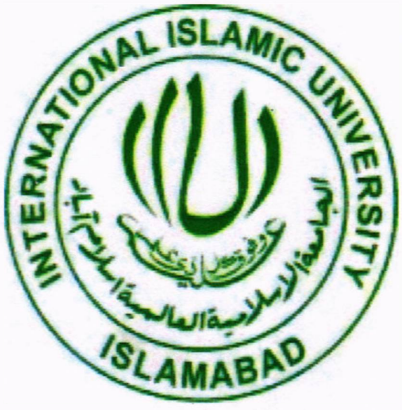
MOLECULAR SCREENING OF FAMILIES WITH CONGENITAL DEAFNESS FROM DISTRICT MARDAN



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(2013)

MOLECULAR SCREENING OF FAMILIES WITH CONGENITAL DEAFNESS FROM DISTRICT MARDAN

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FINAL APPROVAL

It is certified that we have read the thesis submitted by **Mr. Shahid Hussain** and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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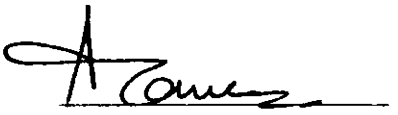

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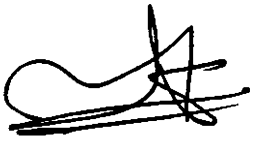

A thesis submitted to Department of Bioinformatics and Biotechnology,
International Islamic University, Islamabad as a partial
fulfillment of requirement for the award of the
degree of MS.

I Dedicate This Work to My Beloved Parents And uncles

DECLARATION

I hereby declare that the work present 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.

Date _____

_____

Shahid Hussain

CONTENTS

ACKNOWLEDGMENTS.....I
LIST OF ABBREVIATIONS III
LIST OF FIGURES IV
LIST OF TABLES V
ABSTRACT VI

1. INTRODUCTION.....1
2. REVIEW OF LITERATURE.....5
2.1. Structure of Human Ear.....5
2.2. Anatomy of Ear.....5
2.2.1. External Ear.....5
2.2.2. Middle ear.....7
2.2.3. Inner ear.....7
2.3. Physiological tests for Hearing.....12
2.4. Molecular Genetics of Deafness.....13
2.4.1. Deafness and its classification.....13
2.4.2. Molecular genetics of hearing loss.....14
2.4.3. Syndromic Deafness.....15
2.4.4. Non Syndromic Deafness.....15
2.4.5. Non Syndromic Autosomal Recessive Deafness.....16
2.4.6. Reported None syndromic ,Recessive deafness genes 2011.....20
2.5. Prevalent of Autosomal Recessive Loci/Genes in Pakistan population.....21
2.6. Basis of Linkage Analysis.....27
2.7. Recombination Fraction reflects Genetic distance.....28
2.8. LOD Score Calculation.....28
3. MATERIALS AND METHODS.....29
3.1. Family Description.....29
3.2. Pedigree Analyses.....29
3.3. Identity (id) Numbers Allotment.....29
3.4. Blood Sampling.....30
3.5. DNA extraction from blood.....30
3.6. Linkage Analyses by using Polymerase chain reaction (PCR).....32
3.6.1. Exclusion of known loci.....32

3.7. Polyacrylamide Gel Electrophoresis (PAGE).....34
3.8. Gel visualization.....35
3.9. Screening of Mutation.....34
4. RESULTS..... 39
4.1. Families Studies.....39
4.1.1. Family DFN10.....39
4.1.2. Family DFN13.....39
4.1.3. Family DFN17.....40
4.2. Linkage Studies.....40
4.2.1. Linkage to known Loci (Exclusion Study).....40
4.3. Linkage of Family DFN13 on DFNB4 (SLC26A4).....62
4.4. Mutation Screening in SLC24A4 gene (Family DFN13).....64
5. DISCUSSION 67
6. REFERENCES.....74

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Shahid Hussain

LIST OF ABBREVATIONS

AA	Amino Acid
ABR	Auditory brain Stem response
Bp	Base pair
cM	Centimorgan
dB	Decibels
DFNA	Deafness, Autosomal Dominant
DFNB	Deafness, Autosomal Recessive
dNTPs	Deoxynucleotide phosphates
EDTA	Ethylenediaminotetraacetic acid, disodium salt
Hz	Hertz
Kb	Kilobases
M	Molar
$MgCl_2$	Magnesium Chloride
Min	Minutes
mm	Millimeter
µl	Microlitre
µM	Micromolar
ng	Nanogram
pmole	Pico moles
SDS	Sodium dodecyl Sulphate
STR	Short Tandem Repeat
TAMRA	Carboxytetramethylrhodamine
USH	Usher

LIST OF FIGURES

S.No	Figure. No.	Family	Page	Marker (s)	Locus
1	4.1a	DFN17	41		DFNB39
2	4.1b	DFN13	42	D7S820, D7S2204	
3	4.1c	DFN10	42		
4	4.2a	DFN17	43		DFNB16
5	4.2b	DFN13	44	D15S659	
6	4.2c	DFN10	44		
7	4.3a	DFN17	45		DFNB48
8	4.3b	DFN13	45	D15S211	
9	4.3c	DFN10	46		
10	4.4a	DFN17	46		DFNB35
11	4.4b	DFN13	47	D14S53, D14S588	
12	4. 4c	DFN10	47		
13	4.5a	DFN17	48	D14S599, D14S306	DFNB5
14	4.5b	DFN13	49		
15	4.6a	DFN17	50		DFNB24
16	4.6b	DFN13	50	D11S2000	
17	4.6c	DFN10	51		
18	4.7a	DFN17	52	D7S2420	DFNB4
19	4.8a	DFN17	53		DFNB44
20	4.8b	DFN13	53	D7S2846	
21	4.8c	DFN10	54		
22	4.9a	DFN17	54		DFNB19
23	4.9b	DFN13	55	D18S976, D18S843	
24	4.9c	DFN10	56		
25	4.10a	DFN17	56		DFNB22
26	4.10b	DFN13	57	D16S764, D16S403	
	4.10c	DFN10	58		
27	4.11a	DFN17	58		DFNB12
28	4.11b	DFN13	59	D10S1432	
29	4.11c	DFN10	59		
30	4.12a	DFN17	60		DFNB23
31	4.12b	DFN13	60	D10S1221	
32	4.12c	DFN10	61		
			FIGURES OF LINKED FAMILY		
33	4.13	DFN13	62	D7S2456, D7S2420	DFNB4
34	4.14	DFN13	62	D7S2456	Gel Image
35	Sequencing Result of Exon2 in a patient (IV-02) of Family DFN13				65
36	Sequencing Result of Exon2 in a normal (III-21) of Family DFN13				66

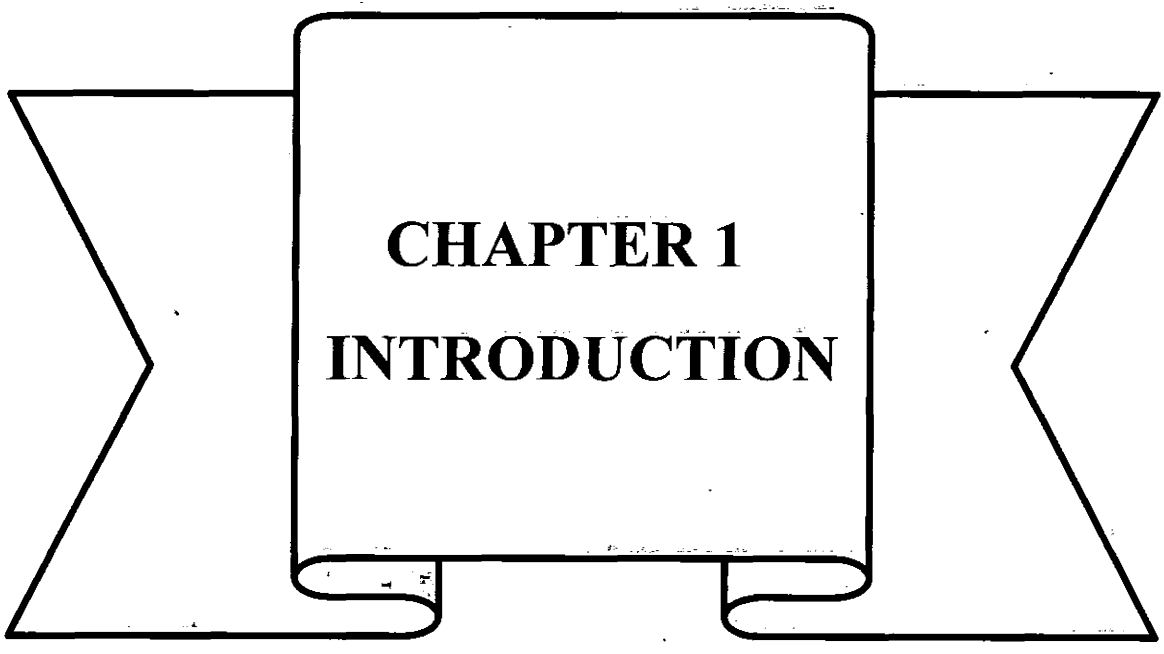
LIST OF TABLES

S.No.	Table	Contents	Page
1	2.1	Degrees of severity in hearing loss	13
2	2.2	Reported Autosomal Recessive Loci (2011)	16
3	2.3	Reported Autosomal Recessive, Non Syndromic Genes (2011)	20
4	3.1	PCR Recipe	33
5	3.2	Master Mix Recipe For Amplification of Exon by a Specific Primer	36
6	3.3	Primers for 21 Exons of SLC26A4 Gene	37
7	4.1	LOD score of linked markers	63

ABSTRACT

Deafness is described as complete or partial loss of hearing. Hearing loss is observed to be due to both environmental and genetic factors. Genetic deafness is either syndromic or non-syndromic. There is a high rate and risk of such genetically inherited disorders in Pakistan; 1.6 per 1000 individuals, as compared to the rest of world which is just 1 per 1000.The accelerated cause of inherited diseases in Pakistan comes off consanguineous marriages. The objective of the current study is to find association of loci involved in deafness in multi-generation families collected Mradan, Khyber Pakhtunkhwa Pakistan. Multi-generation families affected from non-syndromic deafness were ascertained after informed consent from Mardan Khyber Pakhtunkhwa Pakitan. Blood sample (5ml) was drawn from normal and affected individuals of the families. DNA was extracted using standard organic methods. These DNA samples were been amplified through PCR, using loci specific microsatellite markers. The PCR products were run on PAGE for linkage analysis. The linked family was screened for mutation through direct sequencing. One of the families was found to be linked at DFNB4. Sequencing of the candidate gene (SLC26A4) on DFNB4 was done to find out the mutated exon, out of 21 exons present in the candidate gene, Two splicing site mutations (T>G and C>G) were observed on exon 2. This study will provide genetic proof for the screening and identification of hearing loss genes in the population of Pakistan. Furthermore genetic counseling and molecular screening of deafness is trying to become common in Pakistani.

Keywords: Deafness, Autosomal recessive, None-syndromic, Linkage analysis, Mutation, SLC26A4,



CHAPTER 1

INTRODUCTION

INTRODUCTION

Deafness is found to be the most common sensory disorder which effects 1 per 1000 neonates. (Kalay *et al.*, 2005 and Ramshankar *et al.*, 2003). Both environmental (e.g. microbial infection acoustic trauma, toxicity) and genetic factors are involve in causing hearing loss (Morton.1991).Genetic factor is mostly observed major factor for hearing impairment (Marazita *et al.*, 1993 and Rehm, 2003). Congenital hearing loss is highly heterogeneous defect which has been classified into syndromic (30%) and non-syndromic (70%) disorders, non-syndromic is either Autosomal dominant, Autosomal recessive, X-linked and mitochondrial mutations (Petersena and Willemsb 2006; Mukherjee *et al.*, 2003). In syndromic deafness 400 other related inherited abnormalities are found (Gorlin R.J, 1995). It is founded that above hundred genes are involved in hearing loss. More than 130 loci have been studied in previous published data. Loci of hearing impairment are abbreviated by DFN (deafness), Furthermore Autosomal recessive, Autosomal dominant, X-linked are denoted as DFNB,DFNA and DFN respectively (Mukherjee *et al.*, 2003).

