	Homozygous	Affected

Family X

We did genotyping for Five individuals in family X; X1, X2, X3, X4 and X5 by using two markers D9S 1874 and AC (21). The result for affected individual X1 was homozygous for both markers. For the unaffected individual, X2, the result was heterozygous for D9S 1874 marker and homozygous for AC (21) marker. On the other hand, the genotyping result for X3 as unaffected individual was heterozygous for D9S 1874 marker and homozygous for AC (21) marker. Finally, the results for the affected individuals, X4 and X5, were heterozygous for D9S 1874 marker and homozygous for AC (21) marker. The genotyping result for this family shown in table 4.4. These contradicting results show that FBXO10 is not linked to hearing loss in this family.

Table 4.4. shows the result of genotyping for family X by using D9S 1874 and AC (21) markers.

Family members	D9S1874	AC (21)	Affected
X1	Homozygous	Homozygous	Affected
X2	Heterozygous	Heterozygous	Unaffected
X3	Heterozygous	Homozygous	Unaffected
X4	Heterozygous	Homozygous	Affected
X5	Heterozygous	Homozygous	Affected

Family CT

Three genotyping tests have been applied to three different individuals in family CT; CT1, CT2 and CT3 using two markers D9S 1874 and AC (21). For the affected individuals CT3 and CT2 the results were homozygous for both markers and heterozygous for both markers, respectively. On the other hand, the result for the unaffected individual, CT1, was heterozygous for D9S 1874 and

homozygous for AC (21) markers. The genotyping result for this family shown in table 4.5. These contradicting results suggest that FBXO10 is not linked to hearing loss in this family.

Table 4.5. shows the result of genotyping for family CT by using D9S 1874 and AC (21) markers.

Family members	D9S1874	AC (21)	Affected
CT1	Heterozygous	Homozygous	Unaffected
CT2	Heterozygous	Heterozygous	Affected
CT3	Homozygous	Homozygous	Affected

Family AH

Genotyping analysis have been applied on seven different individuals from family AH; AH1, AH2, AH3, AH4, AH5, AH6 and AH7 using two markers D9S 1874 and AC (21). The results for the affected individuals AH2, AH4, AH5 and AH7 were consistent in which all were homozygous for both markers. On the other hand, the results vary among the unaffected individuals. For instance, AH1 and AH3 were homozygous for D9S 1874 marker and heterozygous for AC (21) marker. However the genotyping result for AH6 individual was homozygous for both markers. The genotyping result for this family shown in table 4.6. These results show that FBXO10 is not related to hearing loss in this family.

Table 4.6. shows the result of genotyping for family AH by using D9S 1874 and AC (21) markers.

Family members	D9S1874	AC (21)	Affected
AH1	Homozygous	Heterozygous	Unaffected
AH2	Homozygous	Homozygous	Affected
AH3	Homozygous	Heterozygous	Unaffected
AH4	Homozygous	Homozygous	Affected

AH5	Homozygous	Homozygous	Affected
AH6	Homozygous	Homozygous	Unaffected
AH7	Homozygous	Homozygous	Affected

Family BV

Genotyping analysis have been applied on nine different individuals for family BV; BV1, BV2, BV3, BV4, BV5, BV6, BV7, BV8 and BV9 using two markers D9S 1874 and AC (21). The result for affected individuals; BV3, BV6 and BV7 were not consistent. For instance the results for BV3 and BV6 were homozygous for both marker whereas it was heterozygous for both markers for BV7. In addition, there were also variations in results for the unaffected individuals; BV1, BV4, BV8, BV9, BV2 and BV5. For instance, the results for BV1, BV4, BV8, and BV9 were homozygous for both markers. However the genotyping results for BV2 and were heterozygous forD9S 1874 marker and homozygous for AC (21) marker. The genotyping result for this family shown in table 4.7. These contradicting results show that FBXO10 is not linked to hearing loss in this family.

Table 4.7. shows the result of genotyping for family BV by using D9S 1874 and AC (21) markers.

Family members	**D9S1874**	**AC (21)**	**Affected**
BV1	Homozygous	Homozygous	Unaffected
BV2	Heterozygous	Homozygous	Unaffected
BV3	Homozygous	Homozygous	Affected
BV4	Homozygous	Homozygous	Unaffected
BV5	Heterozygous	Homozygous	Unaffected
BV6	Homozygous	Homozygous	Affected
BV7	Heterozygous	Heterozygous	Affected

BV8	Homozygous	Homozygous	Unaffected
BV9	Homozygous	Homozygous	Unaffected

Family BA

For family BA, we have applied genotyping analysis on four different individuals; BA1, BA2, BA3 and BA4 using D9S 1874 and AC (21) markers. The result for affected individuals; BA3 and BA4 were not consistent. For instance, the result for BA3 was homozygous for both markers whereas the result for BA4 was heterozygous forD9S 1874 and homozygous for AC (21). Similarly, genotyping results for the unaffected individuals; BA1 and BA2 were not consistent. The result for BA1 was heterozygous for D9S 1874 and homozygous for AC (21) whereas the result for BA2 was homozygous for both markers. The genotyping result for this family shown in table 4.8. These contradicting results show that FBXO10 is not linked to hearing loss in this family.

Table 4.8. shows the result of genotyping for family BA by using D9S 1874 and AC (21) markers.

Family members	D9S1874	AC (21)	Affected
BA1	Heterozygous	Homozygous	Unaffected
BA2	Homozygous	Homozygous	Unaffected
BA3	Homozygous	Homozygous	Affected
BA4	Heterozygous	Homozygous	Affected

Family AR

Genotyping analysis have been applied on three different individuals from family AR; AR1, AR2 and AR3 by using D9S 1874 and AC (21) markers. For the affected individual AR3, the result was homozygous for both markers. But again the results were inconsistent with the unaffected parents AR1 and AR2. Result for AR1 was homozygous for both markers whereas results for AR2 were

heterozygous for both markers. The genotyping results for this family are shown in table 4.9. These results show that FBXO10 not linked to hearing loss in this family.

