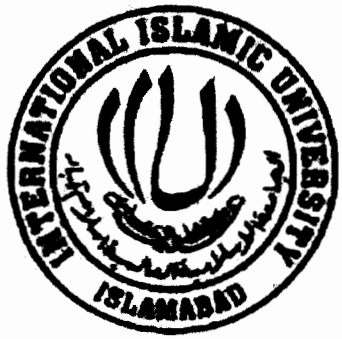
Genetic Analysis and Candidate Genes Prediction in Pakistani Families with Hearing Impairment



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Department of Bioinformatics

Faculty of Basic & Applied Sciences

International Islamic University Islamabad

2009

MS
572-869
TAG

DATA ENTERED

5/3/2012

T06371E2009BIMS
2/7/2010

M.Phil

Accession No TH-6371

Bacterial genetics
Transposons
Bacteriophages

A.E
24-1-11

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Verily, when He intends a thing, His Command is, "be", and it is!

(Ya-Sin: 82)

CERTIFICATE

Title of Thesis: Genetic Analysis and Candidate Genes Prediction in Pakistani Families with Hearing Impairment.

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Accepted by the Department of Bioinformatics, Faculty of Basic and Applied Sciences, International Islamic University Islamabad, in partial fulfillment of the requirements for the Master of Sciences in Bioinformatics.

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(27th August, 2009)

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Tahira Noor

DEDICATION

My Loving Mother

To the one who gave me life
I can give nothing but love
To the star so full of light
to the one sent from up above.
Thank you for the kindness
for everything you do
thank you for the happiness
thanks for seeing me through.
I love you more than anything
I'll love you to the end
you are my mother first
then my very best friend.
It is unusual what we share
everything I do you know,
every beautiful quality
you have in you, I try to show.
Not often enough do I tell you
how much you mean to me
Not often enough do I show you
how your love set me free
To live forever and always
Is what I wish for you.
A long life ever-lasting
for one so sacred and true
You are my special angel
who I always find comfort in
whether life is in a shambles
whether I lose or whether I win
To the one who genuinely is,
sent from heaven above.

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ACKNOWLEDGMENTS

In the name of ALLAH, the most Gracious, the most Merciful, the one and the only, the Creator, Who is perfect in all characteristics, worthy of worship. All praises for Allah Who guides us when we bewilder in darkness of ignorance and help us in enlightening our ways. Countless salutation upon the Holy Prophet Hazrat Muhammad (P.B.U.H), the fortune of knowledge, Who took the humanity out of the abyss of ignorance and elevated it to the zenith of consciousness.

Ordinary words of gratitude don't truly encompass that love and gratitude extended by my loving grand parents, parents, my brothers and sister for their prayers, constant interest in my studies and encouragement, due to which I am here. Infact it is the result of their love, affection and encouragement that kept me going.

I wish to express my warmest, sincerest thanks and profound gratitude to my supervisor **Dr. Abdul Hameed**, Principal Scientific Officer, Institute of Biomedical and Genetic Engineering, Islamabad, who gave me valuable suggestions and also increase my capabilities of research and hard work.

I feel privileged and have great pleasure to place on record my sincere indebtedness to my supervisor **Dr. Asma Gul**, Assistant Professor, International Islamic University, Islamabad under whose sincere guidance and obliging supervision that I prepared this manuscript.

I shall be failing in my duty if I do not put across my thanks and gratitude to Muhammad Ismail, Deputy Director, Institute of Biomedical and Genetic Engineering, Islamabad. It was because of his inspiring guidance, consistent encouragement during the entire work, that I completed this work.

I am indebted to Nasir Malik, Specially Muhammad Ajmal and Ammara Javiad, Scientific officers, Institute of Biomedical and Genetic Engineering, Islamabad for their cooperation, generous help and sympathetic attitude through out my research work.

I would also like to thank Dr. Irfan Khan, Chairman, Department of Environmental Science and Bioinformatics, International Islamic University, Islamabad for solving my all problems regarding chemicals.

I convey my heartiest and sincerest acknowledgements to my worthy and pertinacious friend for indispensable moral support during my whole study programme.

I would like to express my immense affection to my all class fellows Saima Zubair, Sumaira Nishat, Shagufta Kanwal, Amara Jabeen, Memoona Mushtaq, Awaisa Ghazal, Iffat Farzana and Adeela Safder for there memorial company and kind support.

I also venerate the affected families, without their cooperation and support the present research work could not be accomplished.

May Allah Help me in fulfilling dreams of my parents and give me success in both worlds.

Amin

Tahira Noor

LIST OF ABBREVIATIONS

APS	Ammonium Per Sulphate
ARNSHI	Autosomal recessive non-syndromic hearing impairment
bp	Base pair
cDNA	Complimentary DNA
CMV	Cytomegalovirus
Cx26	Connexin 26
dB	Decibels
DBHL	Decibels hearing level
DFN	Deafness
DFNA	Autosomal dominant non-syndromic deafness
DFNB	Autosomal recessive non-syndromic deafness
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDN3	Endothelin 3
EDNRB	Endothelin receptor type B
EDTA	Ethylene di-ammine tetra acetate
ERs	Estrogen receptors
ESRRs	Estrogen-related receptors
EST	Expressed Sequence Tag
EVA	Enlarged vestibular aqueduct
HI	Hearing impairment
kD	Kilo Dalton
LDLRA	Low-density lipoprotein receptor class A
ml	millilitre

mM	millimolar
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NCBI	National Center for Biotechnology Information
NF2	Neurofibromatosis 2
ng	Nanogram
NHR	Nuclear hormone receptor
NSHI	Non-syndromic hearing impairment
NSHL	Nonsyndromic hearing loss
OAEs	Otoacoustic emissions
Ods	Optical Densities
OMIM	Online Mendelian inheritance in Man
OSMED	Otospondylomegaepiphyseal dysplasia
PCR	Polymerase Chain Reaction
RP	Retinitis pigmentosa
Rpm	Revolution per minute
SDS	Sodium Dodecile Sulphate
SRCR	Scavenger receptor cysteine rich
SRs	Steroid receptors
STE	Saline Tris EDTA
TBE	Tris-borate EDTA
TEMED	N'N'N'N-tetra methyl ethylene diamine
Tris	Trizma Base
tTJ	tricellular tight-junction

TTSP	Transmembrane Serine Protease
USH	Usher syndrome
UV	Ultraviolet
WS	Waardenburg syndrome
µl	Microlitre
µM	Micromolar

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ABSTRACT

In humans Hearing impairment (HI) is considered as one of the most common neurosensory deficit. Multiple factors are involved in causing HI and these factors include genetic and environmental factors. Severe or profound hearing loss affects approximately one in 1000 children worldwide and half of these cases are affected because of genetic factors. For non-syndromic hearing impairment, 75-80% of the cases are due to autosomal recessive inheritance, 15-20% display autosomal dominant inheritance, 2-3% of cases show X-linked inheritance and less than 1% are due to mitochondrial inheritance. HI represents extreme genetic heterogeneity. To date in non-syndromic deafness 135 gene loci have been mapped, including 77 loci with autosomal recessive mode of inheritance and 29 of those corresponding nuclear genes have been cloned.

In the present study, two Pakistani families (A, B) with autosomal recessive non-syndromic form of deafness, were ascertained from different regions of Pakistan. In family A seven individual and in family B nine individuals were affected with autosomal recessive form of hearing impairment. Initially both families A and B were screened for linkage to known deafness loci. After the linkage analysis Genotyping results showed that the family A was excluded from linkage to known loci. In the course of screening the affected individuals of family B were homozygous with these markers D14S588 (71.0 cM), D14S53 (82.7 cM) and D14S74 (83.9 cM), thus family B showed linkage to ESRRB on chromosome 14q24.1-24.3.

In the present study, by using Bioinformatics approach we can reduce very large number of candidate genes into a few strong candidate genes, which could be screened for mutation analysis. If the mutation analysis of all genes is done then it will be time taking and labor-intensive process. By using Bioinformatics approach, which is presented here, few strong candidate genes are predicted and these few strong candidate genes can be managed for mutation analysis.

INTRODUCTION

The human ear is one of the more remarkable parts of the human body because of its remarkable sensitivity to sounds. The acquisition and transduction of sound by the mammalian ear is a highly complex process involving numerous tissues and cell types. The complexity of the auditory process is reflected not only in the elaborate organ of Corti and its stereocilia processes but also in a myriad of other cell types that make up the cochlea, many of which are vital for hearing (Brown *et al.*, 2008).

Hearing Pathway

In the mammalian ear, sound is collected by the pinna and travelled to the tympanic membrane. Vibration of the tympanic membrane leads to movement of the ossicular chain in the middle ear, transmitting sound impulses across the middle-ear cavity to the inner ear. The inner ear contains vestibular and cochlear compartments. The vestibular compartments sense movement and gravity, and comprise the sacculus, utriculus and the semi-circular canals (Brown *et al.*, 2008). The snail-shell like cochlea is the primary transducer of the peripheral auditory system. The three functional components of the cochlea are the lateral wall, the organ of Corti and the spiral ganglion (Jin and Duan, 2006). The organ of Corti is composed of a single row of inner hair cells and three rows of outer hair cells. The inner ear is the site of mechanotransduction; it converts stimuli into electrical signals that are then transmitted to auditary centre. The auditory system has proven difficult to access and very small amount of tissue, containing highly specialized cells are available for analysis (Guipponi *et al.*, 2008).

Hearing Impairment

Due to the complexity of the inner ear, alterations in different pathways may result into hearing impairment (HI) phenotype. Hearing impairment, which occurs before or at the crucial stages of language development, leads to an impaired ability to develop speech, language and effective communication skills. Hearing impairment is the common sensory disorder worldwide (Apps *et al.*, 2007; Britner-Glindzicz, 2002). Prelingual hearing impairment exists when the impairment is congenital or otherwise acquired before the individual has acquired speech and language, it is more difficult to treat because the child is unable to access audible/spoken communication from the

outset. In post-lingual hearing impairment, hearing loss is adventitious after the acquisition of speech and language. It may develop due to disease, trauma, or as a side-effect of a medicine. Late-onset hearing impairment, known as presbycusis, is a significant burden on the adult population (Brown *et al.*, 2008; Gratton and Vazquez, 2003).

Hearing impairment is more prevalent in populations where consanguineous marriages are common. As a consequence of the unique socio-cultural practices in Pakistan; approximately 60% of marriages are consanguineous while more than 80% are between first cousins (Elahi et al., 1998).

Classification of Hearing Impairment

On the basis of physiological etiology hearing impairment is classified into three types. These three basic types of hearing loss are: conductive hearing impairment, sensorineural hearing impairment and mixed hearing impairment.

Conductive Hearing Impairment

Conductive deafness is associated with external-ear or middle-ear abnormalities. Conductive forms of deafness result in mild or moderate hearing impairment. Most forms of conductive hearing impairment yield to medical treatments or surgeries (Petit, 2006). Conductive hearing imapairment usually involves a reduction in sound level, or the ability to hear faint sounds. Examples of conditions that may cause a conductive hearing impairment include conditions associated with middle ear pathology such as fluid in the middle ear from colds, allergies, poor eustachian tube function, ear infection, perforated eardrum, benign tumors, impacted earwax, infection in the ear canal, presence of a foreign body, absence or malformation of the outer ear, ear canal, or middle ear.

Sensorineural Hearing Impairment

Sensorineural deafness is caused by a defect located anywhere along the auditory pathway, from the cochlea to the auditory cerebral cortex, the degree of hearing impairment associated with sensorineural forms can vary from mild to profound (Friedman *et al.*, 2007). Sensorineural hearing impairment cannot be medically or surgically corrected. It is a permanent loss. Sensorineural hearing impairment is caused by diseases, birth injury, drugs that are toxic to the auditory system, and

genetic syndromes. Sensorineural hearing impairment may also occur as a result of noise exposure, viruses, head trauma, aging, and tumors.

Mixed Hearing Impairment

Sometimes a conductive hearing impairment occurs in combination with a sensorineural hearing impairment. In this hearing loss damage occurs in the outer or middle ear and in the inner ear or auditory nerve. This type of hearing impairment is referred to as a mixed hearing loss.

Other descriptors associated with hearing impairment are:

Bilateral versus unilateral: Bilateral hearing impairment means both ears are affected. Unilateral hearing impairment means only one ear is affected.

Progressive versus sudden Hearing Impairment: Progressive hearing impairment is a hearing impairment that becomes increasingly worse over time. A sudden hearing impairment is one that has an acute or rapid onset and therefore occurs quickly.

Severity of Hearing Loss

Degree of hearing loss refers to the severity of the loss. The severity of hearing loss is measured by the degree of loudness, a sound must attain before being detected by an individual. When hearing is tested with a clinical audiometer, the measured level is expressed in dBHL (decibels hearing level). Petit, (2006) classified hearing impairment according to the degree of hearing loss compared with normal hearing:

- Mild, loss of 21–40 dBHL (cannot hear whispers).
- Moderate, loss of 41–70 dBHL (cannot hear conversational speech).
- Severe, loss of 71–90 dBHL (cannot hear shouting).
- Profound, loss of 90 dBHL (cannot hear sound when it usually becomes painful).

Frequency of Hearing Loss

The frequency of hearing loss is designated as:

- Low (<500 Hz)
- Middle (501-2000 Hz)

- High (>2000 Hz)

Etiology and Prevalence

Hearing Impairment etiology exhibits extreme genetic heterogeneity. The etiology of profound childhood deafness is markedly diverse and involves numerous environmental and genetic factors or a combination of both. A great deal of success has been achieved during the past decade in identifying new loci and genes causing deafness and it is observed that 1% of protein coding human genes are involved in hearing loss (Friedman and Griffith, 2003; Nance, 2003).

Approximately one in 1000 children is affected by severe or profound hearing loss at birth or during early childhood (Petersen and Willems, 2006). More than 50% of prelingual deafness is genetic, most often autosomal recessive and nonsyndromic. The disorder DFNB1, caused by mutations in the *GJB2* gene (which encodes the protein connexin 26) and the *GJB6* gene (which encodes the protein connexin 30), accounts for 50% of autosomal recessive nonsyndromic hearing loss. The carrier rate in the general population for a recessive deafness-causing *GJB2* mutation is about one in 33. A small percentage of prelingual deafness is syndromic or autosomal dominant nonsyndromic (Smith *et al.,* 2004). In the general population, the prevalence of hearing loss increases with age. This change reflects the impact of genetics and environment, and also interactions between environmental triggers and an individual's genetic predisposition, as illustrated by aminoglycoside-induced ototoxicity (Gantz *et al.,* 2005).

The prevalence of profound bilateral hearing loss is 1.6 in 1,000 in Pakistan and 70% of deafness arises in inbred families (Elahi *et al.*, 1998). Consanguineous marriages decrease the proportion of shared genes in families with increase in consanguinity, making them an excellent resource material for conventional linkage analysis and for the identification of new loci, genes and mutations.