Hearing loss is either sensorneural (inner ear malfunction) or conductive (middle ear abnormalities) (Gorlin R. J, 1995). In Case of sensorneural hearing impairment a high genetic heterogeneity has been observed. To date 139 non-syndromic hearing loss loci have been mapped, out of these 139 loci are found to be inherited in Autosomal recessive pattern. Moreover 33 genes of nuclear genome responsible for deafness have been cloned, (www.uia.ac.be/dnal). In human genome, 1% of the total 30000 genes show expression, which are involved in hearing (Friedman and Griffith, 2003). The inner ear cells are highly sensitive, having a very small size and cellular diversity, which have complicated the physiological and proteomics studies of inner ear (Thalmann, 2006).

The genetic approaches are found to be more beneficial than the biochemical and physiological methods. But due to high heterogeneity it was difficult to study this disorder in routine way (Williams and Smith. 1997). The Pakistani gene pool is the richest source of genetically inherited disease due to high incidence of consanguineous marriages. More than 80% marriages are found to be among the first cousins (Hussain & Battle, 1998). The hearing loss prevalence is found to be 1.6 per 1000 in Pakistani population. (Elahi *et al.*, 1998, and Jabar *et al.*, 1992).Several studies on deafness in Pakistani population have reported many new loci. A good number of families with many affected individuals are present in Pakistan which has helped in identification

of new loci as well as cloning of newly discovered genes. So far 24 new Autosomal recessive linkages and 15 genes of deafness have been reported in Pakistani population.

Hearing loss is measured in decibels (dB).The threshold level of 15 dB is considered normal, while range of 26-40 dB is mild, 44-55 dB is moderate, 56-70 dB is moderately sever and 71-90 dB is sever or profound. Deafness leads to abnormalities in speech, language, psychological and cognitive development. The age of onset greatly influence the hearing impairment.

Genetic studies were started in a Autosomal dominant form of postlingual DFNA1 in a multi generation effected family from Cost Rica (Leon *et al.*, 1992). While non-syndromic type DFNB1 was studied and mapped in Tunisian family (Guilford *et al.*, 1994).

Among Autosomal ressicive loci DFNB1 having GJB2 and GJB6 gene is found to be most prevalent and is found in 50% of recessive cases. Other loci with a high prevalence are DFNB4,DFNB9,DFNB7/11,DFNB 8/10,DFNB3 and DFNB12 (Karina *et al.*, 2008). While in Autosomal dominant mutations GJB2 is less common (Denoyelle *et al.*, 1998).

GJB 2 genes codes for conexin 26 (Cx26), a member of trans-membrane gap-junction proteins, which monitor the metabolic and electrical junction between adjacent cells. In the cochlea, Cx26 is expressed in the supporting cells of the neurosensory epithelium and in cochlear duct fibrocytes (Lautermann *et al.*, 1998), and is presumed to be involved in potassium recycling from depolarized hair cells back to the endolymph. To date, more than 70 *GJB2* mutations have been reported to cause deafness, with a significant difference in the frequency and distribution among different populations (http://www.iro.es/cx26deaf.htlm).

Deafness is globally present but the study of single gene inheriting pattern in various communities is highly complicated. Specially in those societies where open culture and living styles of people are common, so there a lot of heterogeneity are found in phenotypes (Petit 2001, Reardon 1992). Furthermore it is not feasible to identify the causative agents of hearing loss due to very complex structure of few accruing cells in the inner ear. Each portion of the ear has cells with different shapes and structures which also confuse to study (Florenkov *et al.*, 2004). The biochemical and physiological studies are not accurate and sufficient to find out the player behind hearing loss the genetic approaches especially in identifying specific genes responsible for deafness in consanguineous families are easy tools to use associated with deafness (Friedman and Griffith. 2003). In consanguineous families a specific mutation in genes is inherited generation to generation so affecting many individuals. The genes carrying markers from parents

to offspring are favorable tools in identification of disease Locus. Consanguineous marriages are considered to be reason of inheritance of a disease especially in remote areas, in which homozygosis is used to simplify genetic linkages.

The Culture and societies of Asian countries including Pakistan have got much more genetic disorders due to high rate of consanguineous marriages. It has been estimated that rate of consanguineous marriages in Pakistan is 60%, out of which 80% are first cousins (Hussain and Bittels 1998). In Pakistani's Population, 70% hearing loss is due to consanguineous marriages. So hearing loss in Pakistani populations is sought to be 1.6 per 1000 (Elahi et al., 1998). To date 24 non syndromic loci DFNB16, DFNB20, DFNB26, DFNB29, DFNB35, DFNB36, DFNB37, DFNB38, DFNB39, DFNB42, DFNB47, DFNB48, DFNB49, DFNB51, DFNB56, DFNB62, DFNB63, DFNB65, DFNB67, DFNB68 and DFNB72 have been mapped in Pakistani population. Thus Pakistani population is an excellent source to provide information about genes involved in hearing loss. From the studies it has been found that approximately 300 genes are involved in hearing process. Out of these 57 loci are inherited in Autosomal dominant Pattern, in which hearing loss is observed at post lingual and progressive mode. While 77 Loci have been mapped which are involved non syndromic Autosomal recessive hearing loss. Out of which 49 genes have been identified. (Hereditary hearing loss Homepage), therefore to identify the remaining unknown genes these sort of studies are helpful to understand molecular basis at hearing impairments. From the very beginning scientists are working to disclose the reason of genetic disorders. Two main techniques are mostly used, the classical approach and second one is called modern candidate gene approach, in the former techniques already reported Loci are linked with markers for the exclusion studies. If the linkage is observed, it indicates that these Loci are among already reported and in the case if linkage is not found then the study is proceeded to sequencing and mapping, which results in the discovery of new genes while the modern candidate gene approach is more accurate and easy to be handle over than the classical approach. In this method the genes or its location is known in other mammals (mouse) and its orthologous gene is predicted in human. Human genes are estimated to be 80% similar to mouse so it is very helpful to identify new genes in human. In human most of the genes (1) i.e. OTOA, TIME, TMC1, MY06, POU4F3 and EYA4 were identified by using deaf mouse models. These models are also used to identify and specify several genes involved in hearing loss e.g. DIAPH1,

COL11A2, TECTA, GJB2, OTOF, TFCP2L3, MY07A and SLC26A4. (Friedman and Griffith, 2006 ; Avraham, 2003).

The study ascertained here was designed to identify new locus causing deafness in Pakistani population. Three families with the history of deafness were been collected from district Mardan and the study linkage analysis was done via "classical approach".

Blood samples were collected and processed for DNA extraction after written informed consent. All the enrolled families were studied for linkage to known loci. One family (DFN13) was found to be linked to *DFNB4* locus. Initially all the normal individuals and Patients were screened by using Microsatellite markers for exclusion study. In three families one was found to be linked with DFNB4 locus on chromosome 7q31. SLC26A4 gene is present on DFNB4 locus. By screening mutation in the selected gene a novel splice site mutation was found. The benefit of this study on one side is to provide scientific data to scientific community and awareness through screening of carrier status and genetic counseling to reduce the socio-economic burden on the affected families and decrease the incidence of deafness in Pakistan. Identification of already reported deafness locus (*DFNB4*) was screened for mutation by using exon specific markers for 21 exons of SLC26A4 Gene and a novel mutation was identified in a Pakistani family. The current project conducted on Pakistani families will help us to better understand the development and functioning of the auditory system.

CHAPTER 2
REVIEW
OF
LITERATURE

REVIEW OF LITERATURE

2.1. Structure of Human Ear

The human ear is a complicated organ composed of functionally and structurally diverse anatomy. This perceives sounds and controls the balance of the body. The structure is designed in such a manner that can easily percept the waves of sound from our surrounding, recognize it and finally response function is directly performed through brain. The frequency range between 50 and 20,000 cycles per second is tolerable and audible, below or above frequency from these ranges are not perceived by human ears (Dallos, 1996). It transfer sound impulse to the brain so is described as microcomputer and a microphone. Human ear is divided into three major parts outer ear, middle ear and inner ear which collectively control and perform the hearing system.

2.2. Anatomy of Ear

2.2.1. External Ear:

External ear is composed of three main parts, pinna or auricle, auditory canal and ear drum.

Pinna:

Two holes from both sides of the skull lead to ear canals, which is 2.5 cm long, and having a diameter of 0.8mm while shape is like an irregular cylinder. At outer side the canal is opened to a visible structurally important region called pinna or auricle. Pinna is the most outer visible region of the ear made of cartilage surrounded by skin which literally out grown from sides of the head. Through ligaments and muscles it is attached to the head. The bowl like deep region is called cochlea as given in figure 2.1.

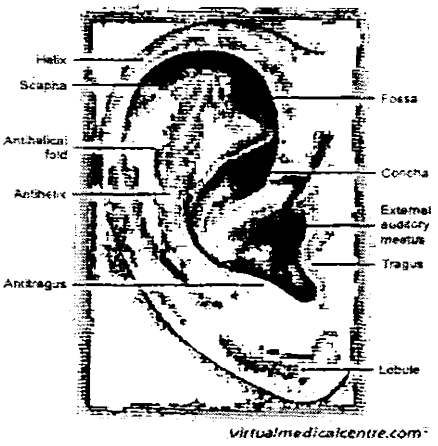


Figure 2.1: Structure of outer ear

Auditory canal:

The canal like region which starts from external auditory meatus and reach to ear drum is called auditory canal.

Tympanic membrane

Tympanic membrane or ear drum is a thin double layered, partition between middle ear and external ear as shown in figure 2.2. This membrane is responsible for preventing inner delicate part of the ear from any of the external hazardous agents like microbes to cause any infectious diseases. It is also useful in proper transmission of sound.