Table 4.9. shows the result of genotyping for family AR by using D9S 1874 and AC (21) markers.

Family members	D9S1874	AC (21)	Affected
AR1	Homozygous	Homozygous	Unaffected
AR2	Heterozygous	Heterozygous	Unaffected
AR3	Homozygous	Homozygous	Affected

Family BN

For family BN, we did genotyping for all family member BN1, BN2, BN3, BN4 and BN5 using two markers D9S 1874 and AC (21). The results were not consistent both for the affected; BN3 and BN4 and unaffected; BN1, BN2 and BN5 individuals. Result for BN3 was homozygous for both marker and it was heterozygous for both markers for BN4. Results for BN1 and BN2 were heterozygous for both markers, but it was homozygous for BN5. The genotyping results for this family are shown in table 4.10. These results show that FBXO10 is not linked to hearing loss in this family.

Table 4.10. shows the resulst of genotyping for family BN by using the two marker

Family members	D9S1874	AC (21)	Affected
BN1	Heterozygous	Heterozygous	Unaffected
BN2	Heterozygous	Heterozygous	Unaffected
BN3	Homozygous	Homozygous	Affected
BN4	Heterozygous	Heterozygous	Affected
BN5	Homozygous	Homozygous	Unaffected

CHAPTER 5

Discussion and Conclusion

Autosomal recessive non-syndromic hearing loss is condidered genetically extremely heterogeneous. Up to this point, 70 autosomal recessive non-syndromic hearing impairment loci have been mapped on human chromosome and ˜ 40 causative genes have been cloned (Borck G 2011). All the autosomal recessive non-syndromic hearing loss loci are typically characterized by congenital, profound sensorineural hearing loss, with only two exceptions. The first is DFNB8 that is characterized with mild hearing loss beginning at 10 to 12 years of age and progressing to profound stage by age of 14 to 16, and its mapped to chromosome region 21q22 (Veske A 1996). The second exception is DFNB13 which is characterized by progressing severe to profound hearing impairment appears during second and third decades of life.

Hearing loss to different degrees, is one of the most prevalent sensory defect, worldwide (Willems 2000). Hearing impairment with severe degree is the highest in terms of its prevalence (D. P. Kelsell 1997), affecting more than 250 million people in the world (Colin Mathers 2000). About 1 in 1000 new born suffer a form of hearing loss (Hashem Shahin 2002), approximately half of all cases have genetic etiology (Nikolay A Barashkov 2011). More than 100 different genes which are estimated to be involved in the inner ear and in the hearing process in general (Klemens Frei 2002; Kramer 2010), and only single- gene mutations can lead to damage the cochlea and cause hearing loss (Willems 2000).

The present study has been founded on the supposition that the human population of Palestine has a special genetic composition, consanguinity, and mutation rates. In Palestinian population, about 44,3% of the marriage are between relatives and the mutation can randomly appear at different loci and even at different time stages like any other population. Prelingual hereditary hearing impairment occur in the Palestinian population at a frequency of approximately 1.7 per 1000 (Hashem Shahin 2002) and may exceed in isolated communities. Furthermore, in Palestinian population until now, twenty nine mutated alleles are known to cause NSHL

FBXO10 is substrate-recognition component of the SCF (SKP1-CUL1-F-box protein)-type E3 ubiquitin ligase complex. FBXO10 gene was mapped on chromosome 9p13.2 and found to have 11 exons and encodes 956 amino acids; a mutation in this gene may lead to deafness. This

finding has been suggested in DE family that has been studied at Hereditary Research Lab HRL at Bethlehem University by Dr. Hashem Shahin. The affected individuals in this family were diagnosed to have bilateral severe to profound SNHL. Deafness phenotype in this family segregated perfectly with a novel homozygous missense mutation in exon 2 of *FBXO10* gene. *FBXO11* is another example of F-box protein family, Fbxo11 is expressed in epithelial cells of the middle ears. *FBXO11* in mice is paraloge to *FBXO10* in human and mutations in *FBXO11* lead to Jeff mouse. This makes FBXO10 an important candidate gene for the study of the genetic path way involve in hearing loss in human

We studied whether this missense mutation or other mutations in *FBXO10* is causing the hearing loss in 72 families with SNHL. Most of these cases resulted from consanguineous marriages. All of the families that we studied are living in different parts of either Hebron or Bethlehem. To show if mutations in this gene are found in the Palestinian deaf community; we carried out linkage exclusion analysis. We started our analysis by running some basic computational biology techniques in order to define some novel candidates that might have a role in hearing impairment. *FBXO10* has been predicted as being a good candidate for our analysis. Also *FBXO11* in mice is paraloge to *FBXO10* and mutation in *FBXO11* in mice lead to hearing impairment and this hinted to us that mutation in *FBXO10* in human also may lead to hearing impairment. But our findings reported after making linkage analysis study indicate that that the novel mutation in *FBXO10*, that has been identified in DE family could be specific for this family.

Linkage study in the 72 probands, presented here, was initially studied by using five markers which we selected it on the basis of their map position (UCSC Genome Browser) and heterozygosity coefficient , and then we chose nine probands with homozygote alleles for at least four markers. Two markers were used to genotype both affected and normal individuals in each family. Genotyping results for these nine families confirm that the *FBXO10* is not likely to cause hearing loss in those families

CHAPTER 6

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