Causes of Hearing Loss

Some of the many causes of deafness include:

- Environmental factors
- Genetic factors

Environmental Factors

Acquired hearing loss in children

It commonly results from prenatal infections from "TORCH" organisms (i.e., toxoplasmosis, rubella, cytomegalic virus, and herpes), or postnatal infections, particularly bacterial meningitis caused by *Neisseria meningitidis, Haemophilus influenzae,* or *Streptococcus pneumoniae*. Meningitis from many other organisms, including *Escherichia coli, Listeria monocytogenes, Streptococcus agalactiae,* and *Enterobacter cloacae*, can also cause hearing loss.

Acquired hearing loss in adults

Aquired hearing loss in adults is most often attributed to environmental factors, especially noise exposure, but susceptibility probably reflects an environmental-genetic interaction. For example, aminoglycoside-induced hearing loss is more likely in persons with an A-to-G transition at nucleotide position 1555 in the mitochondrial genome (mtDNA) (Arnos, 2003).

Genetic Factors

Single-Gene Disorders

- **Syndromic hearing impairment** is associated with malformations of the external ear or other organs or with medical problems involving other organ systems.
- **Nonsyndromic hearing impairment** has no associated visible abnormalities of the external ear, nor are there any related medical problems; however, it can be associated with abnormalities of the middle ear and/or inner ear.

Syndromic Hearing Impairment

Over 400 genetic syndromes that include hearing loss have been described (Gorlin *et al.,* 1995). Syndromic hearing impairment may account for up to 30% of prelingual deafness, but its relative contribution to all deafness is much smaller, reflecting the occurrence and diagnosis of postlingual hearing loss.

Autosomal Dominant Syndromic Hearing Impairment

- Waardenburg syndrome (WS) is the most common type of autosomal dominant syndromic hearing loss. It consists of variable degrees of

sensorineural hearing loss and pigmentary abnormalities of the skin, hair (white forelock), and eyes (heterochromia iridis).

- Branchiootorenal syndrome is the second most common type of autosomal dominant syndromic hearing loss. It consists of conductive, sensorineural, or mixed hearing loss in association with branchial cleft cysts or Fistulae, malformations of the external ear including preauricular pits, and renal anomalies.
- Stickler syndrome consists of progressive sensorineural hearing loss, cleft palate, and spondyloepiphyseal dysplasia resulting in osteoarthritis.
- Neurofibromatosis 2 (NF2) is associated with a rare, potentially treatable type of deafness. The hallmark of NF2 is hearing loss secondary to bilateral vestibular schwannomas. The hearing loss usually begins in the third decade, concomitant with the growth of a vestibular schwannoma, and is generally unilateral and gradual, but can be bilateral and sudden (Gantz, 2005).

Autosomal Recessive Syndromic Hearing Impairment

- Usher syndrome is the most common type of autosomal recessive syndromic hearing loss. It consists of dual sensory impairments: affected individuals are born with sensorineural hearing loss and then develop retinitis pigmentosa (RP). Three types of Usher syndrome are recognized based on the degree of hearing impairment and result of vestibular function testing.

1. Congenital severe-to-profound sensorineural hearing loss and abnormal vestibular dysfunction
2. Congenital mild-to-severe sensorineural hearing loss and normal vestibular function.
3. Progressive hearing loss and progressive deterioration of vestibular function.

- Pendred syndrome is the second most common type of autosomal recessive syndromic hearing loss. The syndrome is characterized by congenital severe-to-profound sensorineural hearing impairment and euthyroid goiter.
- Jervell and Lange-Nielsen syndrome is the third most common type of autosomal syndromic hearing loss.

- Refsum disease consists of severe progressive sensorineural hearing loss and retinitis pigmentosa caused by faulty phytanic acid metabolism.

X-Linked Syndromic Hearing Impairment

The common type of this hearing impairment includes

- Alport syndrome is characterized by progressive sensorineural hearing loss of varying severity, progressive glomerulonephritis leading to end-stage renal disease, and variable ophthalmologic findings (i.e., anterior lenticonus).
- Mohr-Tranebjaerg syndrome (deafness-dystonia-optic atrophy syndrome) was first described in a large Norwegian family with progressive, postlingual, nonsyndromic hearing impairment.

Mitochondrial Syndromic Hearing Impairment

Mitochondrial DNA mutations have been implicated in a variety of diseases ranging from rare neuromuscular syndromes such as Kearns-Sayre MELAS, MERRF, and NARP, to common conditions like diabetes mellitus, Parkinson disease, and Alzheimer disease (Fischel-Ghodsian, 1998).

Nonsyndromic Hearing Impairment

More than 70% of hereditary hearing loss is nonsyndromic (Cremers *et al.*, 1991; van Camp *et al.,* 1997). Disorders discussed in this section are organized by mode of inheritance. The different gene loci for nonsyndromic deafness are designated DFN (for DeaFNess). Loci for genes inherited in an autosomal dominant manner are referred to as DFNA, those for genes inherited in an autosomal recessive manner as DFNB, and those for genes inherited in an X-linked manner as DFN. The number following these designations reflects the order of gene mapping and/or discovery.

Autosomal Dominant Nonsyndromic Hearing Impairment

Family studies of autosomal dominant nonsyndromic hearing loss have shown that heterogeneity is high. Unlike autosomal recessive nonsyndromic hearing loss, which is also extremely heterogeneous but in which the majority of cases are caused by mutations in a single gene in many world populations, a single gene responsible for the majority of cases of autosomal dominant nonsyndromic hearing loss has not been

identified. In spite of this limitation, the audioprofile can be distinctive and useful in predicting candidate genes for mutation screening (Friedman *et al.,* 2003). For example, mutations in *WFS1* are found in 75% of families segregating autosomal dominant nonsyndromic hearing impairment that initially affects the low frequencies while sparing the high frequencies. Autosomal Dominant deafness is designated DFNA.There are 57 Loci of Autosomal Dominant deafness which are identified worldwide up to date.

Autosomal Recessive Nonsyndromic Hearing Impairment

Autosomal recessive is a subtype of nonsyndromic deafness. Most forms of autosomal recessive deafness are characterized by hearing loss from birth. An autosomal recessive inheritance pattern means that two copies of an altered gene are present in each cell. Autosomal recessive deafness is designated DFNB. Each type is numbered in the order in which it was described. For example, DFNB1, DFNB2, and DFNB3 are particular forms of autosomal recessive deafness. There are 77 Loci of Autosomal Recessive deafness, which are identified worldwide up to date. The level of hearing loss varies within and among families. Autosomal recessive deafness is typically caused by changes to structures in the inner ear. The inner ear consists of three parts: a snail-shaped structure called the cochlea that helps process sound, nerves that send information from the cochlea to the brain, and structures involved with balance. Hearing loss caused by changes in the inner ear is called sensorineural deafness. Between 75 percent and 80 percent of all nonsyndromic deafness is inherited in an autosomal recessive pattern. In many populations, mutations in the GJB2 gene cause most cases of nonsyndromic deafness. Certain mutations in this gene are most common in particular ethnic groups, such as people of Ashkenazi (eastern or central European) Jewish, Asian, or Caucasian (white) ancestry. In many world populations, 50% of persons with autosomal recessive nonsyndromic hearing loss have mutations in *GJB2* (Zelante *et al.*, 1997; Estivill *et al.*, 1998; Kelley *et al.*, 1998). The other 50% of cases are attributed to mutations in numerous other genes, many of which have been found to cause deafness in only one or two families (Scott *et al.*, 1998). Extensive phenotype-genotype studies have shown that it is possible to predict the hearing loss associated with *GJB2* mutations based on the specific genotype (Snoeckx *et al.*, 2005). The other 50% of cases are attributed to mutations in numerous other

genes, many of which have been found to cause deafness in only one or two families (Zbar *et al.*, 1998).

X-Linked Nonsyndromic Hearing Impairment

X-linked Nonsyndromic hearing impairment constitutes 1–3% of all Nonsyndromic hearing impairment cases. There are seven DFN loci (Van Camp and Smith, 2009). Hearing loss is usually prelingual except for DFN6. Boys are frequently affected due to presence of single X chromosome. Hearing loss is progressive and severe involving all tones. DFN2 and DFN4 mostly cause severe prelingual sensorineural hearing loss. The type of hearing loss or phenotype is not known very well for DFN5 and DFN8. All forms cause sensorinenral hearing loss except DFN3 that causes mixed hearing loss (Petersen *et al.*, 2008). This type of hearing loss is also referred to as congenital fixation of the stapes footplate with perilymph gusher, mapped to Xq21.1 (DFN3). Affected males have also temporal bone abnormalities. Mutation in the *POU3F4* gene (MIM 300039), which encodes a POU domain containing DNA-binding regulatory protein, is responsible (De kok *et al.,* 1995). So far, it is the only gene identified to be associated with non-syndromic X-linked deafness (Bayazit and Yilmaz, 2006; Petersen *et al.*, 2008).

Mitochondrial Nonsyndromic Hearing Impairment

Mitochondrial DNA mutations are present in less than 1% of the children with pre-lingual deafness but are more frequent at a later age (Kokotas *et al.*, 2007). In the Caucasian population, at least 5% of post-lingual, non-syndromic hearing impairment is caused by known mitochondrial DNA (mtDNA) mutations, representing the most frequent cause of hearing loss after the 35delG mutation in the *GJB2* gene encoding connexin 26 (Jacobs *et al.*, 2005). The frequency of mitochondrial hearing loss is unknown. It has been estimated that 1% of prelingual hearing loss is because of constitutional mutations in mtDNA (Marazita *et al.*, 1993; Kokotas *et al.*, 2007). It has been suggested that around 20% of inherited post-lingual hearing loss may be caused by mutations in the mitochondrial genome, but large ethnic differences might be present The mtDNA mutations in the *MTRNR1* gene and the *MTTS1* gene cause non-syndromic hearing loss. At the RNA level, non-syndromic hearing loss is caused by mutations in mtDNA encoding rRNA (*MTRNR1*) or tRNA *(MTTS1*) but never in protein-encoding mtDNA cochlea. At the DNA level, the transcription of genes such

as the *MTRNR1* gene is lower in the cochlea than that of other genes which makes the cochlea more vulnerable to mutations in these genes (Kokotas *et al.*, 2007).

Connexins, Connexons and Gap Junctions

Twenty-one genes have been recognized in the human genome coding for connexin protein expression (Sohl and Willecke, 2003). Single connexon is a hexamers of connexins join within the cellular plasma membrane (Chang *et al.*, 2003; del Castillo *et al.*, 2003). Connexons from adjacent cells then unite, forming gap junctions, which serve as intercellular communication channels (Sabag *et al.*, 2005; Rabionet *et al.*, 2002). Connexins and gap junctions are very important for normal auditory function and are found in the epithelial and connective tissue cells of the cochlea (Sabag *et al.*, 2005; Kammen-Jolly *et al.*, 2001).

GJB2 (connexin 26)

Cx26 is the most prevalent of the connexins expressed in the cochlea (Martin *et al.*, 1999) along the nonsensory epithelial cells around the outer hair cells. The mutations in the gene encoding Cx26 expression (*GJB2*) disrupt the recycling of potassium ions to the endolymph, resulting in cellular dysfunction and ultimately cell death (Apps et *al.*, 2007). Mutations of the *GJB2* gene, encoding connexin 26 (Cx26) (Erbe *et al.*, 2004), are the most common cause of hereditary, prelingual, nonsyndromic hearing loss (Seeman *et al.*, 2005). Approximately 90 *GJB2* mutations leading to abnormal Cx26 expression have been reported and linked to hearing impairment, although the strength of their association is variable (Azaiez *et al.*, 2004; Cryns *et al.*, 2004). Up to 85% of abnormal Cx26 expression is due to the 35delG mutation (Estivill *et al.*, 1998), a truncating deficit that introduces a premature stop codon (Martin *et al.*, 1999; Smith and van Camp, 1999). Some *GJB2* mutations have high prevalence rates in specific populations,35delG among people of European descent, 167delT in the Ashkenazim, 235delC among East Asians, and 427C>T(R143W) in the Ghanaian population (Santos *et al.*, 2005).

GJB6 (connexin 30)

The *GJB6* gene encoding connexin 30 is located next to the connexin 26 gene, and the deletion extends from the 5 prime end of the *GJB6* gene toward the *GJB2* gene. Therefore, the *GJB6* deletion may also deletes control regions of the *GJB2* gene. Connexin-30 shows 77% identity in amino acid sequence with connexin-26 (Petersen

and Willems, 2000; Lautermann *et al.*, 1998). The Delta (*GJB6*-D13S1830) mutation has a relatively high carrier frequency, albeit variable between populations (Seeman *et al.*, 2005; Frei *et al.*, 2004; del Castillo *et al.*, 2003), and a significant co-contributor to nonsyndromic deafness. Only one dominant *GJB6* mutation related with non-syndromic deafness has been identified in a small number of patients (del Castillo *et al.*, 2005).

***GJB3* (connexin31)**

The *GJB3* gene encoding the gap junction protein connexin 31 is mapped to chromosome 1p35–p33. The *GJB3* protein connexin 31 has four hydrophobic transmembrane domain-like motifs, a structure similar to that of the other connexins and sharing 76% homology with human *GJB2*. A 3-bp deletion in the *GJB3* gene was identified in a Spanish family segregating autosomal dominant mild hearing impairment with peripheral neuropathy (Lopez *et al.*, 2001).

***TRIC* (tricellulin)**

Tricellulin is a tricellular tight-junction (tTJ) protein, which is essential in the formation of barriers between tricellular contacts of epithelial cells throughout the body (Ikenouchi *et al.*, 2005). Riazuddin *et al.* (2006) reported mutations in the *TRIC* gene encoding the tricellulin at DFNB49 locus on chromosome 5q13 in eight families with ARNSHI (Riazuddin *et al.*, 2006; Ramzan *et al.*, 2005). *TRIC* gene sequencing of three families led to the identification of a novel mutation (IVS4 + 1G >A) and the previously described mutation (IVS4 + 2T >C) in three families. About 1.06% of families with ARNSHI in Pakistan manifest hearing impairment (HI) due to mutations in the *TRIC* gene (Chishti *et al.*, 2007).

***TMHS* (tetraspan membrane protein of hair cell stereocilia)**

TMHS, also named *LHFPL5* belongs to the family of tetraspan proteins with four transmembrane domains and two connecting extracellular loops. It is specifically expressed in stereocilia of inner and outer hair cells of the mouse during stereocilia formation, suggesting an important role in hair bundle morphogenesis (Shabbir *et al.*, 2006; Longo-Guess *et al.*, 2005). The homozygous p.Cys161Phe mutation in *TMHS* described to cause deafness and vestibular dysfunction in the ''hurry-scurry'' (hscy) mouse (Longo-Guess *et al.*, 2005). Shabbir *et al.* (2006) reported mapping of the DFNB67 locus and identification of a homozygous deletion, c.246delC, leading to an

unchanged Pro83 followed by a stop codon (p.Pro83ProfsX1), and missense mutation (p.Tyr127Cys) in *TMHS* in two families from Pakistan (Kalay *et al.*, 2006).

***CDH23* (otocadherin)**

Cadherins (calcium dependent cell adhesion molecule) are membrane bound Glycoprotein receptors that function in cell-to-cell contact at adhesions junctions (Nagafuchi, 2001) and possibly form extracellular connections between adjacent hair cell stereocilia. The *CDH23* gene is a very large gene consisting of at least 70 exons encoding 3353 amino acids. Loss of function mutations in the mouse *Cdh23* ortholog causes stereocilia disorganization of the inner and outer hair cells in the waltzer mouse, identifying otocadherin as a critical component for proper hair bundle formation (Petersen and Willems, 2006).