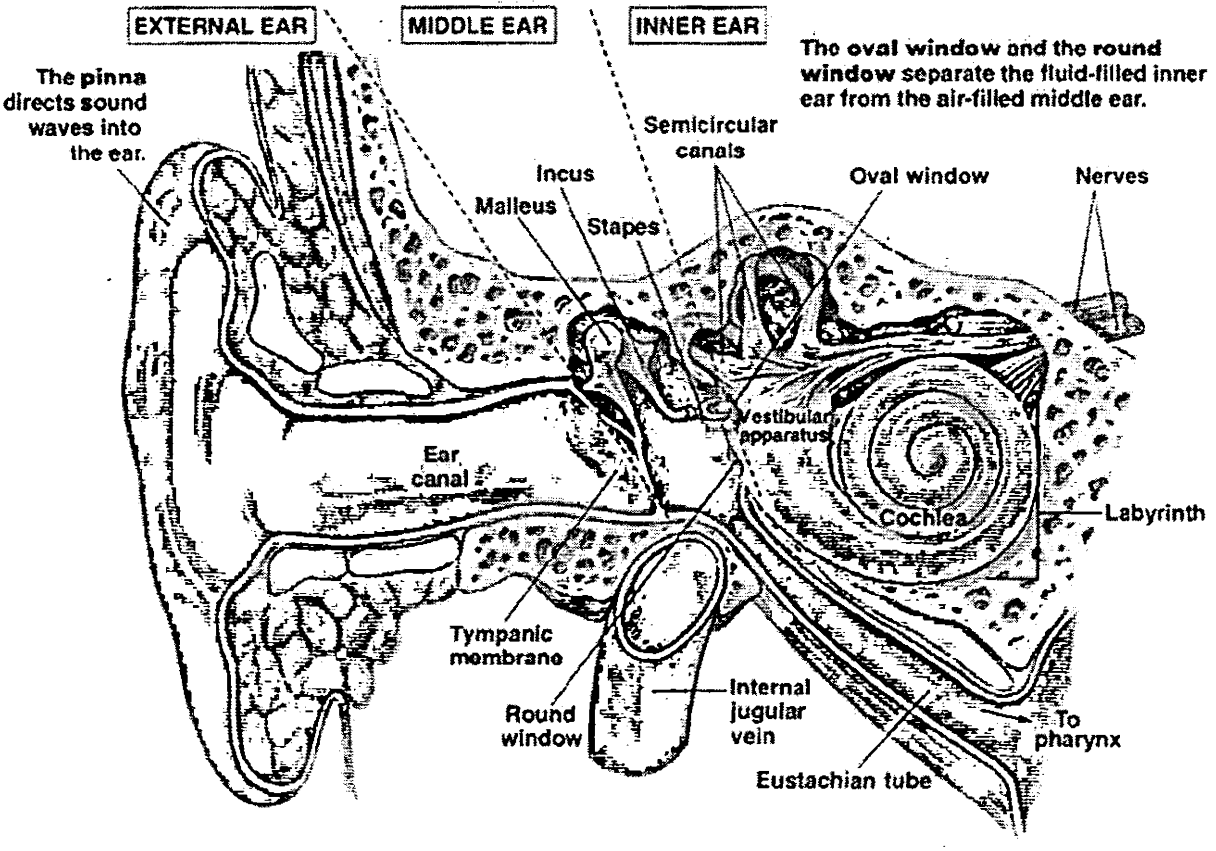


Figure 2.2: structure of human ear showing major parts external, middle and inner ear.

2.2.2. Middle ear:

The tympanic membrane acts as boundary wall between middle ear and external ear. Eustachian tube is a narrow connection way between throat and middle ear and to the back of nose. The eustachian tube equalizes the pressure between the outer and middle ear to keep the eardrum intact. In low air pressure, where the air pressure become greater then air pressure at outer ear, the person feel pain in ears and head. Some of the air is passed from middle ear to throat due to opening of Eustachian tube while when the person yawns or swallows. Adjusting the pressure in middle ear to match the pressure with outer ear. This equalization prevents the tympanic membrane from disruption.

The anatomy of middle ear is too complicated. It is a narrow tube portion which has approximately the same length vertically and horizontally. Three small bones or ossicles are present in middle ear called malleus or hammer, anvil and stapes or stirrup. Stapes is found to be the smallest bone in entire human body.

The malleus is literally embedded in eardrum the stirrup is fixed in oval window. The sound frequency produces movement in eardrum which creates movement in hammer. This series of movement is preceded to the anvil, which vibrate the thin bone stirrup. As the sound in vibration form is passed from eardrum through the involvement of all bones make high concentration of sound which increases the sound frequency before passing from inner ear through oval window. The inner and middle ear is protected from damage during loud noise pollution by two small muscles called tympani and stapactiouse.

2.2.3. Inner ear:

The inner part of the ear has a complicated structure, which resides within a deep portion of the skull called petrous portion of the temporal bone. This part of the ear is termed as labyrinth because of its complexity. The two major parts of this region are membranous labyrinth and bony labyrinth (Hudspeth *et al.*, 1989).

Bony or osseous labyrinth:

This portion is filled with fluid called perilymph, which has resemblance in chemical structure to spinal cord fluid. It has three main parts.

Vestibule: It is located near and next to oval window.

Second part is composed of three semi circular canal called superior, lateral and posterior.

Cochlea: it is a 35mm long spiral bony organ. Its shape has similarity to snail shell. The cochlea name is given to it because in Greek cochlea means snail. The cochlea has three chambers, the scala vestibule which is located just above of the oval window. The scala tympani is a membranous nature its function is to release pressure and the third one is scala media which is an organ of corti (Hudspeth *et al.,* 1989).

Membranous Labyrinth:

This is a membranous portion of the inner ear located with bony or osseous labyrinth. Endolymph fluid is filled inside this region which has a different chemical composition then perilymph. The saculus and utriculus forms the membranous part of the vetibular apparatuse as shown in figure 2.3. The junction between bony and membranous part is termed asscala media which has a receptor region for hearing. It's a part of corti, which is located in asscala media. The media joins the vestibular organ of vestibule, saccule and utricle through a narrow small tube called ductusreunions. Enolymph has a quit different shape and nature its an another fluid of the human body which contains a very lower quantity of sodium and is rich with potassium. The potassium is responsible for the transport through staria vascularis, while perilymph has same nature to extra cellular fluids which can result in the presence of high concentration of sodium. Its osmolarity is same to that of plasma of the blood that's why blood and perlymph how the same osmotic features (Graham *et al.,* 2000).

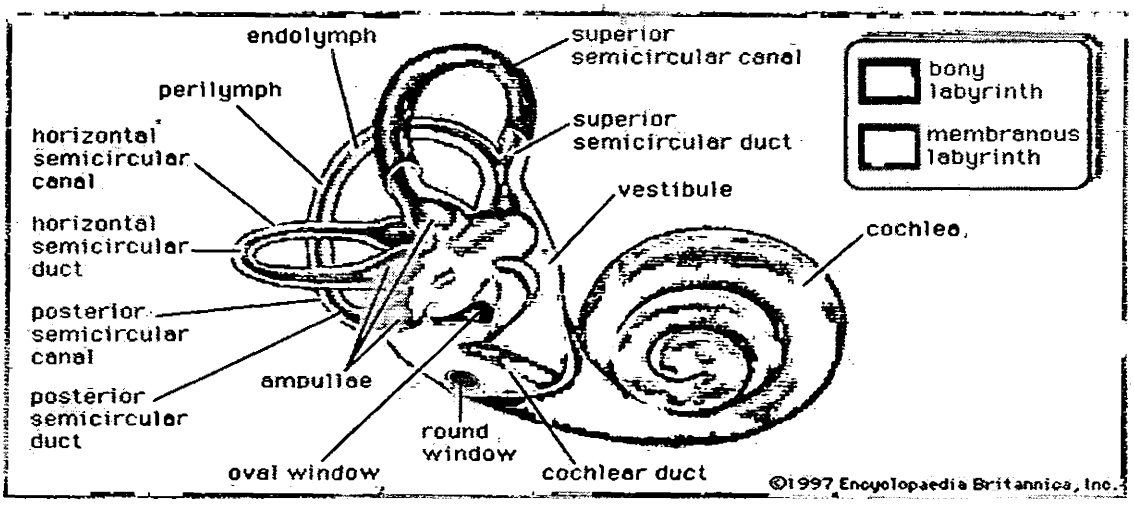


Figure 2.3: Bonny and membranous labyrinth

Organ Of Corti

The organ of corti is a receptor of hearing which convert sound waves into electrical impulses. It is surrounded by protein body called tectorial membrane those receptor cells functions in auditory traduction are called hair cells because their surface is thin like hair so called hair cells. The hair cells are joint together through tip and from a bushy structure which perform the mechano-electrical transduction process. The most lengthy stereocilia of outer hair cells is connected with tectorial membrane as shown in figure 2.4.

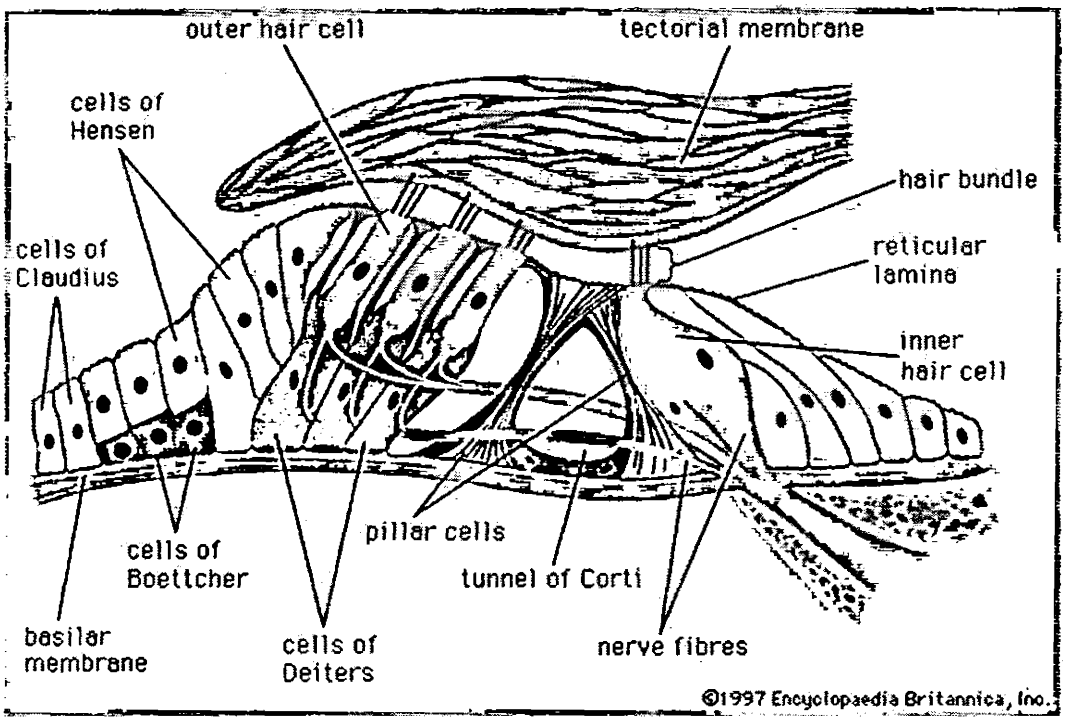


Figure 2.4: Organ of coti

Hair Cells

These are the epithelial cells of inner ear which rise from ectoderm. The hair cells have shapes like cylinder or flask. There are two types of hair cells, the outer hair cells and the inner hair cells.

Outer Hair Cells

These cells have motor elements as well as sensory elements that are responsible in sensitivity of hearing as shown in figure 2.5. These cells also differentiate a sound by amplifying sound

reception. In human ear three to four layers of outer hair cells are found, which are more in number than inner hair cells. These cells also contain fiber muscles filaments which on stimulation are contracted.

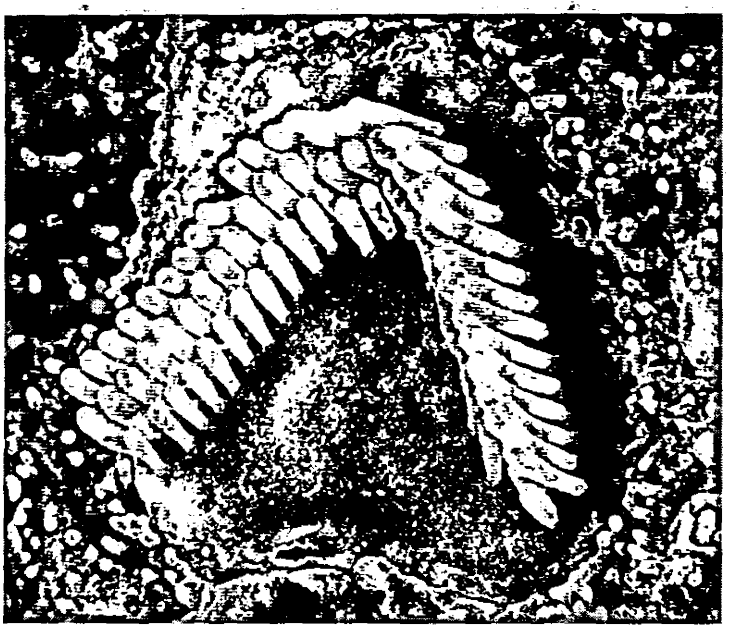


Figure 2.5: Outer hair cells.