***TMIE* (transmembrane Inner Ear Protein)**

TMIE gene encodes protein made up of 156 amino acids is mapped to chromosome 3p, which exhibits no significant similarity to any other gene (Naz *et al.*, 2002). The deaf *Tmie*-mutant spinner mouse shown to have progressive hair cell degeneration with irregular apical surfaces of hair cells and extra rows of maturing stereocilia in inner hair cells (Mitchem *et al.*, 2002). The previously reported variant c.241C>T (p.R81C) was observed in two Pakistani families. Two variants, c.92A>G (p.E31G) and the splice site mutation c.212 −2A>C, identified in one Pakistani and one Jordanian family, respectively (Santos *et al.*, 2005).

***TMC1* (transmembrane Channel-Like 1)**

The *TMC1* protein have an important function in K^+ channels of inner hair cells, which is consistent with the hypothetical structure of protein domains in which sequence variants are identified. *Tmc1* mutations are also identified in the recessive deafness *(dn)* and dominant Beethoven (*Bth)* mouse mutant cause postlingual hearing impairment. Mutation in this gene caused DFNB7/11 in 11 families and DFNB36 in a single family (Kurima *et al.*, 2002). Five putatively functional nonsynonymous sequence variants, were detected in seven families One family has the known splice site mutation c.536 -8T>A (Santos *et al.*, 2005;Kurima *et al.*, 2002).

***TMPRSS3* (transmembrane protease, serine 3)**

TMPRSS3 is a member of the Type II Transmembrane Serine Protease (TTSP) family and encodes for a protease that also contains LDLRA (low-density lipoprotein receptor class A) and SRCR (scavenger receptor cysteine rich) domains. Fourteen pathogenic mutations, which occur not only in the catalytic domain but also in the LDLRA and SRCR domains, have been identified to date that causes the DFNB8/10 forms of deafness. (Guipponi *et al.*, 2008). Linkage analysis in 159 consanguineous Pakistani families segregating congenital deafness identified five families with potential linkage to the DFNB8/DFNB10 locus. Homozygous *TMPRSS3* mutations have been identified in four of these families (Ben-Yosef *et al.*, 2001).

***COL11A2* (collagen 11α2)**

A phenotype–genotype comparison suggests that mutation type and location are critical determinants in defining the phenotype of *COL11A2*-associated diseases (Petersen and Willem, 2006; Chen *et al.*, 2005). Mutation screening of the *COL11A2* gene in the Iranian family identified a missense mutation P621T in exon 21 in homozygous state in affected individuals (Chen *et al.*, 2005). Several other *COL11A2* mutations include recessive mutations in otospondylomegaepiphyseal dysplasia (OSMED) and dominant mutations in non-ocular Stickler syndrome (Petersen and Willem, 2006).

***OTOA* (otoanchorin)**

The *OTOA* gene is mapped to chromosome 16p12.2 (DFNB22) and encodes for the glycocyclophosphatidyl-inositol anchored protein otoancorin (Jovine *et al.*, 2002). Otoancorin mediates attachment of the tectorial membrane in the cochlea,and the otoconial membranes and cupulae in the vestibule. *OTOA* consists of 28 exons. A splice-site mutation at the exon 12/intron 12 junction co-segregates with the hearing impairment in single family (Petersen and Willems, 2006; Zwaenepoel *et al.*, 2002).

***ESPN* (espin)**

The espins are actin-bundling proteins. The human *ESPN* gene consists of 13 exons and encodes 854 amino acid protein with eight ankyrin repeats, two praline rich regions, an actin-binding WH2 domain, and a coiled coil domain important for actin bundling. Two homozygous *ESPN* frameshift mutations in the two Pakistani families are identified (Petersen and Willems, 2006). Naz *et al.* (2004) identified a

homozygous 4-bp deletion (2469delGTCA) in exon 13 of the *ESPN* gene, leading to a stop codon at nucleotide 2533. The resultant protein lack one of the C-terminal actin-binding sites necessary for espin activity.

***SLC264A* (pendrin)**

SLC26A4 encodes a transmembrane protein pendrin, which functions as a transporter of chloride and iodide and is expressed in the thyroid gland, the inner ear, and the kidney (Petersen and Willems, 2006; Scott *et al.*, 1999). A large consanguineous family from India showed linkage to the region (DFNB4). Affected individuals were homozygous for a missense mutation involving a conserved residue in *SLC26A4*, representing both syndromic and non-syndromic forms of deafness (Petersen and Willems, 2006). Enlarged vestibular aqueduct (EVA) is a frequent symptom in patients with Pendred syndrome but it can also be present as an isolated finding together with sensorineural hearing loss. In many cases, one or two *SLC26A4* mutations have been identified (Park *et al.*, 2005; Pryor *et al.*, 2005).

***OTOF* (otoferlin)**

OTOF gene is mapped to the chromosome 2p23, consisting of 28 exons (Mirghomizadeh *et al.*, 2002). This gene encodes otoferlin, which is protein (Yasunaga *et al.*, 2000). *OTOF* mutations are involved in non-syndromic, recessive auditory neuropathy, which is characterized by moderate to profound, sensorineural hearing loss with normal otoacoustic emissions (OAEs), indicating preserved outer hair cell function (Varga *et al.*, 2003; Tekin *et al.*, 2005). Haplotype analysis suggested a common ancestor for the Q829X mutation, which seems responsible for about 3% of all cases of recessive, pre-lingual deafness in the Spanish population (Migliosi *et al.*, 2002).

***MYO7A* (myosin 7A)**

MYO7A is mapped to 11q13.5 at DFNB2. Myosins are a family of actin-based molecular motors that use energy from hydrolysis of ATP to generate mechanical force. The *MYO7A* gene is a typical unconventional myosin consisting of 48 coding exons. They are actin-based motor molecules which transduce chemical energy into the production of a force enabling them to move along actin filaments (Weil *et al.*, 1996). More than 50 distinct *MYO7A* mutations have been identified causing USH1B, four different mutations in *MYO7A* have been implicated at DFNB2, and two in

dominant deafness at (DFNA11). These mutations are dispersed throughout the *MYO7A* gene (Petersen and Willems, 2006).

***MYO15* (myosin 15A)**

The human *MYO15* gene is identified and mapped to the DFNB3 critical region on chromosome 17p. The human *MYO15* gene was identified and sequence analysis of *MYO15* revealed mutations in the three linked families (Petersen and Willems, 2006). Sequencing of the *MYO15* gene identify three homozygous mutations segregating in three of the Pakistani families, suggesting that *MYO15* mutations are responsible for at least 5% of recessive, profound hearing loss in that population (Liburd *et al.*, 2001).

***MYO6* (myosin 6)**

The *MYO6* gene is an unconventional myosin highly expressed at the base of the stereocilia of the inner and outer hair cells. In the Snell's waltzer mouse, giant stereocilia are seen along with degeneration of hair cells (Petersen and Willems, 2006). Two Pakistani families with non-syndromic deafness also linked to the DFNB37 locus (Ahmed *et al.*, 2003). The linkage region included the *MYO6* gene encoding myosin VI. *MYO6* was previously found to be responsible for an autosomal-dominant form of post-lingual progressive deafness in one Italian kindred (DFNA22) (Melchionda *et al.*, 2001), whereas mouse *Myo6* is responsible for deafness and vestibular dysfunction in the Snell's waltzer (sv) mouse.

***CLDN14* (claudin 14)**

The DFNB29 locus maps to chromosome 21q22.1 by linkage analysis in two large consanguineous Pakistani families with pre-lingual, profound deafness. Sequence analysis of CLDN14 identified a homozygous single nucleotide deletion in transmembrane domain 2 in the two DFNB29 families (Wilcox *et al.*, 2001). The absence of claudin 14 from tight junctions in the organ of Corti leads to altered ionic permeability of the paracellular barrier of the reticular lamina. *Cldn14* knockout mice, showed normal endocochlear potential but deafness was due to rapid degeneration of cochlear outer hair cells, followed by slower degeneration of the inner hair cells (Ben-Yosef *et al.*, 2003).

***TECTA* (α-tectorin)**

This gene is mapped to chromosome 11q at DFNB21 (Petersen and Willems, 2006). The α-tectorin protein, which is encoded by the *TECTA* gene, is one of the major components of the tectorial membrane in the inner ear. Six mutations in the *TECTA* gene have been reported in different families. Allelic segregation consistent with possible linkage to the DFNB21 locus was found in one family. By sequencing all 23 coding exons of *TECTA*, a 16 bp deletion (c.6203-6218del16) in exon 21, leading to a frameshift mutation, segregating with hearing loss (Alasti *et al.*, 2008). Homozygosity for functional null alleles of *TECTA* at the DFNB21 locus causes recessive, prelingual, severe-to-profound stable hearing loss (Naz *et al.*, 2003).

***USHIC* (harmonin)**

The *USH1C* gene contains 28 exons (Verpy *et al.*, 2000) and encodes a PDZ domain-containing protein, harmonin. Harmonin has been shown to bind to otocadherin and interact with myosin VIIA suggesting a functional unit underlying the formation of a coherent hair cell bundle (Siemens *et al.*, 2002; Boeda *et al.*, 2002). A splice-site mutation in intron 12 of the *USH1C* gene was detected in homozygous state in the Indian family (Petersen and Willems, 2006). Mutations of the mouse ortholog *Ush1c* gene cause congenital deafness and severe balance deficits (deaf circler), which is characterized by progressive loss of hair cells and a secondary degeneration of spiral ganglion cells (Johnson *et al.*, 2003).

Many of the genes have reported in only single or a few consanguineous deafness families, and their contribution to deafness on a population base is limited or currently unknown (Hutchin *et al.*, 2005). Most of the deafness genes identified are large, with many exons and no mutational hotspots. Only the *GJB2* gene is small with only one coding exon with a very prevalent mutation 35delG associated with HI in Caucasians. The molecular diagnostic possibilities in recessive deafness are limited due to the genetic heterogeneity, specific nature of most mutations, and large size of most deafness genes.

In the present study two Pakistani families with Autosomal Recessive Deafness have been ascertained. The data presented here includes the mapping of DFNB loci in two families with Autosomal Recessive mode of inheritance and identification of candidate gene for deafness by using Bioinformatics Approach.

MATERIALS AND METHODS

Families Study

Two families, with autosomal recessive, non-syndromic hearing impairment were ascertained in the current study. One family was collected from Wah Cantt, blood samples of half of its branch were collected from Karachi. Second family was collected from Pindi Ghaib. These families were visited at their residential places. Pedigrees of the families were drawn using a standard method (Bennett *et al.*, 1995). All the necessary information were checked and completed by observing and interviewing different members of the families. In the pedigrees males were symbolized by squares and females by circles. The normal individuals were designated with unfilled, while the affected with filled symbols (circles or squares). Each generation was denoted by Roman numerals, while individuals within a generation were denoted by Arabic numerals. Double lines between the partners showed cousin marriages. Mode of inheritance was deduced through pedigree analysis.

Blood sampling

The blood samples from both normal and affected individuals including their parents were collected by veni puncture technique. For DNA extraction, 8-10 ml of whole blood was collected from each individual. To preserve, the blood samples were collected in containers containing potassium EDTA anticoagulant that inhibits the clotting of blood by removing calcium ions from blood. Each blood sample was assigned unique identity number. Before the DNA extraction was started the blood samples were stored at 4°C.

Genomic DNA Extraction

Before the genomic DNA extraction from blood samples was started, a log sheet having columns for family ID, sample ID, individual names, age, sex, sample OD and DNA concentration was created. DNA extraction was carried out in thermo control centrifuge by using standard Phenol Chloroform DNA Extraction protocol, explicated as under:

Day 1

- Each tube was labeled appropriately with sample ID.
- Three times of cell lysis buffer-I (KHCO3, NH4Cl, and 0.5 M EDTA) was added to each blood sample. Then sample tubes were placed on ice for 30min.
- Samples were centrifuged at 3200 rpm for 20 minutes at 4°C.
- Supernatant was discarded and the pellet was re-suspended, if the pellet was reddish then 10ml of cell lysis buffer was added. Again samples were centrifuged at 2000 rpm at 4°C for 20 minutes.
- Supernatant was discarded and pellet was re-suspended.
- To the re-suspended, 4.75ml of STE (Saline Tris EDTA) was added and then 250µl of 10%SDS (Sodium Dodecile Sulphate) was added drop wise while vortex the mixture.
- To this mixture, 10µl of proteinase K (20mg/ml) was added. Sample tubes were placed in water bath at 55°C for overnight incubation.

Day 2

- Samples were extracted with 5ml (equal quantity) of equilibrated phenol; samples were kept on ice for 10min and gentle shaking with intervals. Then centrifuged at 3200 rpm for 30min at 4°C. Aqueous layer from each sample tube was removed with cut tips in to new appropriately labeled tubes.
- To the supernatant removed, 5ml of chilled chloroform-isoamylalcohol (24:1) was added. Sample tubes were shaked for 10 minutes on ice. Then centrifuged at 3200 rpm for 30min at 4°C. Aqueous layer from each tube was removed with cut tips in to new appropriately labeled tubes.
- To this aqueous layer, 500µl of 10M-ammonium acetate and 5ml of chilled isopropanol (or 10ml of chilled absolute ethanol) was added and gently shaked until DNA precipitated as visible white threads. Precipitated DNA was placed overnight at -20°C (or for 15min at -70°C).

Day 3

- Precipitated DNA samples were centrifuged at 3200 rpm for 60min at 4°C, supernatant was discarded and pellet was re-suspended.
- DNA was washed with 5ml of chilled 70 % ethanol for 40 minutes at 3200rpm at 4°C.
- The supernatant was discarded and pellet was dried. Samples were re-suspended in 10mM Tris-HCL (pH 8.0, volume of Tris added according to the size of pellet).
- Re-suspended samples were transferred into appropriately labeled eppendorf tubes. DNA samples were stored at 4°C as Stock.

DNA Dilution

Optical Densities (ODs) of stock DNA were analyzed on spectrophotometer and DNA concentrations were calculated for each stock sample. The dilution of 100 ng/µl of each stock sample was prepared for PCR amplification.

Polymerase chain reaction (PCR)

Genotyping was performed by PCR (polymerase chain reaction), followed by resolving the alleles on 8% non-denaturing polyacrylamide gel by electrophoresis. PCR was performed in a total volume of 10 µl with 2 µl template DNA sample from DNA dilutions, 0.3 µl of each primer (20 µM stock), 1 µl 10X PCR buffer with $MgCl_2$, (500 mM KCl, 100 mM of Tris-HCl pH 8.3, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), 0.8 µl dNTPs (2.5mM stock) and 0.3µl of Taq DNA Polymerase (3U/µl) (BIOMATIK) in 5.3 µl PCR water. The master mix was centrifuged for 10 seconds for thorough mixing. PCR was carried out in Gene Amp PCR system 9700 (Applied Biosystems, USA).

Thermal cycling conditions

Step 1 (1 cycle)			
Initial denaturation	95°C	×	5min
Step 2 (35 cycles)			
Denaturation	95°C	×	45sec

Annealing	55°C	×	1min
Extension	72°C	×	1min
Step 3 (1 cycle)			
Final extension	72°C	×	10min

Polyacrylamide Gel Electrophoresis (PAGE)

1. Inner plate of the gel assembly was siliconized by siliconization solution (Sigmacote)
2. Front plate was wiped with 70% ethanol.
3. After siliconization, glass plates were assembled with (0.75mm) spacers and clamps.
4. Assembled plates were fixed into base of the apparatus.
5. Gel solution (8%) recipe

De-ionized water	175 ml
40% acryl amide solution	62 ml
10X TBE buffer	25 ml

6. From the above solution, 50ml was transferred into a cylinder and 300µl of APS (Ammonium Per Sulphate) and 300µl of TEMED were added.
7. Solutions were mixed and immediately poured into the base.
8. To the remaining 200ml of solutions, 850µl of APS and 150µl of TEMED were added and mixed.
9. As the gel in the base was polymerized, plates were laid down against a Styrofoam rack in a tilled position and 200ml gel solution was poured into plate (inner) slowly and continuously avoiding air bubble formation.
10. After pouring the gel solution comb was inserted between two plates carefully not to allow any air bubble trapped under teeth.
11. Gel was allowed to polymerize overnight.
12. Next day gel plates were taken out from the base and were placed in electrophoretic tank.

13. Electrophoretic tank was filled with 1X TBE buffer and gel was allowed to pre-run at 100W for about 10-15 minutes.
14. Comb was removed from the polymerized gel.
15. To the PCR products, 5µl of loading dye was added, mixed and whole mixture was loaded into each wells.
16. In the first well DNA size ladder/marker was loaded.
17. The gel was allowed to run at 100W for about 4-5 hr depending on size of the PCR Products that were tracked by the dye.
18. After electrophoresis, gel was stained for about 1 minute in a tray containing ethidium bromide staining solution.
19. Gels were analyzed in UV-illumination for the presence / absence of alleles and then photographed and saved.

Reagents used in gel solution

1- 10X TBE

TBE (Tris Boric Acid EDTA) buffer (pH 8.2)

	Mol.wt	gm/L	Final Conc
Tris (Trizma Base)	121	108	0.89 M
Borate	61.83	55	0.889 M
EDTA	372.2	7.44	0.02 M

1. 108gm Tris, 55gm Boric Acid and 7.44gm EDTA were weighted and transferred into a beaker.
2. De-ionized water was added in a beaker up to 0.8 L and dissolved on hot stirrer.
3. Volume of solution was raised up to 1 liter in a cylinder.
4. pH of solution was adjusted at 8.03.
5. Filter and transferred to labeled bottles and stored at room temperature.

2- APS 25% (w/v)

25grams of ammonium per sulphate was dissolved in 100 ml of distilled water labeled and stored in the refrigerator.

Genotyping and Primer Database Analysis

Microsatellite markers mapped by Cooperative Human Linkage Center (CHLC) were obtained from Research Genetics, Inc. (USA). From LDB Genetic Map (H.H. Stassen, Psychiatric University Hospital Zurich, Switzerland) the cytogenetic locations of these markers, their heterozygosity as well as the sizes of the amplified products were obtained.

Linkage Studies

Several candidate loci were tested in the current study by typing microsatellite markers linked to these loci. The autosomal recessive non-syndromic hearing impairment loci initially investigated and the microsatellite markers with their cytogenetic location are shown in the table 2.1.

Table 2.1: A list of microsatellite markers used to test linkage to known DFNB loci

Locus	Markers	Location (cM)	Polymorphism
DFNB1	D13S787	19.4	TetraNR
	D13S1493	30.5	TetraNR
DFNB2	D11S2371	88.4	TetraNR
	D11S2002	102	TetraNR
DFNB3	D17S947	15.5	DNR
	D17S2196	25.7	TetraNR
	D17S1294	28.3	TetraNR
	D17S1303	12.7	TetraNR
DFNB4	D7S1799	115.6	TetraNR
	D7S3061	132.2	TetraNR
DFNB5	D14S306	41.9	TetraNR
	D14S288	43.2	DNR
DFNB6	D3S2409	60.6	TriNR
	D3S1766	66.9	TetraNR
DFNB7	D9S1118	26.8	TetraNR
	D9S922	92.3	TetraNR
	D9S1122	92.3	TetraNR
	D9S273	55.7	DNR
	D9S301	60.0	TetraNR
	D9S175	73.2	DNR
DFNB8	D21S1440	46.8	TriNR
	D21S1446	49.7	TetraNR
	D21S2055	46.3	TetraNR
DFNB9	D2S1360	25.8	TetraNR
	D2S405	43.5	TetraNR
DFNB10	D21S1440	46.8	TriNR
	D21S1446	49.7	TetraNR
	D21S2055	46.3	TetraNR
DFNB11	D9S1118	26.8	TetraNR
	D9S922	92.3	TetraNR
	D9S1122	92.3	TetraNR
	D9S273	55.7	DNR
	D9S301	60.0	TetraNR
	D9S175	73.2	DNR
DFNB12	D10S1432	78.8	TetraNR
	D10S2327	82.7	TetraNR
DFNB13	D7S1824	153.2	TetraNR
	D7S1805	160.4	TetraNR
DFNB14	D7S515	109.3	DNR
	D7S1799	115.6	TetraNR
DFNB15	D3S1764	150.2	TetraNR
	D3S1569	158.1	DNR
	D3S1744	161.1	TetraNR
	D3S1279	169.1	DNR

	D19S216	5.4	DNR
	D19S1034	5.8	TetraNR
	D19S586	11.3	TetraNR
	D19S221	13.3	DNR
	D19S591	3.8	TetraNR
DFNB16	D15S65	54.5	TetraNR
	D15S643	59.6	TetraNR
DFNB17	D7S1799	115.6	TetraNR
	D7S2847	128.3	TetraNR
DFNB18	D11S1981	15.4	TetraNR
	D11S1999	10.9	TetraNR
	ATA34E08	31.3	TriNR
	D11S904	29.6	DNR
DFNB19	D18S452	4.7	DNR
	D18S976	5.9	TetraNR
	D18S843	7.6	TriNR
DFNB20	D11S968	143.4	DNR
	D11S2359	143.9	TriNR
DFNB21	D11S1998	121.3	TetraNR
	D11S4464	131.5	TetraNR
	D11S1986	114.1	TetraNR
DFNB22	D16S764	13.4	TetraNR
	D16S403	30.3	DNR
	D16S769	37.3	TetraNR
DFNB23	D10S220	49.1	DNR
	D10S1227	54.0	TriNR
	D10S1225	66.8	TriNR
	D10S1208	41.7	TriNR
DFNB24	D11S2000	111.7	TetraNR
	D11S1986	114.1	TetraNR
	D11S908	116.9	DNR
	D11S1998	121.3	TetraNR
DFNB25	D4S2408	33.4	TetraNR
	D4S405	34.9	DNR
	D4S1627	47.2	TetraNR
	D4S428	59.6	DNR
	D4S3248	61.7	TetraNR
	D4S391	32.5	DNR
DFNB26	D4S1644	142.8	TetraNR
	D4S424	143.5	DNR
	D4S1625	144.1	TetraNR
	D1S210	188.3	DNR
	D1S1589	189.1	TriNR
DFNB27	D2S326	173.0	DNR
	D2S364	191.7	DNR
	D2S1391	193.2	TetraNR
	D2S1776	168.1	TetraNR
DFN28	D22S683	43.2	TetraNR

	D22S283	43.6	DNR
	D22S423	44.2	DNR
	D22S274	45.1	DNR
DFNB29	D21S1442	35.9	TetraNR
	D21S2055	46.3	TetraNR
DFNB30	D10S1423	19.1	TetraNR
	D10S1426	23.4	TetraNR
	D10S1208	41.7	TriNR
	D10S2474	19.0	TetraNR
	D10S197	20.3	DNR
DFNB31	D9S930	119.8	TetraNR
	D9S934	123.7	TetraNR
	D9S282	131.3	DNR
	D9S302	123.4	TetraNR
DFNB32	D1S1588	102.2	TriNR
	D1S206	106.3	DNR
	D1S1631	107.0	TriNR
	D1S3723	107.2	TetraNR
DFNB33	ATA63D01	138.0	TriNR
	D9S158	139.5	DNR
DFNB35	D14S588	71.0	TetraNR
	D14S258	71.6	DNR
	D14S53	82.7	DNR
	D14S606	85.9	TetraNR
	D14S592	63.5	TriNR
	D14S63	65.9	DNR
	D14S74	83.9	DNR
DFNB36	D1S468	2.5	TriNR
	D1S1612	4.7	TetraNR
	D1S1597	9.1	TetraNR
DFNB37	D6S1053	67.0	TetraNR
	D6S1031	70.9	TriNR
	D6S462	95.1	DNR
DFNB38	D6S305	170.3	DNR
	D6S1277	175.4	TetraNR
DFNB39	D7S1802	68.1	TetraNR
	D7S2204	72.1	TetraNR
	D7S2212	79.0	TetraNR
	D7S820	79.6	TetraNR
DFNB40	D22S1685	33.0	TriNR
	D22S315	36.7	DNR
	D22S689	40.5	TetraNR
	D22S685	42.2	TetraNR
DFNB42	D3S2460	136.6	TetraNR
	D3S1292	144.3	DNR
	D3S1764	150.2	TetraNR
	D3S1278	135.1	DNR
DFNB44	D7S1818	62.0	TetraNR

	D7S1830	64.4	TetraNR
	D7S1802	68.1	TetraNR
DFNB45	D1S235	252.3	DNR
	D1S547	260.9	TetraNR
	D1S1609	261.6	TetraNR
DFNB46	GATA178F11	0.5	TetraNR
	D18S481	1.2	DNR
DFNB47	D2S2952	7.3	TetraNR
	D2S162	9.3	DNR
	D2S423	10.5	TetraNR
	D2S1400	14.9	TetraNR
DFNB48	D15S131	79.5	DNR
	D15S211	86.4	DNR
	D15S153	67.7	DNR
	D15S205	90.0	DNR
DFNB49	D5S647	59.9	DNR
	D5S424	67.7	DNR
	D5S1501	71.7	DNR
	D5S2500	57.5	TetraNR
DFNB50	PAH	108.0	TetraNR
	D12S2070	122.0	TriNR
	D12S395	130.5	TetraNR
	D12S2078	139.6	TetraNR
	D12392	142.1	TetraNR
DFNB51	D11S1392	35.3	TetraNR
	D11S935	38.3	DNR
	ATA34E08	31.3	TriNR
	D11S905	40.6	DNR
	D11S1985	60.2	TetraNR
DFNB53	D6S2439	28.8	TetraNR
	D6S1051	35.2	TetraNR
	D6S1017	42.4	TetraNR
DFNB55	D4S3248	61.7	TetraNR
	D4S2367	63.9	TetraNR
	D41627	47.2	TetraNR
DFNB57	D10S2470	92.1	TetraNR
	D10S677	96.1	TetraNR
	D10S1239	104.3	TetraNR
	D10S1237	113.5	TetraNR
	D10S1230	124.2	TriNR
	D10S1213	128.1	TetraNR
	D10S1248	140.2	TetraNR
DFNB58	D2S410	125.3	TetraNR
	D2S1328	141.4	TetraNR
	D2S112	142.5	DNR
DFNB59	D2S1776	168.1	TetraNR
	D2S1391	193.2	TetraNR
	D2S1384	212.1	TetraNR

DFNB60	D5S471	133.4	DNR
	D5S1505	135.6	TetraNR
	D5S816	142.7	TetraNR
	D5S1480	151.3	TriNR
	D5S436	152.5	DNR
DFNB62	D12S391	12.0	TetraNR
	D12S364	16.6	DNR
	D12S373	18.9	TetraNR
	D12S1042	24.6	TriNR
DFNB63	D11S1314	85.0	DNR
	D11S2371	88.4	TetraNR
DFNB65	D20S480	59.3	TetraNR
	D20S100	63.9	DNR
DFNB66	D6S2439	28.8	TetraNR
	D6S2427	38.4	TetraNR
	D6S1019	39.1	TetraNR
DFNB67	D6S2427	38.4	TetraNR
	D6S1019	39.1	TetraNR
DFNB68	D19S226	13.9	DNR
	D19S714	14.4	TetraNR
DFNB72	D19S1034	5.8	TetraNR
	D19S591	3.8	TetraNR

Linkage to Known DFNB Loci

To date 77 autosomal recessive non-syndromic deafness loci are mapped. To exclude the 77 autosomal recessive non-syndromic deafness loci, an initial search for linkage was carried out by using polymorphic microsatellite markers mapped within these 77 autosomal recessive non-syndromic deafness loci. Table 2.1 summarizes microsatellite markers located in the region of known deafness loci, which were used as first pass analysis for genetic linkage in families with non-syndromic recessive deafness.

Linkage to DFNB1 Locus (*GJB2*)

For the exclusion of DFNB1 locus from linkage, following approach was followed:

- Linkage of the families to DFNB1 locus was investigated by typing the microsatellite markers D13S787 and D13S1493 of chromosome 13, mapped in the linkage interval of the locus.

Bioinformatics Approach

Bioinformatics approach was used to find out strong candidate genes, which are associated with nonsyndromic hereditary hearing loss. The approach that was followed is given below.