Inner Hair Cells

These cells have only one layer or row of 3500-3600 cells shown in figure 2.6. The major function of these cells is to produce sense of hearing, when these cells are damaged, so cause profound haring loss The Inner cells are in straight line or U-Shaped so appears in the form of continuous fence along the Inner aspect of the corti. These cells does not appear to contact the over lying tectorial membrane. These cells get 95% differing innervations from the nerve fibers which comes from the acoustic portion of the VIII nerve. These cells are responsible for creating sensations of hearing and cause severe deafness on minute damage.

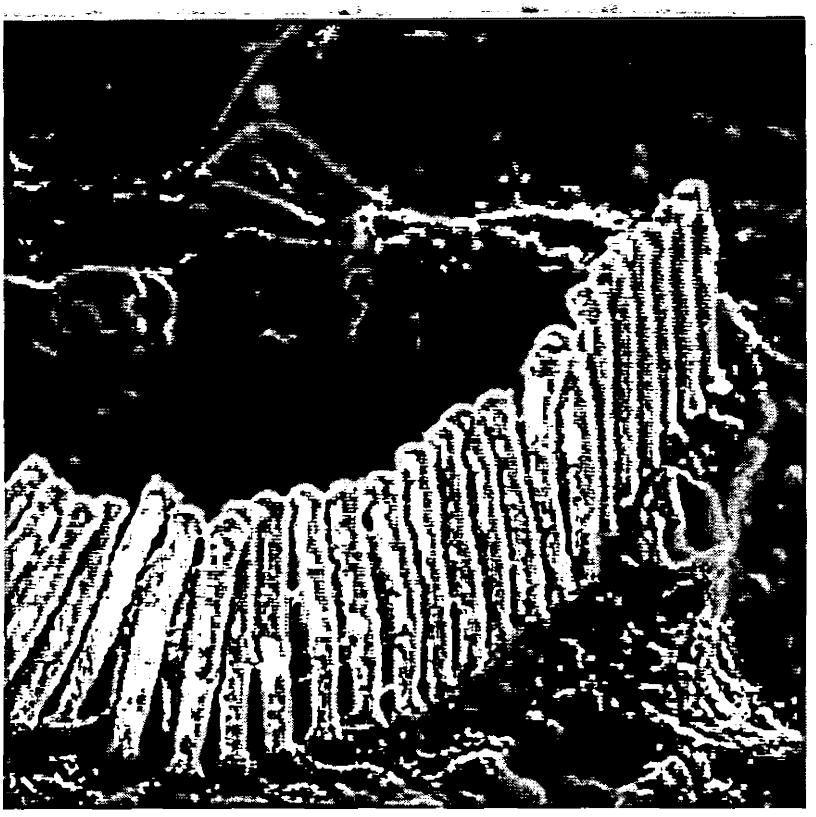


Figure 2.6: Arrangement of Hair Cell Stereocilia And Mechanotransduction

Hairs bundles are present in the form of rows of stereocilia which enlarges in one specific direction across the bundle. These hair cells are arranged in three rows of different lengths and behind the row of longest stereocilia a single kinocilium is located. These hair cells bounds to plasma membrane, embedding filaments of the cytoskeletal protein called actin, while kinocilium is similar to motile cilia and it is a true cilium. Kinocilium is found to be present only during development. The kinocilium and the longest row of stereocilia show the polarity of hairs bundles. The sterocilia of outer hair cells is indirectly connected to tectorial membrane through tectorial membrane crown. The sterolium tips are in contract with the near occurring shaft by thin tip. These take part in the process of mechano-transduction. The Sterocillia are also in contact with transverse links both in the row to row and in the same row manner. Another type of Linkage is ankle Links in which are not present in the corti but present in the vestibular organ.

Inner and outer hair cells transport the sound waves to the electrochemical energy. Hair cells consist of mechanosensitive ion channels. The regulation (Opening or closing) of these ion

channels play a basic role in hearing. Ions channels are opened when the sterocillia bundles deflect toward tall edge endolymph have a high concentration of potassium. When the ion channel opens the potassium ions enters. The potassium modifies the hearing cells and activates their potential. Thus the hair cells convert the sound waves to electrical signals. These signals reach to the brain via auditory nerves. (Corey and Hudspeth, 1979; Ohmori, 1985).

2.3. Physiological tests for Hearing:

To determined the auditory status of a person several physiological tests are performed at any stage of life. Audiometer is the most commonly using for this purpose, which give information about the processing of sound waves in hearing system commonly the following system are used in audiometric analysis.

Auditory Brainstem Response Testing (ABR):

In ABR System a click is used to evoke electrophysiological responses, which awake the VIII cranial nerve and auditory brain.

Evoked Otoacoustic Emissions (EOAEs):

Evoked otoacoustic emissions are those sounds which originate in cochlea. Sound impulses are measured in external auditory canal with the help of transducer and microphones. EOAEs reflect basically the activity of outer hair cells of the cochlea across a high frequency range and are present in ear with better sensitivity than 40-50 dB HL.

Behavioral test:

These tests include Behavioral Observation Audiometry (BOA) and visual reinforcement audiometry (VRA). BOA is used only for the infancy stage. It is highly dependent on the tester experience and is subject to error. VRA is used for those children whose age ranges from six months to 2 years. It is reliable, accurate and complete audiogram.

Pure-tone Audiometry (PTA):

PTA (air and bore conduction), is the lowest intensity or pitch on which the human ears is capable of hearing. Earphones are used to test frequencies ranges from 250-8000 Hz the normal

hearing of adults give result of zero dB HL which is an average threshold of frequency. While the highest frequency is 120 dB HL, which give pain to the listener.

2.4. MOLECULAR GENETICS OF DEAFNESS

2.4.1. Deafness and its classification:

The complete or partial loss of hearing is termed as deafness. It is classified by several ways.

- The first criteria used in classification of deafness is according to the nature of disease that either it is environmental, genetic or a combination of these both.
- The second criteria is that which part of the ear is defected due to which it has used its function e.g. middle or inner ear, conductive or sensorneural.
- The third one is to classify the diseases on the basis of its severity, normal, middle or high as given the table 2.1.

DEGREE OF SEVERITY	HEARING LOSS
Normal	0-25 dB
Mild	26-40dB
Moderate	41-55dB
Moderate severe	56-70dB
Sever	71-90dB
Profound	>90dB

Table 2.1: Degrees of severity in hearing loss

- The fourth criteria is to categorize the deafness on the basis of the age of onset congenital, pre lingual or post lingual and progress of the impairment.

- And the last criterion to classify deafness is either it is syndromic or non syndromic. (Kalatzis and petit 1998, Riazuddin *et al.,* 2000; Khovskaya *et al.,* 2000; Schultz *et al.,* 2005).

2.4.2. Molecular genetics of hearing loss:

Molecular hearing impairment is a highly heterogeneous trait. Classical approaches for the diagnosis of hearing mechanism are not sufficient to study congenital deafness due to low accuracy of biochemical methods. The main reason is that a very small number of each cell is present in the hearing organized body (ear). However Genetic approaches have high accuracy and are most beneficial and promising techniques to study hereditary deafness (Friedman and Griffith 2003, Petit 1996).

The identification and localization of genes involved in syndromic deafness started in 1990s. But up to 1994 only three responsible genes for syndromic deafness had been mapped from human genome. In syndromic deafness classification symptoms were used to categorize its different forms. For this purpose positional cloning and linkage analysis were most feasible. Especially Linkage analysis for non syndromic deafness is more difficult because it needs a single family for linkage analysis. However for these studies many deaf families have been analyzed through linkage analysis. By using molecular genetics technologies a lot of involved genes have been localized, identified an mapped. From a variety of researches it has been concluded that 1% genes of the total genes (30,000). in human for the hearing. Approximately 300 genes are predicted to be involved in hearing mechanism in human (Friedman and Giffith, 2003).

The development and advancement in molecular tool and techniques has made it possible to understand Genetic of deafness. As 1 per 1000 persons are affected with deafness, so a huge amount of families are available for these sorts of studies. Several available databases are serving as strong tools to provide data about the hereditary deafness e.g. polymorphic genetic markers, physical maps, genetic maps, transcription database, human and mice DNA sequences databases, mouse and zebra fish models for human hearing. So from the above discussion it can be concluded that many defected genes involved in disorders of hearing will be identified and mapped in future.

2.4.3. Syndromic Deafness:

More than 400 different symptoms are associated with deafness, Therefore it is estimated that approximately 30% other associated disease are found along with deafness, which may be in the form of sensorineural, conductive, or mixed (Grolin *et al.,* 1995).

2.4.4. Non Syndromic Deafness NSD):

NSD is that form of hereditary hearing loss in which no other associated symptoms are observed. NSD is found to be more than syndromic deafness. It is more prevalent and account for 70% of all hereditary deafness and is almost exclusively sensorineural (Mortan, 1991; Reardon, 1992; Marazita *et al.,* 1993).

The identification of causative genes for deafness is more challenging and required further steps in deafness research. Deafness is highly complicated because of its heterogeneity and limited clinical differentiations (Petit *et al.,* 2001). Other feature also make it complicated e.g. very similar clinical phenotypes are generated by different mutated genes, even within a family (Masmoudi *et al.,* 2000).

Mostly consanguineous marriages are playing a key role in hereditary deafness, because two carriers give birth to affected individuals and in this way the mutated genes are properly inherited from generation to generation making the structure of mutated genes more complicated and cause severe hearing loss (Balciuniene *et al.,* 1998). These studies are becoming more complex due to genetic and environmental factors interplay. The consistent families are best tools to study their segregation and pattern of inheritance (Guilford *et al.,* 1994).

Linkage analysis is helpful in refining intervals in deafness causing loci and mapping new genes. This analysis is feasible especially for identification studies of many recessive Loci. The non Syndromic deafness is further divided in different classes e.g. X-Linked (DFN), Autosomal dominant (DFNA)., Autosomal recessive (DFNB) and mitochondrial hearing loss with prevalence of DFNB is 80%, DFNA is 20%, DFN is 1% and mitochondrial is less than 1%. (Mortan, 1991; Mortanand and Nancy, 2006).

2.4.5. Non Syndromic Autosomal Recessive Deafness:-

The most common form of deafness is non syndromic Autosomal recessive (NSRD). In it 24 genes and 95 Loci causing deafness have been mapped and identified (Hereditary Hearing loss Homepage) as given in table 2.2. The mapped loci of NSRD had been designated as DFNB1, DFNB2, DFNB3 and so on, in the same order in which they are reported and reserved.

Reported Autosomal recessive loci (up to 2011):

Locus (OMIM).	Location	Gene (OMIM).	Important reference
DFNB1	13q12	GJB2	Guilford *et al.*, 1994 ; Kelsell *et al.*, 1997.
DFNB2	11q13.5	MYO7A	Guilford *et al.*, 1994 ; Liu *et al.*, 1997 ; Weil *et al.*, 1997.
DFNB3	17p11.2	MYO15A	Friedman *et al.*, 1995 ; Wang *et al.*, 1998.
DFNB4	7q31	SLC26A4	Baldwin *et al.*, 1995 ; Li *et al.*, 1998.
DFNB5	14q12	unknown	Fukushima *et al.*, 1995.
DFNB6	3p14-p21	TMIE	Fukushima *et al.*, 1995 ; Naz *et al.*, 2002.
DFNB7/11	9q13-q21	TMC1	Jain *et al.*, 1995 ; Scott *et al.*, 1996 ; Kurima *et al.*, 2002.
DFNB8/10	21q22	TMPRSS3	Veske *et al.*, 1996 ; Bonné-Tamir *et al.*, 1996 ; Scott *et al.*, 2001.
DFNB9	2p22-p23	OTOF	Chaib *et al.*, 1996 ; Yasunaga *et al.*, 1999.
DFNB10	see DFNB8		
DFNB11	see DFNB7		
DFNB12	10q21-q22	CDH23	Chaib *et al.*, 1996 ; Bork *et al.*, 2001.
DFNB13	7q34-36	unknown	Mustapha *et al.*, 1998.
DFNB14	7q31	unknown	Mustapha *et al.*, 1998.
DFNB15	3q21-q25		Chen *et al.*, 1997.