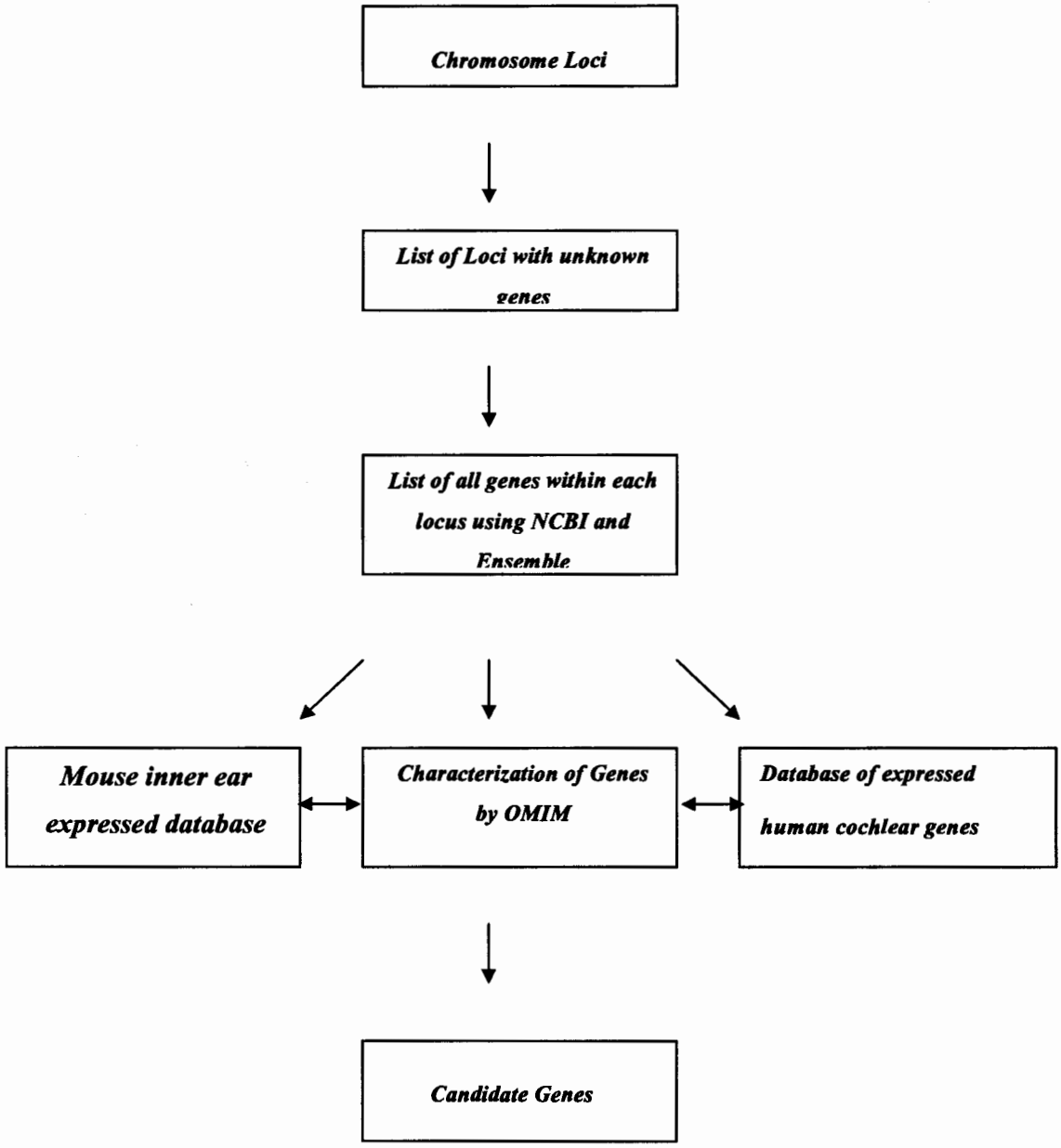


Figure 2.1: Scheme of predicting candidate genes. The rectangles show tasks that were processed in the sequence as indicated by arrows.

For the various nonsyndromic loci, which are identified so far, Hereditary Hearing Loss Homepage (http://webh01.ua.ac.be/hhh/) was used. Same web based source was used to make a list of deafness loci with unknown genes for autosomal recessive forms of hearing imapairment identified in Pakistan.

NCBI (http://www.ncbi.nlm.nih.gov/) and Ensemble databases (http://www.ensembl.org/index) were used to make Lists of all cloned and identified genes within each of the listed genomic intervals .To make a List of Genes, which are present in Human Cochlear database, fetal cochlear cDNA library and EST database, which was updated in 2002, was used. After it List of Genes, which are present in mouse inner ear expression database was made.

A standard was made for gene to be considered as candidate gene. This standard was that if genes are there in one of the two mentioned databases then these genes will be considered as candidate genes. Genes, which were not in both databases, were not considered. Through this standard for different loci possible candidates genes was found. After it Gene Characterization was done using OMIM.

It was found that there were few genes, which are not characterized yet (Table 3.18), and also there were few genes, which are present in OMIM, but shown no disorder (Table 3.19). List of characterized genes, which was obtained from OMIM, was then used to find details of characterized genes and from these details it was concluded that *KIAA119* and *EDN3* are candidate genes for deafness.

RESULTS

Autosomal recessive is one of the subtypes of nonsyndromic deafness. Mostly hearing loss from birth is identification of all types of autosomal recessive deafness. In autosomal recessive inheritance in each cell two copies of an altered gene are present. DFNB is used for representing Autosomal recessive deafness. Each type is numbered in the same order in which it was explained. For example, DFNB1, DFNB2, DFNB3 and DFNB4 are particular types of autosomal recessive deafness. Worldwide 77 Loci of Autosomal Recessive deafness are identified up to date. Within and among families the level of hearing loss differs.

Alterations of structures in the inner ear are usually caused of Autosomal recessive deafness. The inner ear has three parts. Snail-shaped structure called the cochlea is the first part, which helps in processing sound, nerves are the second part, which send information from the cochlea to the brain, and structures are third part, which are involved in balance (Brown *et al.*, 2008). 75 to 80% of all nonsyndromic deafness is inherited in an autosomal recessive pattern.

Mutation in *GJB2* effects 50% of persons with autosomal recessive nonsyndromic hearing loss in many world populations (Zelante *et al.*, 1997; Estivill *et al.*, 1998; Kelley *et al.*, 1998). Mutations in numerous other genes effects rest of the 50% of cases (Scott *et al.*, 1998; Zbar *et al.*, 1998). Based on the specific genotype it is feasible to predict the hearing loss linked with *GJB2* mutations (Snoeckx *et al.*, 2005).Hearing impairment is relatively more common in those geographical areas where strong caste system acts as strong social bonds and produced solidarity in such society with high consanguinity, which makes genetic linkage analysis, the method of choice for gene identification. Homozygosity mapping is based on the assumption that a mutation is inherited from both parents because of recessive mode of inheritance. This method efficiency maps recessive conditions in consanguineous families.

Description of the Families Studied

Family A

Family A was present in wah cantt and half of its branch was present in Karachi. A genetic pedigree was drawn after complete investigation about the history of the family. The pedigree (Figure 3.1) showed four generations consisted of 25 members out of whom seven were diseased and status of one individual was unknown having

age of one year. Analysis of pedigree showed that through parents disease appeared in second generation and it was found that trait was transmitted in autosomal recessively. From all the family members Blood samples were collected

Family B

Family B was present in Pindi Ghaib. A genetic pedigree was drawn after complete investigation about the history of the family. The pedigree (Figure 3.2) showed five generations consisted of 32 members out of which nine were diseased and status of one individual was unknown having age of one year. Analysis of pedigree showed that through parents disease appeared in third generation and it was found that trait was transmitted in autosomal recessively. From all the family members Blood samples were collected.

Linkage Studies

Autosomal recessive nonsyndromic hearing impairment loci, which are already known and mentioned in table 2.1, were tested through homozygosity mapping. Microsatellite markers were used in the candidate intervals for this purpose. Hereditary hearing loss home page (www.uis.ac.be/dnalab/hhh) was used to have Information about genetic location of recessive deafness. For exclusion mapping microsatellite markers, which were used, are given in table 2.3 and 2.4. A standard PCR reaction and electrophoresis on 8% non-denaturing polyacrylamide gel was used to analyse microsatellite markers as explained in Materials and Methods. Gels were stained with ethidium bromide to visualize amplified PCR products and by visual inspection genotypes were assigned.

In family A (Figure 3.1), for genmotyping six DNA samples including three affected and three normal individuals were used. For linkage several deafness loci were tested. Most of the loci were mapped previously in Pakistani families showing non-syndromic hearing impairment. Results of the genotyping showed that family A was not linked to any locus and thus excluded.

In family B (Figure 3.2), for genmotyping six DNA samples including four affected and two normal individuals were used. For linkage several deafness loci were tested.

Results of the genotyping showed that family B was linked to locus DFNB35. DNA analysis with polymorphic microsatellite markers showed that in al affected

individuals markers D14S588 (Figure 3.43), D14S53 (Figure 3.44) and D14S74 (Figure 3.45) were homozygous but in normal they were heterozygous. Family B thus established convincing linkage to DFND35 locus and this locus has *ESRRB* on chromosome14q24.1-24.3.

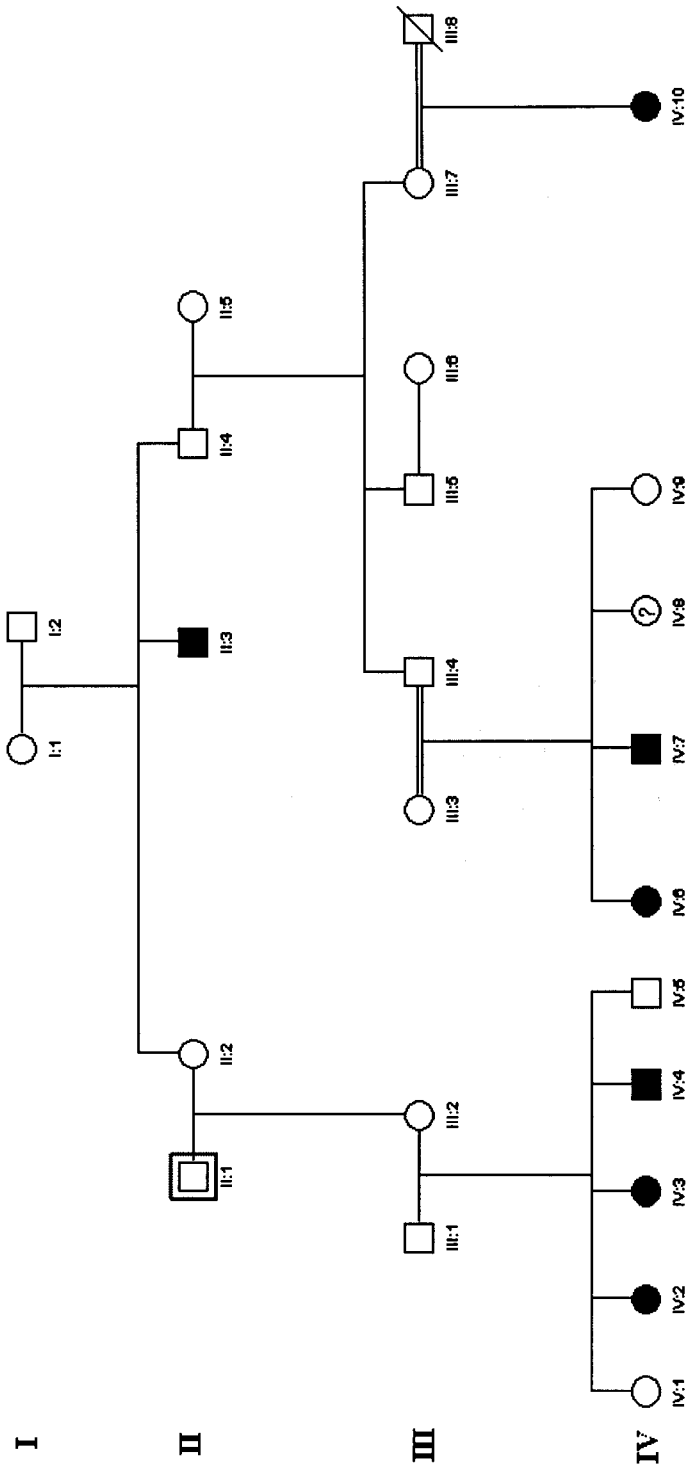


Figure 3.1: Pedigree of family A with autosomal recessive non-syndromic hearing impairment. Circles and squares represent females and male, respectively. Filled circles and squares represent affected individuals. Double lines indicate cousin marriage ss.

Family A

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.2: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D7S1799 at 115.6cM on chromosome 7q31, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.3: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D14S306 at 41.9cM on chromosome 14q12, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.4: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D3S2409 at 60.6cM on chromosome 3p14-p21, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.5: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D9S1122 at 92.3cM on chromosome 9q13-q21, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

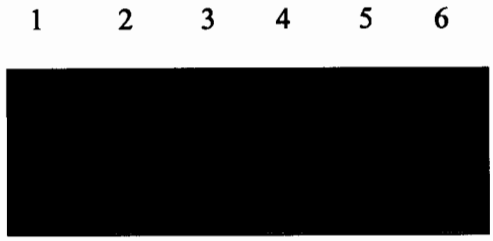
Figure 3.6: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D21S2055 at 46.3cM on chromosome 21q22, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

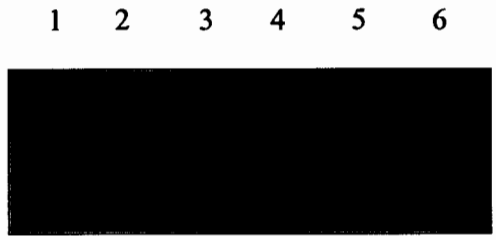
Figure 3.7: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D10S1432 at 78.8cM on chromosome 10q21-q22, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.8: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D7S1805 at 160.4cM on chromosome 7q34-36, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.9: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D7S1799 at 115.6cM on chromosome 7q31, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.10: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D3S1764 at 150.2cM on chromosome 3q21-q25, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.11: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D3S1744 at 161.1cM on chromosome 3q21-q25, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

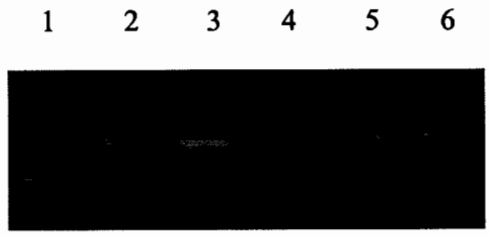
Figure 3.12: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D15S659 at 54.5cM on chromosome 15q21-q22, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

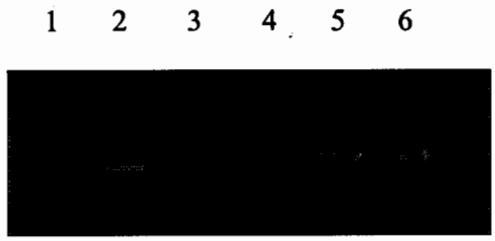
Figure 3.13: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D11S2000 at 111.7cM on chromosome 11q23, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.14: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D9S930 at 119.8cM on chromosome 9q32-q34, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

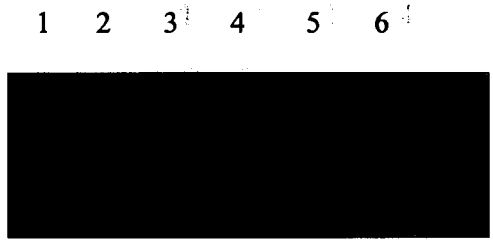
Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.15: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D9S302 at 123.4cM on chromosome 9q32-q34, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

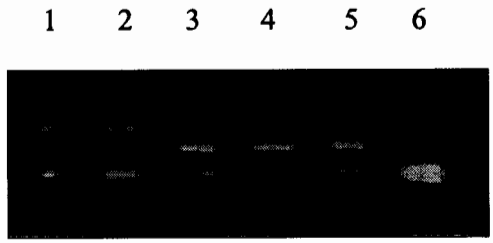
Figure 3.16: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker ATA59H06 at 138.0cM on chromosome 9q34.3, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

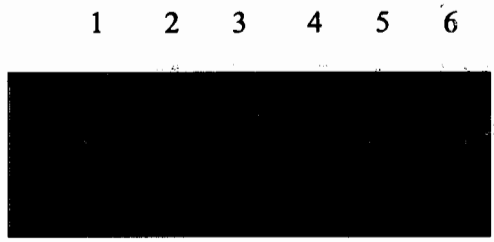
Figure 3.17: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D14S606 at 85.9cM on chromosome 14q24.1-24.3, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