	19p13	GIPC3	Charizopoulou *et al.*, 2011.
DFNB16	15q21-q22	STRC	Campbell *et al.*, 1997 ; Verpy *et al.*, 2001.
DFNB17	7q31	unknown	Greinwald *et al.*, 1998.
DFNB18	11p14-15.1	USH1C	Jain *et al.*, 1998 ; Ouyang *et al.*, 2002 ; Ahmed *et al.*, 2002.
DFNB19	18p11	unknown	The Molecular Biology of Hearing and Deafness meeting Bethesda, October 8-11, 1998 (Green *et al.*, abstract 108).
DFNB20	11q25-qter	unknown	Moynihan *et al.*, 1999.
DFNB21	11q	TECTA	Mustapha *et al.*, 1999.
DFNB22	16p12.2	OTOA	Zwaenepoel *et al.*, 2002.
DFNB23	10p11.2-q21	PCDH15	Ahmed *et al.*, 2003.
DFNB24	11q23	RDX	Khan *et al.*, 2007.
DFNB25	4p13	GRXCR1	Schraders *et al.*, 2010.
DFNB26	4q31	unknown	Riazuddin *et al.*, 2000.
DFNB27	2q23-q31	unknown	Pulleyn *et al.*, 2000.
DFNB28	22q13	TRIOBP	Walsh *et al.*, 2000 ; Shahin *et al.*, 2006 ; Riazuddin *et al.*, 2006.
DFNB29	21q22	CLDN14	Wilcox *et al.*, 2001.
DFNB30	10p11.1	MYO3A	Walsh *et al.*, 2002.
DFNB31	9q32-q34	WHRN	Mustapha *et al.*, 2002 ; Mburu *et al.*, 2003.
DFNB32	1p13.3-22.1	GPSM2	Masmoudi *et al.*, 2003 ; Walsh *et al.*, 2010.
DFNB33	9q34.3	unknown	Medlej-Hashim *et al.*, 2002.
DFNB35	14q24.1-24.3	ESRRB	Ansar *et al.*, 2003 ; Collin *et al.*, 2008.
DFNB36	1p36.3	ESPN	Naz *et al.*, 2004.

DFNB37	6q13	MYO6	Ahmed *et al.*, 2003.
DFNB38	6q26-q27	unknown	Ansar *et al.*, 2003.
DFNB39	7q21.1	HGF	Schultz *et al.*, 2009.
DFNB40	22q	unknown	Delmaghani *et al.*, 2003.
DFNB42	3q13.31-q22.3	ILDR1	Aslam *et al.*, 2005. Borck *et al.*, 2011.
DFNB44	7p14.1-q11.22	unknown	Ansar *et al.*, 2004..
DFNB45	1q43-q44	unknown	Bhatti *et al.*, 2008.
DFNB46	18p11.32-p11.31	unknown	Mir *et al.*, 2005.
DFNB47	2p25.1-p24.3	unknown	Hassan *et al.*, 2005.
DFNB48	15q23-q25.1	unknown	Ahmad *et al.*, 2005.
DFNB49	5q12.3-q14.1.	MARVELD2	Ramzan *et al.*, 2004 ; Riazuddin *et al.*, 2006.
DFNB51	11p13-p12	unknown	Shaikh *et al.*, 2005.
DFNB53	6p21.3	COL11A2	Chen *et al.*, 2005.
DFNB55	4q12-q13.2	unknown	Irshad *et al.*, 2005.
DFNB59	2q31.1-q31.3	PJVK	Delmaghani *et al.*, 2006.
DFNB61	7q22.1	SLC26A5	Liu *et al.*, 2003.
DFNB62	12p13.2-p11.23	unknown	Ali *et al.*, 2006.
DFNB63	11q13.2-q13.4	LRTOMT/COMT2	Du *et al.*, 2008 ; Ahmed *et al.*, 2008.
DFNB65	20q13.2-q13.32	unknown	Tariq *et al.*, 2006.

DFNB66	6p21.2-22.3	LHFPL5	Tlili *et al.*, 2005 ; Shabbir *et al.*, 2006 ; Kalay *et al.*, 2006.
DFNB67	See DFNB66		
DFNB68	19p13.2	unknown	Santos *et al.*, 2006.
DFNB71	8p22-21.3	unknown	Chishti *et al.*, 2009.
DFNB72	19p13.3	GIPC3	Ain *et al.*, 2007 ; Rehman *et al.*, 2011.
DFNB73	1p32.3	BSND	Riazuddin *et al.*, 2009.
DFNB74	12q14.2-q15	MSRB3	Waryah *et al.*, 2009 ; Ahmed *et al.*, 2011.
DFNB77	18q12-q21	LOXHD1	Grillet *et al.*, 2009.
DFNB79	9q34.3	TPRN	Rehman *et al.*, 2010.
DFNB81	19p	unknown	Rehman *et al.*, 2011.
DFNB82	see DFNB32		
DFNB83	see DFNA47		
DFNB84	12q21.2	PTPRQ	Schraders er al., 2010.
DFNB85	17p12-q11.2	unknown	Shahin *et al.*, 2010.
DFNB91	6p25	SERPINB6	Sirmaci *et al.*, 2010.
DFNB93	11q12.3-11q13.2	unknown	Tabatabaiefar *et al.*, 2011.
DFNB95	19p13	GIPC3	Charizopoulou *et al.*, 2011.

Table 2.2: Reported Autosomal recessive loci (Hereditary hearing loss homepage).

2.4.6. Reported none syndromic Autosomal recessive deafness genes 2011:

Locus (OMIM).	Gene (OMIM).	Reference (OMIM).
DFNB1A	GJB2	Kelsell *et al.*, 1997.
DFNB1B	GJB6	Del Castillo *et al.*, 2002.
DFNB2	MYO7A	Liu *et al.*, 1997 ; Weil *et al.*, 1997.
DFNB3	MYO15A	Wang *et al.*, 1998.
DFNB4	SLC26A4	Li *et al.*, 1998.
DFNB6	TMIE	Naz *et al.*, 2002.
DFNB7/11	TMC1	Kurima *et al.*, 2002.
DFNB8/ 10	TMPRSS3	Scott *et al.*, 2001.
DFNB9	OTOF	Yasunaga *et al.*, 1999.
DFNB12	CDH23	Bork *et al.*, 2001.
DFNB15/72/95	GIPC3	Ain *et al.*, 2007 ; Rehman *et al.*, 2011.
DFNB16	STRC	Verpy *et al.*, 2001.
DFNB18	USH1C	Ouyang *et al.*, 2002 ; Ahmed *et al.*, 2002.
DFNB21	TECTA	Mustapha *et al.*, 1999.
DFNB22	OTOA	Zwaenepoel *et al.*, 2002.
DFNB23	PCDH15	Ahmed *et al.*, 2003.
DFNB24	RDX	Khan *et al.*, 2007.
DFNB25	GRXCR1	Schraders *et al.*, 2010.
DFNB28	TRIOBP	Shahin *et al.*, 2006 ; Riazuddin *et al.*, 2006.
DFNB29	CLDN14	Wilcox *et al.*, 2001.
DFNB30	MYO3A	Walsh *et al.*, 2002.
DFNB31	WHRN	Mburu *et al.*, 2003.
DFNB35	ESRRB	Collin *et al.*, 2008.
DFNB36	ESPN	Naz *et al.*, 2004.
DFNB37	MYO6	Ahmed *et al.*, 2003.
DFNB39	HGF	Schultz *et al.*, 2009.

DFNB42	ILDR1	Borck *et al.*, 2011.
DFNB49	MARVELD2	Riazuddin *et al.*, 2006.
DFNB53	COL11A2	Chen *et al.*, 2005.
DFNB59	PJVK	Delmaghani *et al.*, 2006.
DFNB61	SLC26A5	Liu *et al.*, 2003.
DFNB63	LRTOMT/COMT2	Ahmed *et al.*, 2008 ; Du *et al.*, 2008.
DFNB66/67	LHFPL5	Tlili *et al.*, 2005 ; Shabbir *et al.*, 2006.
DFNB74	MSRB3	Waryah *et al.*, 2009 ; Ahmed *et al.*, 2011.
DFNB77	LOXHD1	Grillet *et al.*, 2009.
DFNB79	TPRN	Rehman *et al.*, 2010 ; Li *et al.*, 2010.
DFNB82	GPSM2	Walsh *et al.*, 2010.
DFNB84	PTPRQ	Schraders *et al.*, 2010.
DFNB91	GJB3	Liu *et al.*, 2000.

Table 2.3: Reported deafness genes hereditary hearing loss home page 2011

Many of these deafness loci are mapped in families or in endogamous populations. (Friedman and Griffith 2003). Almost all recessive loci range from severe to profound hearing loss, which is prelingual (congenital) by nature. However mostly those population are targeted for these studies who have got profound hearing loss in recessive mode. While in some cases only one kindred is described so the range of phenotypic expression may be unknown.

2.5. Prevalent of Autosomal Recessive Loci/Genes in Pakistan population:

According to data available, twenty four non syndromic Loci and fifteen genes have been reported to date.

DFNB1:

For the first time a locus of non syndromic Autosomal recessive was reported in Tunisian families affected with prelingual deafness. This was mapped to 13q12 of the chromosome (Guilford *et al.*, 1994). In 1997 from a study in three Pakistani families two distinct non sense mutations were identified and was demonstrated that Cx26 is the causative gene (Kelsell *et al.*, 1997). Mutation at DFNB1 became more apparent from many studies conducted on families and

general population of different region of the world (Carrasqollio *et al.,* 1997 and Scott *et al.,* 1998). In Cx26 gene seventy different mutations have been identified (Rabionet *et al.,* 2000).The prevalence of DFNB1 in India is 13.3% (Masheswari *at al.,* 2003), in Iran it accounts for 16.7% (Najmabadi *et al.,* 2005)., in Japan these are 20% (Kudo, *et al.,* 2000), while Pakistan has lower prevalence as compared to India (Santos *et al.,* 2005). Furthermore it was concluded that a complex DFNB1 locus mutation which have GJB2 and GJB6 genes can result in digenic or monogenic pattern of inheritance of prelingual deafness (Del Castillo *et al.,* 2002). The pathophysiology of DFNB1 reveals that it play a key role in potassium (K+). Recycling, which fasten the transport of K+ ions in staria vascularis, from where these ions are rapidly pumped to endolymph thus keeping K+/Na+ balance in endolymph (Tekin *et al.,* 2001).

DFNB2:

This was the second locus for Autosomal recessive hearing loss located at 11q13.5, in a Tunisian family (Guilfor *et al.,* 1994b). In later MYO 7A gene was identified on this locus which encodes myosin VII A. (Gibson *et al.,* 1995). Forty nine exons were mapped in two Chinese families linked to DFNB2 (Liu *et al.,* 1997). Approximately hundred alleles have been described showing mutations of different types at different region (Ouyang *et al.,* 2005). The encoded protein Myosin VIIA is involved in activating filaments using activating ATPase. Myosin VII A is expressed in many tissues but most commonly at sensory cilia and is present in the entire length of stereocilia of inner hair cells (Hasson *et al.,* 1995). Myosin VIIA is also found active at opsin transport (Liu *et al.,* 1999). These proteins also roles in transduction channel adaptation of inner hair cells of the ear and these are also involved in resting tension in the gate spring of transduction channel.

DFNB3:

In a population study at Benkala, DFNB3 was identified on chromosome 17 q 11.2 (Friedman *et al.,* 1995). While familial linkages were observed and missense and non sense mutations were mapped (Wang *et al.,* 1998). MYO15A is the largest among several splice site isoform. This has sixty five exons and encodes Myosin XVa. These proteins play a role in the formation of sterocilia, especially elongation of stereocilia-bundle stair case (Belyanfeseva *et al.,* 2003). In the

latest reports it is shown that Belyantsev and co-workers have transported whirlin to the tips of stereocilia by Myosin XVa in the hair cells (Belyantseva *et al.*, 2005).

DFNB4:

SLC26A4 Gene was mapped on this Locus having 21 exons, mutation in this gene leads to Autosomal recessive deafness with goiter termed as pendred syndrome and non syndromic hearing loss DFNB4. SLC26A4 is located on chromosome 7q22-31.1 (Everett *et al.*, 1997). It is estimated that 10% of the deaf patients are due to mutation at this gene in south and East Asian. Each ethnic population has a different and a diverse mutant allele series, with one or more founder mutations (Everett *et al.*, 1997, Li *et al.*, 1998). SLC26A4 codes for pendrin which is an 86-KDa polypeptide chain, expressed in thyroid and kidney as well as in cochlea (Everett *et al.*, 1997). Pendrin have nine membrane spanning domains and are multipass trans-Membrane protein. But its topology is not been determined experimentally. Pendred syndrome and non syndromic DFNB4 deafness are caused due to two different mutations at SLC26A4 gene (Scott *et al.*, 2000). They also demonstrated that complete loss of pendirn leads to pendred syndrome, in which chloride and iodide transport is entirely blocked while alleles unique to people with DFNB4 are able to transport iodide and chloride but at a very low level as compare to normal function (Scott *et al.*, 2000).

DFNB6:

For the first time this locus was identified in a consanguineous family of India at chromosome 3 q 21 (Fukushima *et al.*, 1995). Because of chromosomal homology with the linked region, the mouse mutant spinner is a candidate for DFNB6 (Naz *et al.*, 2002). They also cloned the human TMIE ortholog and found five different types of mutations in DFNB6 locus. The data about TMIE as RNA and protein is not yet available from which it is not clear to demonstrate it function in deafness.

DFNB7/11:

DFNB7/11 was mapped to chromosome 9q 13 in two consanguineous families of India (Scott *et al.*, 1996). Trans-membrane channel like gene I (TMC1) were identified in eleven families segregating DFNB 7/11 deafness from Pakistan and India (Jain *et al.*, 1995). The actual function

of TMC1 is still not clear but is predicted to encode a multipass Trans-membrane protein most precisely involved in ion exchange. But in mouse TMC1 mutation were found to be involved recessive deafness segregating hearing loss and postnatal hair cells degeneration so concluded that TMC1 are involved in maintain hair cells or hair cells development (Kurima *et al.*, 2002). In a recent study TMC1 was sequenced in a Sudanese family which showed mutation at 1165c>7 in exon 13 leading to stop codon, Arg 389 X and splice site mutation 19+5G>A.

DFNB8/B10:

These loci were identified in a Palestinian family (DFNB10). Also DFNB8 in a Pakistani consanguineous family on the chromosome 21 q 22.3 (Veske *et al.*, 1996). Bonne – Tamir *et al.*, 1996). These genes encode serine protease showed by a study conducted on sequencing of this locus. Serin protease, TMPRSS3 is a only protease found to be involved in hearing loss with no syndrome this gene is present on chromosome 21 having 24 kb size. Thirteen exons are reported on TMPRSS3 gene. (Scott *et al.*, 2003). Four transcripts of this gene re reported in human, (TMPRSS3 a, b, c and d) encoding polypeptides of 454,327, 327 and 344 amino acids respectively (Scott *et al.*, 2001). But recently a fifth transcript which encodes a 538 amino acid polypeptide is reported by Ahmed *et al.*, 2004. In vivo no active function of TMPRSS3 is observed but in vitro it regulate the activity of epithetical Amiloride sensitive channel (ENaC)., which regulate and control signal pathways in the inner ear and may also control sodium level of endolymph (Guipponi *et al.*, 2002).

DFNB12:

This locus was mapped on chromosome 10 q21-q22 in a consanguineous family of Syria which causes non syndromic recessive deafness (Chaib *et al.*, 1996). CDH23 gene is localized on this locus encoding cadherin 23 proteins (Bork *et al.*, 2001). Substitution of some amino acids due to mutation in CDH23 leads to leaking or hypomorphs cadherin 23 causing loss function and non syndromic deafness is established. While null alleles of CDH23 cause vestibular dysfunction as well deafness (Astuto *et al.*, 2002). Cadherin 23 is membrane of cadherin superfamily of integral membrane protein (Jamura and Fuchs 2002). Stereacilia is interconnected into bundles through homophile Interaction of this protein, so it is thought that steraocilia bundles are normally kept organized through CDH23.

DFNB18:

This Locus was mapped in a consanguineous family of india at chromosome 11P 15.1 (Jain *et al.,* 1998). In this family mutation analysis showed defect in harmonin, exon 12 in the result which affects a stop codon region in exon 13 which show it effects both in ear and retina. The splice site mutation is also reported (216 G>A) at exon 3.

DFNB21:

This locus was reported in a Lebanese family on chromosome 11q23-25 TECTA gene is located mutation at TECTA leads to encode D-Tectorin leading to Autosomal non syndromic deafness D-tectorin are non collageneous glycoprotein components of the tectorial membrane, which is an extracellular matrix that overlies the stereocillia of the outer hair cells in the organ of corti a null allele of TECTA at DFNB 21 leads to prelingual non syndromic recessive hearing loss (Naz *et al.,* 2003), While missense mutation in TECTA leads to dominant hearing loss with various other syndromes depending upon type of amino acid mutated (Alloisio *et al.,* 1999).

DFNB23:

DFNB 23 locus is mapped in chromosome 10q 21-22 (Camp and smith 2002). St this locus PCDH15 gene was reported so mutation at PCDH15 showed recessive hearing loss in three families of Pakistan (Ahmed *et al.,* 2003). While serve mutation leads to USHIF (Ahmed *et al.,* 2003). PCDH 15 is a member of cadherin molecules (Alagramam *et al.,* 2001). Alteration in this genes/ proteins shows effects in entire stereocilia enlargement (Ahmed *et al.,* 2003).

DFNB26:

DFNB 26 was reported in a consanguineous Pakistan family with non syndromic deafness at chromosome 4q31. The family defining DFNB26 is unique as a dominant modifier.(Riazuddin *et al.,* 2000)

DFNB29:

DFNB 29 was mapped on chromosome 21 q 22 mutation its gene encodes tight junction Claudine 14 which causes Autosomal recessive hearing loss (Wilcox *et al.,* 2001). Mutation in this gene has same phenotype in mouse and human. These gene with null mutation cause

profound hearing loss without effecting the normal vestibular function (Ben-Yousef *et al.*, 2003). mutation in CLDN14 are a relatively infrequent cause of non syndromic recessive deafness in the Pakistani population while the contribution of CLDN14 mutation to recessive deafness in other population is unknown and may significantly differ from Pakistani population.

DFNB36:

This locus for the first was identified in two consanguineous families on chromosome 1p36.3 (Naz *et al.*, 2004). ESPN gene is present at this locus been reported to be involved in recessive hearing loss. Two mutation at ESPN 2469 del GTCA and 1988 del AGAG have been mapped these encodes protein called ESPN (Naz *et al.*, 2004). Donauding *et al.*, 2005 has demonstrated that dominant mutation at ESPN also leads to dominant haring loss.

DFNB37:

This locus was identified in a large consanguineous family of Pakistan at chromosome 6q13 (Ahmed *et al.*, 2003). Avraham *et al.*, 1995 reported two null mutations of MY06 in mouse. These mutations cause dominant, progressive, non syndromic hearing loss in a family (Melchionda *et al.*, 2001). Mutation analysisof My06 gene have shown three types of mutations, a single base pair insertion (36-37 ins T), a transversion mutation (647A>T) and a transition mutation (647A>T) (Ahmed *et al.*, 2003).

DFNB39:

Wajid *et al.*, reported this locus in 2003, on chromosome 7q.11.22-q21 in a consanguineous family of Pakistan.

DFNB48:

In 2005 Ahmad *et al.*, Published their novel locus which was identified in five Pakistani families, on chromosome 15q 23-q25.

DFNB49:

Ramzan *et al.*, 2004 reported DFNB 49 as a novel locus on chromosome 5q 12.3-14.1 in two consanguineous Pakistani families.

DFNB51:

A novel Autosomal non syndromic deafness locus DFNB51 was identified on chromosome 11p 13-p12 in two families from Pakistan (Sheikh *et al.*, 2005).

DFNB67:

This locus was identified and mapped on chromosome 6p21.1-p22.3. TMHS gene was found which causes DFNB67 deafness (Shabbir *et al.*, 2006)

DFNB68:

Santos *et al*, demonstrated DFNB68 as novel mutation on chromosome 19P13.2 in two unrelated consanguineous families of Pakistan in 2006.

DFNB72:

Three Pakistani consanguineous families were studied and DFNB72 was mapped on chromosome 19P 13.3 by (Ain *et al.*, 2007).

2.6. Basis of Linkage Analysis:-

The human genome is very complex and large, containing thousands of genes organized in the form of chromosome. There are 46 (23 pairs) of chromosome in a normal human. So to find out a disease gene in such a noisy environment is a tricky and daunting task. Its is just like to find a coin in a lake. The searching of a particular genes starts with linkage analysis. It is a technique for developing a relationship between the loci. i.e. if two loci are present on a same chromosome, these are said to be linked, excluding the condition of crossing over which separate them. Crossing over occurs during meiosis when the chromosomes do exchange of genetic segments. When tow loci are naturally present on the tow homologous chromosome, it is said to be original arrangement or parental combination while the new combination which is produced due to crossing over is termed as recombination. The recombination or crossing over separates the two closed loci very rarely. Set of alleles for different genes or markers of the same chromosome are called haplotypes. So alleles on the same haplotype are passed in pedigree as a block, which are

broken only by the process of recombination. More over the word linkage refers to the loci not to specific alleles at these loci. The linkage analysis is a powerful classical approach used to find the location of the disease genes on the chromosome.

2.7. Recombination Fraction reflects Genetic distance:-

Alleles at Loci on the same chromosome for different genes co-segregate at a rate that is associated to a physical distance between them on the chromosome this rate is a recombination fraction of a crossing over event occurring between two loci. When the recombination fraction θ is less than 0.5 between two loci then these are said to linked one of these loci is a distance locus while the other is a polymorphic marker i.e. microsatellite repeats (Strachan *et al.*, 1996). The recombination fraction is said to be $\theta = 0$ is the loci are right closed to each other while the loci present apart or on different chromosome then ranges as $\theta = 0.5$. So this distance measurement is beneficial to measure the map distance between the loci of a gene. Two loci who have got 1% recombination are defined as being 1 centi Morgan (cM), far away on the genetic map and 1 cM represents 0.9 Mbp on the averaged physical map (Foroud *et al.*, 1997).

2.8. LOD Score Calculation:

Logarithm of the odd (LOD) is used in the calculation of a parametric linkage analysis. The score provides the strength of evidence in favor of linkage.

$$\text{Lod Score} = \frac{\text{Log 10x Probability of the date if disease and marker are linked}}{\text{Probability of the data if marker and disease are unlinked}}$$

CHAPTER 3
MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Family Description:

For the current study three families suffering with congenital deafness belong to different regions of Mardan, Khyber Pakhtunkhwa (KPK) were selected. The information of these families was obtained from special children school Mardan and all the families were categorized by providing different numbers to each family to make the study more precise. The numbers were allotted to these families as DFN10, DFN13 and DFN17 respectively. The enrolled families belong to different castes. All members of the affected families were found to be available at their villages at the time of sampling. The selection was based on affected members in each family, willingness and availability of the families. All the families were observed to be with none-syndromic hearing loss impairment.