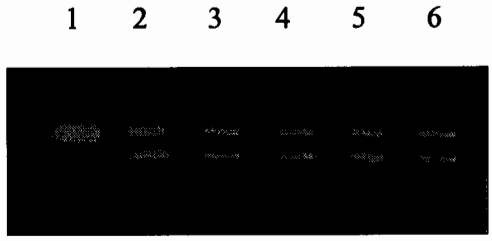
Figure 3.18: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D6S1031 at 70.9cM on chromosome 6q13, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

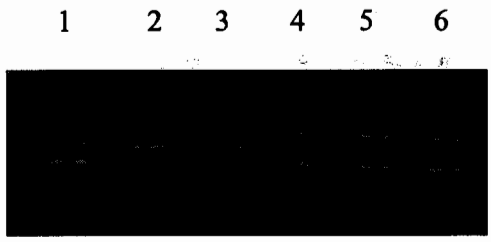
Figure 3.19: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D7S1802 at 68.1cM on chromosome 7q11.22-q21.12, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.20: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D7S2212 at 79.0cM on chromosome 7q11.22-q21.12, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.21: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D7S1830 at 64.4cM on chromosome 7p14.1-q11.22, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

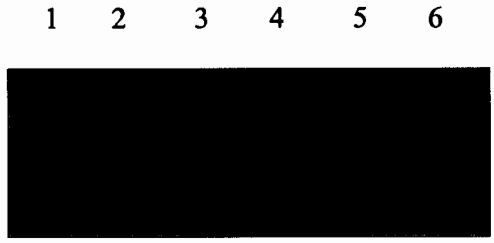
Figure 3.22: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D1S547 at 260.9cM on chromosome 1q43-q44, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.23: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker GATA178F11 at 0.5cM on chromosome 18p11.32-p11.31, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

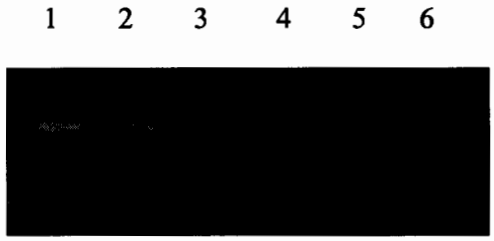
Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.24: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D5S2500 at 57.5cM on chromosome 5q12.3-q14.1, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

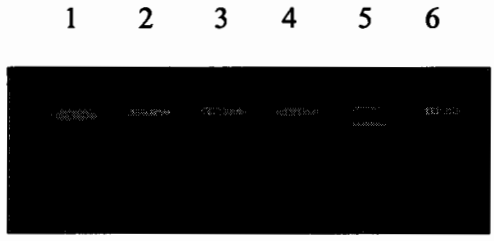
Figure 3.25: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker PAH at 108.0cM on chromosome 12q23, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

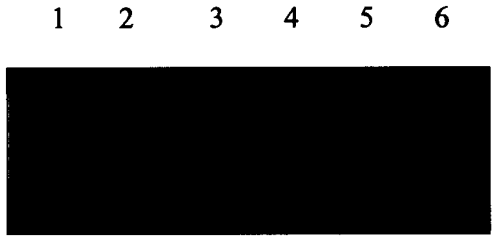
Figure 3.26: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D12S395 at 130.5cM on chromosome 12q23, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.27: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D12S2078 at 139.6cM on chromosome 12q23, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

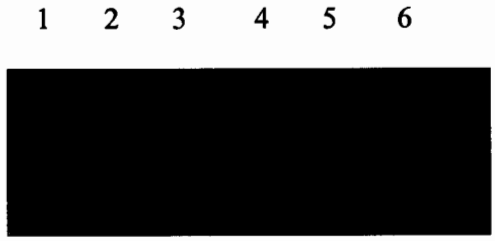
Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.28: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D11S1392 at 35.3cM on chromosome 11p13-p12, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

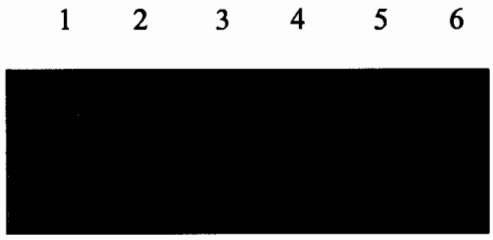
Figure 3.29: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D11S935 at 38.3cM on chromosome 11p13-p12, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.30: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D4S1627 at 47.2 cM on chromosome 4q12-q13.2, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.31: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D10S2470 at 92.1 cM on chromosome 10q23.1-q26.11, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.32: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D10S1237 at 113.5 cM on chromosome 10q23.1-q26.11, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.33: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D10S1230 at 124.2 cM on chromosome 10q23.1-q26.11, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.34: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D5S1505 at 135.6 cM on chromosome 5q22-q31, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.35: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D5S816 at 142.7 cM on chromosome 5q22-q31, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

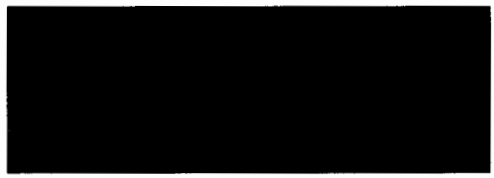
1 2 3 4 5 6

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.36: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D5S1480 at 151.3 cM on chromosome 5q22-q31, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6



Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.37: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D11S2371 at 88.4 cM on chromosome 11q13.2-q13.4, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

Family A

Lane 1. III-2
Lane 2. IV-1
Lane 3. IV-2
Lane 4. IV-3
Lane 5. IV-4
Lane 6. IV-5

Figure 3.38: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D19S714 at 14.4 cM on chromosome 19p13.2, of family A. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

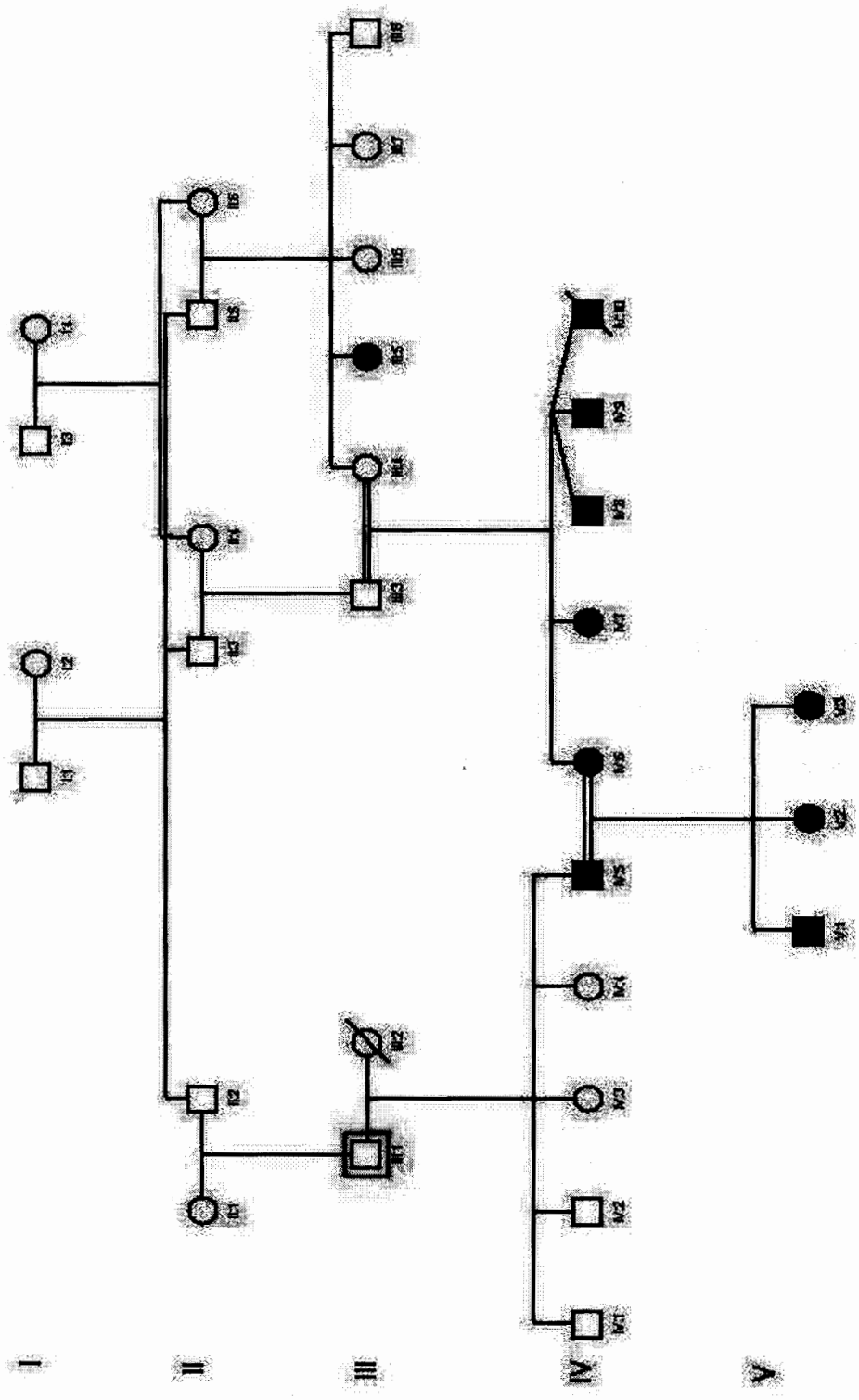
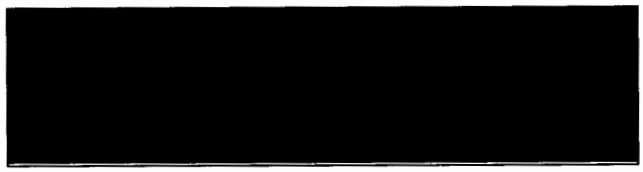


Figure 3.39 Pedigree of family B with autosomal recessive non-syndromic hearing impairment. Circles and squares represent females and males, respectively. Filled circles and squares represent affected individuals. Double lines indicate cousin

FAMILY B

1 2 3 4 5 6 7 8 9 10 11 12 13 14



Family B

Lane 1. III-3	Lane 6. IV-7	Lane 11. IV-2
Lane 2. III-4	Lane 7. IV-4	Lane 12. IV-1
Lane 3. III-5	Lane 8. V-2	Lane 13. III-1
Lane 4. IV-5	Lane 9. IV-3	Lane 14. III-6
Lane 5. IV-6	Lane 10. V-1	

Figure 3.40: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D14S592 at 63.5 cM on chromosome 14q24.1-24.3, of family B. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

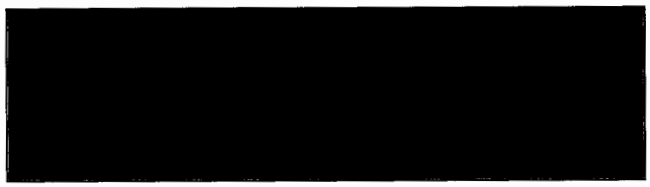
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Family B

Lane 1. III-3	Lane 6. IV-6	Lane 11. V-1
Lane 2. III-4	Lane 7. IV-7	Lane 12. IV-2
Lane 3. III-5	Lane 8. IV-4	Lane 13. IV-1
Lane 4. IV-5	Lane 9. V-2	Lane 14. III-1
Lane 5. II-5	Lane 10. IV-3	Lane 15. III-6

Figure 3.41: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D14S63 at 65.9 cM on chromosome 14q24.1-24.3, of family B. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7 8 9 10 11 12 13 14



Family B

Lane 1. III-3	Lane 6. IV-6	Lane 11. V-1
Lane 2. III-4	Lane 7. IV-7	Lane 12. IV-2
Lane 3. III-5	Lane 8. IV-4	Lane 13. IV-1
Lane 4. IV-5	Lane 9. V-2	Lane 14. III-1
Lane 5. II-5	Lane 10. IV-3	

Figure 3.42: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D14S258 at 71.6 cM on chromosome 14q24.1-24.3, of family B. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Family B

Lane 1. III-3	Lane 6. IV-6	Lane 11. V-1
Lane 2. III-4	Lane 7. IV-7	Lane 12. IV-2
Lane 3. III-5	Lane 8. IV-4	Lane 13. IV-1
Lane 4. IV-5	Lane 9. V-2	Lane 14. III-1
Lane 5. II-5	Lane 10. IV-3	Lane 15. III-6

Figure 3.43: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D14S588 at 71.0 cM on chromosome 14q24.1-24.3, of family B. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Family B

Lane 1. III-3	Lane 6. IV-6	Lane 11. V-1
Lane 2. III-4	Lane 7. IV-7	Lane 12. IV-2
Lane 3. III-5	Lane 8. IV-4	Lane 13. IV-1
Lane 4. IV-5	Lane 9. V-2	Lane 14. III-1
Lane 5. II-5	Lane 10. IV-3	Lane 15. III-6

Figure 3.44: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D14S53 at 82.7 cM on chromosome 14q24.1-24.3, of family B. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Family B

Lane 1. III-3	Lane 6. IV-6	Lane 11. V-1
Lane 2. III-4	Lane 7. IV-7	Lane 12. IV-2
Lane 3. III-5	Lane 8. IV-4	Lane 13. IV-1
Lane 4. IV-5	Lane 9. V-2	Lane 14. III-1
Lane 5. II-5	Lane 10. IV-3	Lane 15. III-6

Figure 3.45: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D14S74 at 83.9 cM on chromosome 14q24.1-24.3, of family B. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Family B

Lane 1. III-3	Lane 6. IV-6	Lane 11. V-1
Lane 2. III-4	Lane 7. IV-7	Lane 12. IV-2
Lane 3. III-5	Lane 8. IV-4	Lane 13. IV-1
Lane 4. IV-5	Lane 9. V-2	Lane 14. III-1
Lane 5. II-5	Lane 10. IV-3	Lane 15. III-6

Figure 3.46: Gel Electropherogram of ethidium bromide stained 8% non-denaturing polyacrylamide gel for marker D14S606 at 85.9 cM on chromosome 14q24.1-24.3, of family B. The Roman numerals indicate generation of the individuals within pedigree while Arabic numerals indicate their positions within generation.

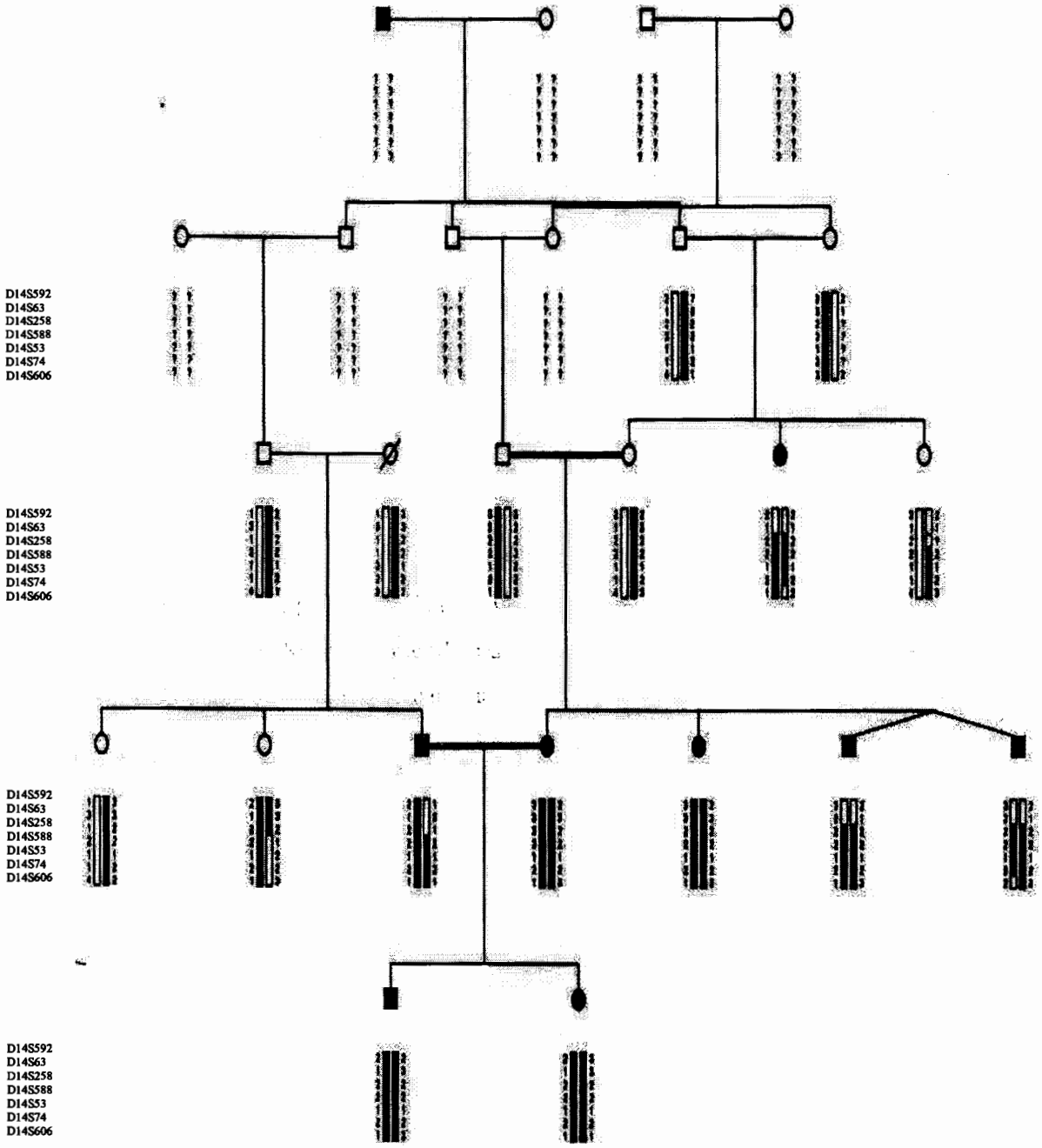


Figure 3.47: Drawing of the pedigree of family B segregating Autosomal Recessive Deafness.Haplotypes for the most closely linked markers are shown below each symbol. The alleles are donated according to their sizes.

Results of Bioinformatics Approach

For the autosomal recessive nonsyndromic deafness, which are found in Pakistan, following approach was followed to make a list of candidate genes.

For the various nonsyndromic loci, which are identified so far, Hereditary Hearing Loss Homepage (http://webh01.ua.ac.be/hhh/) was used. Same web based source was used to make a list of deafness loci with unknown genes for autosomal recessive forms identified in Pakistan (Table 3.1).

NCBI (http://www.ncbi.nlm.nih.gov/) and Ensemble databases (http://www.ensembl.org/index) were used to make Lists of all cloned and identified genes within each of the listed genomic intervals (Table 3.2-Table 3.15). To make a List of Genes, which are present in Human Cochlear database, fetal cochlear cDNA library and EST database, which was updated in 2002, was used (Table 3.16). After it List of Genes, which are present in mouse inner ear expression database was made (Table 3.17).

A standard was made for gene to be considered as candidate gene. This standard was that if genes are there in one of the two mentioned databases then these genes will be considered as candidate genes. Genes, which were not in both databases, were not considered. Through this standard for different loci possible candidates genes was found. After it Gene Characterization was done using OMIM.

It was found that there were few genes, which are not characterized yet (Table 3.18), and also there were few genes, which are present in OMIM, but shown no disorder (Table 3.19). List of characterized genes which was obtained from OMIM (Table 3.20) was then used to find details of characterized genes (Table 3.21) and from these details it was concluded that *KIAA119* and *EDN3* are candidate genes for deafness.

Table 3.1: List of Autosomal Recessive Nonsyndromic Loci.

Locus Name	**Location**	**Locus Name**	**Location**
DFNB26	4q31	DFNB48	15q23-q25.1
DFNB38	6q26-q27	DFNB51	11p13-p12
DFNB42	3q13.31-q22.3	DFNB55	4q12-q13.2
DFNB44	7p14.1-q11.22	DFNB62	12p13.2-p11.23
DFNB45	1q43-q44	DFNB63	11q13.2-q13.4
DFNB46	18p11.32-p11.31	DFNB65	20q13.2-q13.32
DFNB47	2p25.1-p24.3	DFNB72	19p13.3

Table 3.2: List of Genes Location at DFNB26 (4q 31).

PCDH18	*LOC729407*	*TIGD4*
LOC100128578	*IL15*	*ARFIP1*
LOC644879	*LOC100130178*	*ANXA2P1*
LOC100129986	*INPP4B*	*TLR2*
LOC729350	*GYPE*	*RNF175*
RAB33B	*LOC345041*	*SFRP2*
SETD7	*OTUD4*	*LOC729558*
LOC644914	*LOC100131639*	*SH3D19*
LOC100131429	*LOC729497*	*LOC729830*
LOC152594	*LOC100129572*	*PET112L*
LOC729350	*ARHGAP10*	*FBXW7*
LOC644914	*ASSP8*	*DKFZP434I0714*
LOC100129858	*LOC285423*	*TIGD4*
LOC152586	*FBXW7*	*LOC729870*
ELMOD2	*LOC391706*	*TRIM2*

Table 3.3: List of Genes Present at DFNB 38 (6q26-q27).

IGF2R	*LOC100127984*	*LOC100132188*
LOC729603	*FAM103A2P*	*PRR18*
SLC22A2	*UNC93A*	*SFT2D1*
SLC22A3	*TTLL2*	*LOC100130465*
PLG	*TCP10*	*LOC154449*
LOC100129958	*LOC100128124*	*LOC732174*
PACRG	*KIF25*	*PSMB1*
MAP3K4	*DACT2*	*OR4F7P*
AGPAT4	*THBS2*	*LOC729277*
LOC100129958	*WDR27*	*LOC100128405*
PACRG	*PHF10*	*QKI*
LOC729658	*C6orf70*	*LOC728275*

Table 3.4: List of Genes Located at DFNB42 (3q13.31-q22.3).

VSTM3	*PIK3R4*	*RBP2*
LOC645207	*GSTO3P1*	*ACTGP1*
LOC100129275	*ATP2C1*	*SPSB4*
GSK3B	*NUDT16P*	*KRT18P35*
LOC442087	*ACPP*	*GK5*
ARGFX	*LOC729534*	*PCOLCE2*
DTX3L	*KY*	*C3orf25*
PDIA5	*MSL2L1*	*IFT122*
MUC13	*NCK1*	*ARVP6125*
LOC100129984	*LOC646560*	*AMOTL2*
KLF15	*A4GNT*	*PPP2R3A*
MCM2	*CEP70*	*DZIP1L*
RPN1	*LOC646641*	*LOC100129696*
LOC100128615		

Table 3.5: List of Genes Located at DFNB44 (7p14.1-q11.22).

LOC100128370	*LOC644159*	*RCP9*
LOC389493	*ZNF680*	*TCEB1P*
LOC442309	*LOC644387*	*LOC729126*
LOC728348	*ZNF117*	*LOC643353*
LOC731631	*LOC346329*	*CALN1*
LOC100129000	*LOC100130351*	*LOC100129000*
LOC100132308	*ZNF138*	*LOC100131975*
LOC100132838	*LOC100130721*	*ZNF679*

Table 3.6: List of Genes Located at DFNB45 (1q43-44).

NID1	*LOC391183*	*OR2T11*	*EDARADD*
LOC100130485	*LOC284701*	*OR2T35*	*LOC100130331*
HEATR1	*SCCPDH*	*OR2T27*	*LOC645884*
MTR	*LOC343165*	*ZNF672*	*LOC645939*
LOC440737	*LOC441931*	*TRIM58*	*CHML*
MT1P2	*NLRP3*	*OR2L8*	*WDR64*
RYR2	*OR2W5*	*OR2L2*	*LOC391183*
KRT18P32	*C1orf150*	*OR2M5*	*LOC284701*
LOC100130099	*OR2G2*	*OR2M2*	*SCCPDH*
EDARADD	*OR6F1*	*OR2T6*	*LOC343165*
LOC100130331	*TRIM58*	*OR2T7*	*LOC441931*
LOC645884	*OR2L8*	*OR2T11*	*NLRP3*
FMN2	*OR2L2*	*OR2T35*	*OR2W5*
LOC645939	*OR2M5*	*OR2T27*	*C1orf150*
CHML	*OR2T6*	*ZNF672*	*OR2G2*
WDR64	*OR2T7*	*LOC653789*	*OR6F1*

Table 3.7: List of Genes Present at DFNB 46 (18p11.32-p11.31).

LOC727758	*FLJ35776*	*EMILIN2*
USP14	*LOC284215*	*LPIN2*
COLEC12	*PPIAP14*	*LOC727918*
CETN1	*LOC642597*	*MRCL3*
CLUL1	*C18orf18*	*MRLC2*
C18orf56	*EPB41L3*	*IGLJCOR18*
TYMS	*TTMA*	*TTMA*
ENOSF1	*LOC729309*	*L3MBTL4*
ADCYAP1	*L3MBTL4*	*ARHGAP28*
LOC100130247	*LOC100130480*	*TXNDC2*
NDC80	*ARHGAP28*	*LAMA1*
SMCHD1		

Table 3.8: List of Genes Located at DFNB47 (2p25.1-p24.3).

RNF144A	*RRM2*	*LOC653602*	*LOC100129296*
LOC100130731	*C2orf48*	*NAG*	*RAD51AP2*
LOC100129581	*NOL10*	*LOC100128329*	*VSNL1*
MBOAT2	*LOC100130910*	*LOC130678*	*MSGN1*
LOC644651	*LOC645054*	*MYCNOS*	*KCNS3*
RNF144A	*FLJ33534*	*MYCN*	*CISD1B*
LOC100130731	*ROCK2*	*LOC100128475*	*TTC32*
C2orf46	*LOC646050*	*KCNS3*	*SDC1*
KIDINS220	*LOC650157*	*CISD1B*	*LOC151457*
LOC644651	*E2F6*	*TTC32*	*MYCNOS*
ITGB1BP1	*LOC729285*	*LOC151457*	*LOC100129296*
GRHL1	*TRIB2*	*DTNB*	*RAD51AP2*
LOC100131506	*FAM84A*	*ITSN2*	*VSNL1*
ASXL2	*SUPT7L*	*KLHL29*	*MSGN1*
CENPA			

Table 3.9: List of Genes present at DFNB 48 (15q23-q25.1).

ANP32A	*IDH3A*	*LOC647020*
NOX5	*IREB2*	*C15orf26*
KIF23	*CTSH*	*TMC3*
LOC645296	*LOC100129540*	*LOC653643*
ARIH1	*RASGRF1*	*IMP3*
HIGD2BP	*MTHFS*	*NRG4*
NEO1	*LOC647020*	*LOC440292*
LOXL1	*STARD5*	*TBC1D2B*
LOC440287	*LOC440292*	*LOC729911*
ISLR2	*LOC646892*	*ZFAND6*
ISLR	*IDH3A*	*LOC730126*
UBL7	*PSMA4*	*ARNT2*
CYP1A2	*CHRNA5*	*KIAA1199*
COX5A	*LOC646938*	*MORF4L1*
RPP25		

Table 3.10: List of Genes Located at DFNB51 (11p13-p12).

DCDC1	*LOC729605*	*RAG1*
DPH4	*EIF3M*	*RAG2*
ELP4	*PRRG4*	*LOC100131023*
LOC729605	*QSER1*	*LRRC4C*
WT1	*TCP11L1*	*LOC100131020*
WIT1	*LOC338739*	*LOC387761*
EIF3M	*LOC390107*	*LOC100128134*
RPL34P2	*C11orf41*	*LRRC4C*
DEPDC7	*FBXO3*	*LOC387761*
TCP11L1	*LOC100129531*	*ARHGAP1*
CSTF3	*EHF*	*LRP4*
LOC338739	*LOC100132286*	*IMMP1L*
LOC390107	*LOC729738*	*TRAF6*
HIPK3	*SLC1A2*	*DCDC1*
DCDC5	*LDLRAD3*	*FLJ14213*

Table 3.11: Genes Located at DFNB55 (4q12-q13.2).

LOC100132928	*LOC100128865*	*LOC644759*
SGCB	*LOC644196*	*TMPRSS11B*
SPATA18	*SRP72*	*UGT2B17*
USP46	*LOC285453*	*LOC728160*
LOC100130982	*POLR2B*	*LOC728811*
FIP1L1	*IGFBP7*	*TMPRSS11E2*
LNX1	*LOC100129547*	*LOC441018*
LOC441016	*LOC100131441*	*UGT2A3*
SRD5A3	*LOC644548*	*UGT2B27P*
PDCL2	*LOC100131356*	*UGT2B28*
LOC644173	*LOC644682*	*SULT1E1*
LOC339977	*EPHA5*	*LOC442108*
LOC441016	*LOC728048*	*UBA6*
LOC100129728	*CENPC1*	

Table 3.12: List of Genes Located at DFNB62 (12p13.2-p11.23).

TAS2R12	*FGFR1OP2*
TAS2R50	*MED21*
PRH1	*LOC100130082*
PS5	*FGFR1OP2*
BHLHB3	*LOC728858*
C12orf11	

Table 3.13: List of Genes Located at DFNB63 (11q13.2-q13.4).

LOC731511	*LOC100127*	*PHOX2A*
TBX10	*RELT*	*LRRC51*
LOC729213	*LOC100132697*	*OR7E128P*
LOC441616	*SPCS2*	*PGM2L1*
CHKA	*LOC645544*	*LOC729523*
LRP5	*LOC285407*	*PPME1*
MRGPRD	*LOC100131539*	*LOC255620*
TPCN2	*C11orf59*	*LOC100129925*
LOC100129779	*PHOX2A*	*KIAA0280*
SHANK2	*LOC220077*	*KRTAP5-11*
NADSYN1	*CLPB*	*ARHGEF17*
KRTAP5-8	*FCHSD2*	

Table 3.14: List of Genes Located at DFNB 65 (20q13.2-q13.32).

ERP28P	*LOC728902*	*TPD52L2*
NFATC2	*HMG1L1*	*GCNT7*
SALL4	*CTCFL*	*C20orf106*
ZFP64	*TMEPAI*	*RPS4L*
RPL36P1	*LOC100129869*	*PTMAP6*
TSHZ2	*C20orf85*	*C20orf86*
ZNF217	*PPP4R1L*	*RAB22A*
PFDN4	*VAPB*	*TUBB1*
DOK5	*NESPAS*	*ATP5E*
CBLN4	*C20orf66*	*CSTF1*
MC3R	*EDN3*	*C20orf43*
C20orf108	*GCNT7*	*SPO11*
HMG1L1		

Table 3.15: List of Genes Located at DFNB 72 (19p13.3).