3.2. Pedigree Drawing:

To study human genetic disease it is an important step to make pedigree of the affected families. This provides full information about the family and pattern of inheritance of the disease. For this purpose head members of each family were contacted to get full and accurate details. A Performa was designed to get preliminary information like names of normal and abnormal person, postal address and contact number. The head members were also given the information about the number of affected persons, associated defects, onset age of the disease, number of generations affected, consanguineous marriages and both normal and abnormal dead persons were also recorded. Information regarding to each affected individual like ages, level of deafness and association of other diseases was also collected. For making pedigrees, the method described by Benett *et el.,* 1995, was followed in which males are denoted by squares and female are shown by circles. Affected male and female are represented by shaded squares and circles while normal individuals with non shaded circles and squares respectively. Pedigrees were drawn by using Cyrillic software.

3.3. Identity (id) Numbers Allotment:

Three families were given their numbers as DFN10,DFN13 and DFN17.Moreover each normal and affected individual whose sample was collected in each family was also given his ID. The

member of the first family were 10-DFN-01, 10-DFN-02, 10-DFN-03,10-DFN-04,10-DFN-05,10-DFN-06,10-DFN-07,10-DFN-08 and 10-DFN09. In this family blood was collected from four normal and five patients. Similarly the members of the second family were 13-DFN-01, 13-DFN-02, 13-DFN-03,13-DFN-04,13-DFN-05,13-DFN-06,13-DFN-07 and 13-DFN-08, in which five normal persons were collected and three patients with hearing loss. The members of the third family were 17-DFN-01, 17-DFN-02, 17-DFN-03,17-DFN-04,17-DFN-05,17-DFN-06,17-DFN-07,17-DFN-08 and 17-DFN-09. In which blood samples were collected from five normal and four abnormal individuals.

3.4. Blood Sampling:

Blood (5ml) was collected from the patient's radial vein by using disposable Syringe under strict aseptic conditions and was transferred to ACD (acid citrate dextrose) vacutainer tubes. Each tube was labeled by using the ID given to each individual. The tubes were kept at 4°C, until the DNA extraction was started.

3.5. DNA extraction from blood:

DNA was prepared by following the modern organic extraction method as described by Maniatis et al., 1982.Three days were given to each batch (20 samples) of blood samples to get reliable results and large amount of pure DNA.

Day 1:

All the samples were arranged in order according to their ID, s. Total blood sample were transferred to labeled (50 ml) falcon tubes and quantity of each sample was recorded.

Cell lysis buffer:

Cell lysis buffer was added to each sample to lysis red blood cell and remove hemoglobin. Cell lysis buffer was added three times of the blood samples i.e. in 5ml blood 15 ml of cell lysis buffer was added. After addition of cell lysis buffer all tubes were kept on ice for 30 minutes to stop metabolic activities of the cells. Then the samples were centrifuged at 1200 rpm for 10 minutes at 4°C, the supernatant was discarded and pallets were re-suspended. The pallets were observed redish, so each sample was again washed with 15 ml of cell lysis buffer in the same

manner as mentioned above to entirely remove the hemoglobin and lysis the remaining cells. The samples were centrifuged at 1200 rpm for 10 minutes at 4°C. Supernatant was discarder and pallets were resuspended in tubes.

STE (Saline Tris EDTA):

4.75 ml of STE was added to each sample.

Then 250 µl of 10% Sodium deodosyle sulphate (SDS) was added while vertexing the sample.

And 10 µl protienase K was added to each sample.

All the samples were kept at 55°C in water bath for overnight.

- *Cell lysis buffer preparation:*

One liter of cell lysis buffer was prepared by weighting 8.29 gm of ammonium chloride (NH_4CL) and 2 gm of potassium carbonate ($KHCO_3$) was added to a graduated beaker. De-ionized water was added and the volume was raised to 800 ml. Total 200 µl of 0.5M ethylene diamine tetra acetic acid (EDTA) was added to the beaker and the whole solution was dissolved by using magnetic stirrer until the reagents were fully dissolved and particles were disappeared. The volume of solution was raised up to 1000 ml by adding deionized water. Its pH was recorded and was brought to pH 7.4 by the addition of acids and basis as required. Cell lysis buffer was stored in 1 liter bottle at 4°C.

- *STE preparation:*

One liter of STE was prepared by adding 33.3ml of 3M NaCl,100 ml of 1M Tric-Hcl and 2 ml of 0.5M EDTA to a beaker and were dissolved in 1000ml of dH_2O. Its pH was adjusted on 8.0. The prepared solution was filtered with 0.4µl filter paper and stored at 4°C.

Day 2:

Equilibrated phenol:

5ml equilibrated phenol was added to each sample. The samples were shacked slowly for 10 minutes and were kept in ice or freezer for 10 minutes. Then samples were centrifuged at 3200

rpm for 30 minutes at 4°C. Aquouse phase was transferred to labeled (15ml) falcon tubes with cut tips method and 500µl of 10M amonium acetate followed by 5ml chilled iso-propanol was added. The tubes were shaked until the pallets appeared in whitish cluster form. All the samples were stored for overnight at -2°C.

- *Equilibration of phenol:*

1 kg phenol was melted with the addition of 1gm 8-hydroxy quinoline and then was extracted with equal volume of 1M Tris (1L, pH-8.0). Extraction was revised with an equal volume of 0.1M Tric until the pH of the aqueous layer was reached to 8.0.Finaly 100ml of 0.1M Tris containing 0.2% β-macaptoethanol (2ml) was added and stored at -4°C.

Day 3:

The stored samples were centrifuged at 3200 rpm for 1 hour at 4°C and supernatant was discarded while pallets were resuspended. After that 5ml of chilled 70% ethanol was added to wash the samples and again was centrifuged at 3200rpm for 40 minutes at 4°C. The supernatant were discarded and DNA was dried until the last drop disappeared. After drying the pallets 10mM Tris Hcl having pH 8.0 were added to all samples according to the size of pallets.

DNA Storage under proper temperature:

The Tris-Hcl containing DNA was kept at -4°C for overnight and was taken into water bath to provide suitable temperature (55°C) to DNA to expand freely. Then whole solution was transferred to (1.5ml) tubes and were labeled as stock solution

Dilution preparations:

20µl of each DNA was added to 80 µl dH2O to make 20% DNA dilution as working solution in a labeled (1.5ml) tubes.

3.6. Linkage Analysis by using Polymerase chain reaction (PCR)

3.6.1. Exclusion of known loci:

All the samples (each 1.5µl) in diluted form were added to the labeled PCR tubes. Master Mix according to the number of microsatellite marker to be used was prepared. i.e. daily four markers

were used for the exclusion of known loci using twenty seven DNA samples of three families, so master mix was prepared in four separate labeled eppandorf tubes (0.5µl) adding separate marker to each tube. All the contents of PCR were kept on ice tray to avoid degradation of enzymes and other contents while preparing master mix. PCR recipe was prepared by using the contents given below in table 3.1.(describes the amounts used for one marker in twenty seven samples).

Table 3.1: PCR Recipe

Reagents	Stock Conc	Required Conc	Final Conc for 1 reaction	Final Conc for 27 reactions
dH2O			4.6 µl	124.2 µl
PCR Buffer	10X	1X	1 µl	27 µl
MgCl2	25mM	1.5mM	1 µl	27 µl
dNTP's	0.8 µ/µl	0.2mM	1 µl	27 µl
Taq Polymerase	2 µ/µl	0.8 µ/µl	o.3 µl	8.1 µl
forward Primer	2O µM	600nM	0.3 µl	8.1 µl
Reverse Primer	20 µM	600nM	0.3 µl	8.1 µl

8.5 µl of PCR Master Mix was added to each sample of DNA (1.5 µl) been added to PCR tubes so total volume of DNA and Master mix was reached to 10µl.The caps were tightly closed on strips and were kept in PCR machine for 30 cycles at following temperatures.

PCR Conditions:

95°C for 5 minutes.

95°C for 1-2 minutes for 30 cycles.

54-57°C for 2 minutes.

72°C for 1 minutes and final extension at 72°C for 10 minutes.

After completion of polymerase chain reaction the strips were taken out of PCR and were stored at 4°C till next process.

3.7: Polyacrylamide Gel Electrophoresis (PAGE):

- **Preparation of 40% polyacrylamide stock solution (1 liter):**

This solution was prepared with high care and safety because it contains acrylamide which is highly carsenogenic and its dust may create problems in respiration. Proper mask and gloves were ensured. The following standard ratio of acrylamide and Bis acrylamide was used.

Acrylamide	:	Bisacrylamide
39gm	:	1gm

Acrylamide (389.6 gm) was taken in a beaker and 10.4 gm bisacrylamide was added. This mixture was then dissolved with deionized water (1000 ml) until all the particles of solute disappeared by using magnetic stirrer. The prepared solution was then transferred to light tight bottles and was labeled and was stored at 4°C.

Preparation of 10X TBE (Tric. Borate. EDTA.) 1 Liter:

Tric = 108 gm

Borate= 55 gm

EDTA= 7.4 gm

These reagents were dissolved in 1000 ml deionized water and were stored in a labeled bottle.

Preparation of 17% polyacrylamide solution from 40%:

10X TBE (25ml) was added to 40% acrylamide (106 ml) and volume was raised to 250 ml by adding deionized water in a graduated cylinder. So 17% acrylamide solution was prepared. Following formula was applied for 40% acrylamide.

$$n1v1 = n2v2$$

Preparation of 25% ammonium persulfate (APS) (5 ml):

1 gm ammonium per sulfate was added to 4 ml deionized water and was dissolved by shaking. Similarly 25% APS (5 ml) was prepared.

Gel Pouring:

Procedure

The gel apparatus was assembled and pouring solution was prepared as following.

17% acrylamide solution = 250 ml

TEMED = 150 ml

Ammonium per sulfate = 850 ml

The above mentioned solutions were added to a graduated beaker and was dissolved by hand shaking and was immediately transferred to gel assembly. Care was taken to avoid bubbles formation inside the assembly to get smooth and proper gel flap. Sigma coated comb was adjusted at the top of the assembly. After the gel was solidified, preheating of the gel was done for 10 minutes and the comb was withdrawn carefully to get deep wells.

Sample loading and running:

Gel loading dye (5ml) was added to each PCR product and was dissolved with tip sucking method. Then 8 ml of each DNA sample was loaded into gel wells by using separate tips for each sample. All the samples were transferred and electric current (100 watts) was applied for a mean time depending upon the size of the product sequence.

3.8. Gel visualization:

There are a number of buffers used for electrophoresis. The most common being, for nucleic acids Tris/Acetate/EDTA (TAE),Tris/Borate/EDTA (TBE). After the electrophoresis was completed, the molecules in the gel were stained to make them visible. DNA was visualized by using ethidium bromide which, when intercalated into DNA, make it fluorescent under ultraviolet light, By running DNA through an EtBr-treated gel and visualizing it with UV light, the entire bands, each containing more than ~20 ng DNA became distinctly visible.The image was saved for further reading and processing.

3.9. Screening of Mutation:

The family DFN13, in which linkage was observed on DFNB locus, was entitled to screen mutated exon by using exon specific primers at the candidate gene (SLC26A4). For the

screening of mutation sequence analyzer was used. The following procedure was done for PCR product purification and direct sequencing.

The PCR products were purified by taking 25µl PCR product, 2.5 µl ammonium acetate (10M) was added to the sample and then 50 µl chilled absolute ethanol was added, the solution was mixed and was kept at -4°C for 20 minutes. Then the solution was centrifuged at 14000 rpm for 10 minutes, the supernatant was discarded and washing of palett was done with 100 µl of 70% chilled ethanol, this solution was then slowly vertex and again spinning was done at 14000 rpm for 10 minutes finally resuspension of the pallet was done with 15 µl deionized water. Then water was discarded and tubes containing pallet was dried in a oven at 55°C.then the dispersed pallet was centrifuged to settle down at high rpm.50 µl sample was added to PCR tubes. The following master mix as given in table 3.2 was prepared for amplification of exon with its primer (as shown in table 3.3).