OR4F8P	*PIAS4*	*RDH8*
FSTL3	*CREB3L3*	*QTRT1*
PALM	*KIAA1881*	*ZNF20*
PRG2	*SAFB*	*LOC100128569*
AZU1	*P117*	*ITGB1BP3*
CFD	*TMEM146*	*PNPLA6*
C19orf22	*FUT5*	*ADAMTS10*
KISS1R	*ASAH3*	*TIMM13*
SBNO2	*KHSRP*	*LOC100132469*
STK11	*SLC25A41*	*TNFSF9*
EFNA2	*TNFSF14*	*ARHGEF18*
DAZAP1	*MGC24975*	*LOC728187*
MEX3D	*CLPP*	*DENND1C*

Table 3.16: List of Genes, which are present in Human Cochlear database.

Locus	Location	Genes from Cochlea			
DFNB26	4q 31	*PCDH18*	*FBXW7*	*ARFIP1*	*FBXW7*
		TRIM2			
DFNB 38	6q26-q27	*SLC22A3*	*MAP3K4*	*AGPAT4*	*PSMB1*
		QKI	*THBS2*	*PHF10*	
DFNB42	3q13.31-q22.3	*NCK1*	*AMOTL2*	*PPP2R3A*	
DFNB44	7p14.1-q11.22	*ZNF117*			
DFNB45	1q43-44	*RYR2*	*FMN2*		
DFNB 46	18p11.32-p11.31	*COLEC12*	*MRCL3*	*MRLC2*	*EPB41L3*
DFNB 48	15q23-q25.1	*ANP32A*	*KIF23*	*NEO1*	*KIAA1199*
		IREB2	*CTSH*	*PSMA4*	*ARNT2*
		MORF4L1			
DFNB51	11p13-p12	*ELP4*	*TRAF6*	*LRP4*	
DFNB55	4q12-q13.2	*SGCB*	*USP46*		
DFNB63	11q13.2-q13.4	*SHANK2*	*FCHSD2*	*KIAA0280*	
DFNB 65	20q13.2-q13.32	*PFDN4*	*CTCFL*	*TMEPAI*	*ATP5E*
		EDN3	*RAB22A*	*TUBB1*	
DFNB 72	19p13.3	*KHSRP*	*ZNF20*		

Table 3.17: List of Genes, which are present in mouse inner ear expression databases.

Locus	**Location**	**Genes From Mouse**
DFNB42	3q13.31-q22.3	*RBP2*
DFNB 48	15q23-q25.1	CHRNA5
DFNB47	2p25.1-p24.3	*MYCNSDC1*
DFNB55	4q12-q13.2	*EPHA5*

Table 3.18: List of Genes, which are not characterized yet.

Locus	**Location**	**Genes from Cochlea**			**Genes from Mouse**
DFNB26	4q 31	*ARFIP1*	*TRIM2*		Nil
DFNB 38	6q26-q27	*MAP3K4*	*AGPAT4*	*QKI*	Nil
		PHF10			
DFNB42	3q13.31-q22.3	*NCK1*	*AMOTL2*	*PPP2R3A*	*RBP2*
DFNB45	1q43-44	*RYR2*			Nil
DFNB 46	18p11.32-p11.31	*MRCL3*	*MRLC2*		Nil
DFNB47	2p25.1-p24.3	*KIDINS220*	*CPSF3*	*NAG*	*MYCN*
DFNB 48	15q23-q25.1	*ANP32A*	*NEO1*	*MORF4L1*	Nil
DFNB55	4q12-q13.2	*USP46*	*POLR2B*		Nil
DFNB63	11q13.2-q13.4	*FCHSD2*	*KIAA0280*		Nil
DFNB 65	20q13.2-q13.32	*CTCFL*	*RAB22A*	*TUBB1*	Nil

Table 3.19: List of Genes, which are present in OMIM but shown no disorder.

Locus	Location	Genes from Cochlea			Genes from Mouse
DFNB26	4q 31	*PCDH18*	*FBXW7*	*FBXW7*	Nil
DFNB 38	6q26-q27	*SLC22A3*	*PSMB1*		Nil
DFNB44	7p14.1-q11.22	*ZNF117*			Nil
DFNB45	1q43-44	*FMN2*			Nil
DFNB 46	18p11.32-p11.31	*COLEC12*	*EPB41L3*		Nil
DFNB47	2p25.1-p24.3	*KIDINS220*	*CPSF3*	*NAG*	*MYCN*
DFNB 48	15q23-q25.1	*KIF23*	*IREB2*	*PSMA4*	Nil
		CTSH	*ARNT2*		
DFNB51	11p13-p12	*ELP4*	*TRAF6*	*LRP4*	Nil
DFNB55	4q12-q13.2	*IGFBP7*	*EPHA5*	*UGT2B17*	*EPHA5*
DFNB63	11q13.2-q13.4	*SHANK2*	*PFDN4*		Nil
DFNB 65	20q13.2-q13.32	*TMEPAI*	*ATP5E*		Nil
DFNB 72	19p13.3	*KHSRP*	*ZNF20*		Nil

Table 3.20: List of Genes, which have been characterized.

Locus	Location	Genes from Cochlea	Genes from Mouse
DFNB 38	6q26-q27	*THBS2*	Nil
DFNB 48	15q23-q25.1	*KIAA1199*	*CHRNA5*
DFNB_55	4q12-q13.2	*SGCB*	Nil
DFNB 65	20q13.2-q13.32	*EDN3*	Nil

Table 3.21: Details of Genes, which have been characterized.

Gene symbol	Location	Gene Name	Entrez number	Disease
THBS2	6q27	Thrombospondin 2	188061	Lumbar disc herniation, susceptibility to
KIAA1199	15q24	KIAA1199 gene	608366	Deafness, nonsyndromic
EDN3	20q13.2-q13.3	Endothelin-3	131242	Shah-Waardenburg syndrome, congenital, Hirschsprung disease
SGCB, LGMD2E	4q12	Sarcoglycan, beta (43kD dystrophin-associated glycoprotein)	600900	Muscular dystrophy, limb-girdle, type 2E,
CHRNA5, LNCR2	15q25.1	Cholinergic receptor, neuronal nicotinic,	118505	Lung cancer susceptibility 2

After characterization it has been concluded that *KIAA119* and *EDN3* are candidate genes for deafness.

DISCUSSION

In countries like Pakistan recessively inherited diseases are more prevalent where cousin marriages are common. These large consanguineous families provide a beneficial resource for studing linkage analysis of those disorders, which are recessively inherited like hearing impairment. These linkage studies provide a way to understand the molecular and cellular biology of hearing.

Hearing loss is a genetically heterogeneous trait and one of the most common neurosensory disorders in human (Friedman and Griffith, 2003; Morton and Nance, 2006). Congenital hearing impairment affects about 0.16% of the newborns (Mehl and Thomas, 2002). Approximately half of these cases are estimated to be genetically determined, and the remaining cases are likely to have an environmental cause (Roizen, 2003).

Hereditary hearing impairment (HHI) can be classified as non-syndromic and syndromic hearing loss. Almost 70% of HHI are non-syndromic, and 30% are syndromic. There are more than 70 genes involved in non-syndromic forms and more than 400 syndromes are associated with hearing loss (Bayazit and Yalmaz, 2006). According to the pattern of inheritance, HHI can also be classified as autosomal recessive, autosomal dominant, X-linked and mitochondrial (Bayazit *et al.*, 2003).

Nonsyndromic hearing loss (NSHL) is the most genetically heterogeneous trait known. It is estimated that 85% of nonsyndromic hearing loss is caused by autosomal recessive defects (Cremers *et al.*, 1991; van Camp *et al.,* 1997). For autosomal recessive nonsyndromic hearing impairment, usually HHI has prelingual onset, involves all the frequencies and is severe to profound. Autosomal recessive non-syndromic hearing impairment is genetically heterogeneous and is the most common form of inherited hearing loss, accounting for >70% of the cases (Morton, 1991).

In recent years, improved molecular genetic techniques have led to the identification of 71 loci for autosomal recessive non-syndromic hearing impairment and to date 23 of the corresponding genes have been identified (Hereditary Hearing loss Homepage; http://webhost.ua.ac.be/hhh/). Many of these deafness loci have been mapped either in endogamous populations or in families with children of consanguineous marriages (Friedman and Griffith, 2003).

In different populations, only a few GJB2, CDH23 and WFS1 genes are well characterized in terms of occurrence and sequence variants (Santos *et al.,* 2005). Bitner-Glindzicz, (2002) found that 50% of recessive non-syndromic hearing loss in the Caucasian population attributed to mutations in the *GJB2* encoding Cx26, demonstrated that most of these recessive forms of hearing impairment cause a phenotypically identical severe to profound prelingual hearing loss.

In the present study, for searching a locus that contains the candidate gene, responsible for autosomal recessive non-syndromic hearing loss in two families (A, B), linkage studies were performed by a method known as homozygosity mapping. This method searches a region of the genome that is autozygous in inbred individuals affected by a given disease. To quantify the evidence of linkage provided by such a region, a LOD score could be computed for the marker observations by comparing the likelihood of being at the disease locus with the likelihood of being at a random point on the genome (Lander and Botstein, 1987).

Homozygosity mapping, as other mapping methods, exploits the fact that the human genome is replete with polymorphic sequences that vary from one individual to the other (Elegren, 2004). On average, $1/16^{th}$ of the genome of offspring of first cousin marriages would be expected to be homozygous. The region of homozygosity would be expected to be random between different offspring of these marriages, except at a common disease locus shared by the affected offsprings.

For the present work, two families (A and B) with autosomal recessive non-syndromic hearing impairment (ARNSHI) were tested for linkage.

In family A, selected individuals were tested for the linkage to known DFNB loci. Analysis of the results of the genotyped microsatellite markers (Figure 3.3-3.39) showed that the affected individuals were heterozygous with different combinations of parental alleles, thus excluding the family from linkage to these known genetic intervals.

In family B both normal and affected individuals were tested for linkage to all known DFNB loci by using microsatellite markers from their candidate linkage intervals including DFNB1. Family B showed linkage at DFNB35 locus located on chromosome 14q24.1-24.3 containing *ESRRB* gene. *ESRRB* encodes the estrogen-related receptor protein beta that is a member of the nuclear hormone receptor (NHR)

family. One of the NHR subfamilies is composed of the estrogen receptors (ERs), the estrogen-related receptors (ESRRs), and the steroid receptors (SRs) (Laudet V, 1997)

Within the developing inner ear of the mouse, *Esrrb* expression is found in the nonsensory epithelia of the vestibulum, the developing stria vascularis, and lateral wall of the cochlea and in the vestibular ganglion (Collin *et al.*, 2008). Chen and Nathans, 2007 showed a similar distribution of *Esrrb* postnatally, namely in the marginal cells of the stria vascularis in the cochlea and in analogous structures in the vestibular system, both by in situ hybridization and by immunohistochemistry.

ESRRB and other ESRRs bind to both estrogen-responsive elements and steroid-responsive elements, and estrogen has a role in preservation of hearing during aging in human adults. Such a role is consistent with the decline in hearing observed in the estrogen receptor β knockout mouse (Hultcrantz M *et al.*, 2006).

In the present study Bioinformatics Approach is used to predict candidate genes which are associated with nonsyndromic hearing impairment.For the various nonsyndromic loci, which are identified so far, Hereditary Hearing Loss Homepage (http://webh01.ua.ac.be/hhh/) was used. Same web based source was used to make a list of deafness loci with unknown genes for autosomal recessive forms identified in Pakistan.

NCBI (http://www.ncbi.nlm.nih.gov/) and Ensemble database (http://www.ensembl.org/index) was used to make a list of all genes, which are present in genomic intervals. After it all the genes and transcripts of each locus were compared against databases of mouse inner ear genes and human cochlear genes.

If gene-by-gene approach is used for mutation analysis then there are too many genes and mutation analysis of all these genes will be time taking and labor-intensive approach. So we used the human cochlea (http://hearing.bwh.harvard.edu/estinfo.htm) and mouse inner ear expression database (http://www.ihr.mrc.ac.uk/Hereditary/genetable) to eradicate certain genes that were not expressed in these organs.

Mouse inner ear expressed database contains numerous genes present in two animal species and human cochlear database is taken from fetal cochlear cDNA library and EST database, which was updated in 2002 (http://hearing.bwh.harvard.edu/estinfo.htm). The data present in this set was taken

from Unigene (Pontius *et al.,* 2003). The database consists of 14,805 ESTs and 12,624 ESTs are sorted into 4,519 independent clusters by Unigene.

Genes, which were present in one of the previously mentioned databases, were considered for candidacy. Those genes were not considered which were not present in both databases. So for various loci we compiled a list of possible candidate genes. To further narrow down and refine this list, characterization of candidate genes was done by OMIM (http://www.ncbi.nlm.nih.gov/Omim/getmap.cgi). After characterization it was concluded that KIAA119 and EDN3 are candidate genes.

In the present study there were total 14 loci and two genes KIAA119 and EDN3 were identified as candidate genes in locus 48 and locus 65 respectively. So in remaining 12 loci no gene was identified as candidate gene because of the reason that there are many genes, which are not characterized yet according to OMIM Database. With the passage of time OMIM database will be updated and data about the characterization of genes will be available, more genes in the remaining loci will be identified in the future.

KIAA1199 is expressed in the cochlea and vestibule tissues so it is one of the inner ear expressed genes. For the proper functioning of ear KIAA1199 protein is very vital. Non-syndromic hearing loss is caused due to mutation in it (Abe, 2003). Cellular mortality is caused by upregulation of the KIAA1199 gene (Michishita, 2005).

Endothelin 3 is a human gene, which is also called EDN3 (Calderone *et al.,* 1994). This gene encodes a protein, which comes under the family of endothelin. Endothelins perform variety of biological functions and these are endothelium-derived vasoactive peptides. The active form of this protein is a 21 amino acid peptide, which is a ligand for endothelin receptor type B (EDNRB). The relation of EDNRB with this endothelin is very important for neural crest-derived cell lineages development, such as melanocytes and enteric neurons.

Waardenburg syndrome (WS) is caused by Mutations in EDN3 and EDNRB (Arinami *et al.,* 1991). Waardenburg syndrome is a genetic disorder having symptoms of varying degrees of deafness, few defects in structures which arise from the neural crest, and anomalies related to pigmentation (Edery *et al.,* 1996).

The map locations of a large number of nonsyndromic autosomal recessive deafness phenotypes are known, but the specific genes responsible for all these phenotypes have not been identified (http://webh01.ua.ac.be/hhh/). The cloning of genes involved in such phenotypes needs short genomic interval, which can be done by linkage analysis.

If the mutation analysis of all genes is done then it will be time taking and labor-intensive process. Boinformatics approach that is presented here can reduce very large number of genes into a number, which can be managed for mutation analysis.

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