Table 3.2.: Master Mix recipe for amplification of exon by a specific primer.

S:NO	Reagents	For 01 Reaction
01	PCR product	3 µl
02	primers	1 µl
03	Big dye	4 µl
04	Deionized Water	2 µl

PCR cycles

96 °C for 10 second

96 °C for 5 second

60 °C for 4 minutes

Supplementary table 3.3.: *Sequences of the primers for quantitative PCR of SLC26A4 (21exons)*

S.NO	Exons	Sequences 5-3 (Forward/Reverse)	Product size bp
1	Exon 1	F-CCCTTCGACCAAGGTGTCTGT R-ACCCTTCCGCTGCCTTTATAG	80
2	Exon2	F-TCTTCCCCTCCGATCGTCCT R-CCTCCCCAAGGCGTGGAC	292
3	Exon3	F-AAGAGAGCCTTTGGTGTGCTAAA R-TCACTAAGCAGCCATTCCTTGA	85
4	Exon4	F-CATATGCCCTACTAGCTGCAGTTC R-GATGTTCCAAAGATAAAGTATGTCAGGAT	87
5	Exon5	F-GGTGAGTTTAATGGTGGGATCTG R-GCTGCTGGATACGAGAAAGTGTT	69
6	Exon6	F-GTGGCTTGCAGATTGGATTCAT R-TGGAAGGCAGCAGCTGTTG	78
7	Exon7	F-GCGTGTAGCAGCAGGAAGTA R-CCTTGTTTGTCAACCAAATAATG	250
8	Exon8	F-GAAAGTTCAGCATTATTTGGTTG R-GGAGTATCAGTGAAATGAAGCTTG	257
9	Exon9	F-TCACTAGGTTTTTGCCTCCTGAA R-CAGCGATGGAAAATGATGCA	77
10	Exon10	F-CAGTCTCTTCCTTAGGAATTCATTGC R-GGCCGTGCGGGAAAG	98
11	Exon11	F-GACACAAGGGAGAAGGACGA R-AGGGAATGGTTTTCCATGTG	236
12	Exon12	F-AACAATCATCACATGGAAAACC R-TTATTTCAGATAAATATAGGC	193
13	Exon13	F-TTTTCCCTAGGTTATCTGGGTGTTT R-AACTCTCAGGACCACAGTCAACAG	111
14	Exon14	F-TCCAAAATACGGCTGTTCC R-ATGGAGCTGCTGAAACTTCAGG	187
15	Exon15	F-CCTTGCTAAGTAGCCAGAAATG R-TTGGACCCCAGTAAATACTTGT	254
16	Exon16	F-CCTTTGAGAAATAGCCTTTCCAG R-GCTCTCATCAGGGAAAGGAA	241
17	Exon17	F-AAATAATGCTTTTGAGCCTGATGA R-CCAATCCACTTGAATCTCTATTTCCT	87

18	Exon18	F-TCCTGAGCAAGTAACTGAATGC R-GAAAGGGCTTACGGGAAAGT	190
19	Exon19	F-ACGACAACATTAGAAAGGACACATTC R-ACCTTGACCCTCTTGAGATTTCAC	91
20	Exon20	F-CAGTGGAGCATCAGGTGGG R-GTTCCCTGACAGTTCTTAATCAG	246
21	Exon21	F-ACAGGCTATGCGTACACTTGCA R-CATTGAGGAAGTTTTGTCTTGTATTCC	83

Purification of PCR products for sequencing:

The PCR product was transferred to new labeled tubes, 2.5 µl EDTA (125mM) was added to it, then 30µl absolute ethanol was added and vertexing was done. The solution was kept at -4°C for 15 minutes, then its spinning was done at 13000 rpm for 20 minutes. The supernatant was discarded and pallet was vigorously vertexed after adding 100µl (70%) chilled ethanol. then its spinning was done for 10 minutes at 13000 rpm, again supernatant was discarded and pallets was dried in oven at 55°C. finally 10µl Hi Di formamide was added. the pallet was then denatured by PCR at 95°C for 4 minutes. Orange dye (250 µl) was added to sample and sequencer machine was switched on the samples were kept in tray of sequencer and software was monitored for sequencing the samples.

The two genomic substitution mutations at exon 2, one was at +2 splice site (T>G) and the second was at +5 intronic region (C>G) were detected by PCR amplification and sequencing with primers 5-TCTTCCCCTCCGATCGTCCT -3 and 5- CCTCCCCAAGGCGTGGAC -3.

CHAPTER 4

RESULTS

RESULTS

4.1. Families Studies:

In the present project three families (DFN10, DFN13 and DFN17) were selected for the study of congenital deafness from different regions of Mardan, Khyber Pakhtunkhwa Pakistan. The families were ascertained from populations, where the consanguineous marriages are very common due to socio-ethnic reason and thus are suitable for locating the defective genes by genetic linkage studies.

4.1.1. Family DFN10:

Family DFN10 was collected from rural area of KPK, Pakistan. This was a large family; traditionally they prefer to marry within the family that's why consanguineous marriages are common among them. Four individuals including three males and one female are suffering with congenital deafness. All the children have non syndromic hearing loss. This family contains several consanguineous marriage and the pedigree showed Autosomal recessive mode of inheritance. This family was given a number DFN10 due to catalogue number of the working laboratory. The patients are present only at IV generations of the pedigree. In this Generation one female (IV-12) and three males (IV-9, IV-3 and IV-07) are affected. All the effected individuals were communicated through sign to confirm hearing loss. The ages of these patients ranges from 2 years to 12 years. Other any abnormalities were not found so non-syndromic deafness was confirmed.

All the information about the family was collected from the head of the family. Blood samples of 5ml was collected from patients (IV-3, IV-7, IV-9 and IV-12) as well as from normal (III-09, III-20, III-21, IV-08 and IV-05) and were processed for genomic DNA extraction. All the patients were available at the sampling time.

4.1.2. Family DFN13:

This family was allotted a number DFN13 by the lab catalogue. This family was collected from village Thoro district Mardan, KPK Pakistan. Like family A this family was also involve in high rate of consanguineous marriages. This family contains four individuals with hereditary hearing loss, out of which one was female and two were males. One patient was not available for

sampling. In the pedigree blood samples was collected from patients (III-19, IV-02 and IV-03) and normal (III-20, III-21, III-24, IV-04 and IV-05). This disease was found in two generations III and IV of the pedigree. Patients ages rages from 5 years to 27 years. This family was also observed as non syndromic and pattern of inheritance was Autosomal recessive.

4.1.3. Family DFN17:

Family DFN17 belongs to city Mardan. This was a wide family with large number of Patients. In the pedigree many consanguineous marriages were observed. Some patients of this family were in adult age. Like already mentioned families this was also non syndromic Autosomal recessive family. Total patients were eight, out of which only six were available for the study and rest of two were not present. Blood samples were collected from patients (III-02, III-03, IV-08, IV-09, IV-10 and IV-12) and normal (III-04, III-09, III-10 and IV-11) as well this was found to be non syndromic Autosomal recessive family. Patients were found in two generations (III and IV). The ages of the effected vary from 02-years to 32-years. Member and blood was collected for genomic DNA extractions.

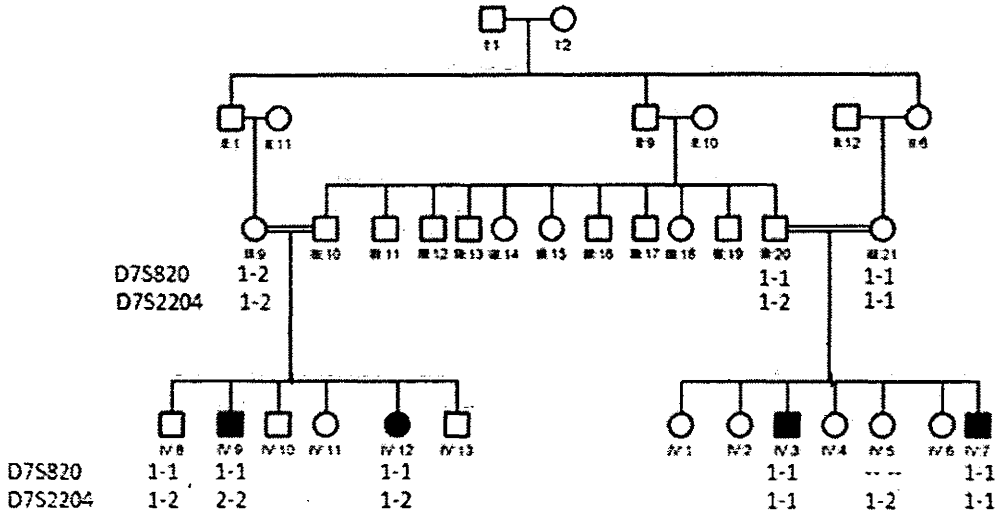
4.2. Linkage Studies:

4.2.1. Linkage to known Loci (Exclusion Study):

Genetic linkage analysis is a method that is used to associate functionality of gene to their location on chromosome. On the basis of genetic linkage studies in other forms of hereditary hearing impairment, it is clear that at least some candidate intervals should be tested for either genetic linkage or exclusion prior to embarking on genome wide search. For exclusion study several known DFNB loci were screened with the help of polymorphic microsatellite markers, which were used for exclusion mapping in the present project. Genotyping using these microsatellite markers was performed as described in material and method. The exclusion results were as follow:

DFNB39 (D7S820 AND D7S2204)

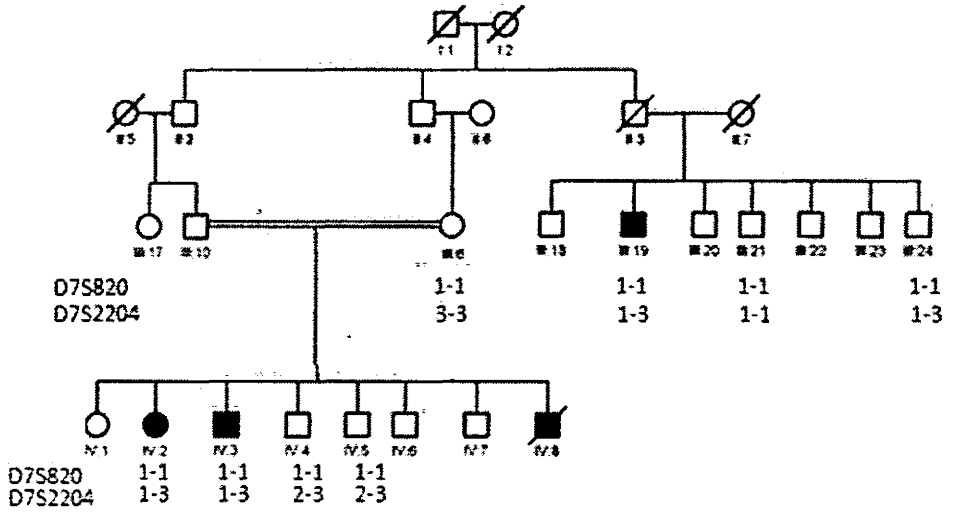
(1) Family DFN17:



The markers D7S820 and D7S2204 were applied on five normal and four effected individuals for screening DFNB39 locus to find linkage. For D7S820 among normal individuals III-09 was found heterozygous and rests of the three (III-20,III-21 and IV-08) were homozygous while IV-05 showed no amplification. Similarly among patients all (IV-09, IV-12,IV-03 and IV-07) were homozygous.

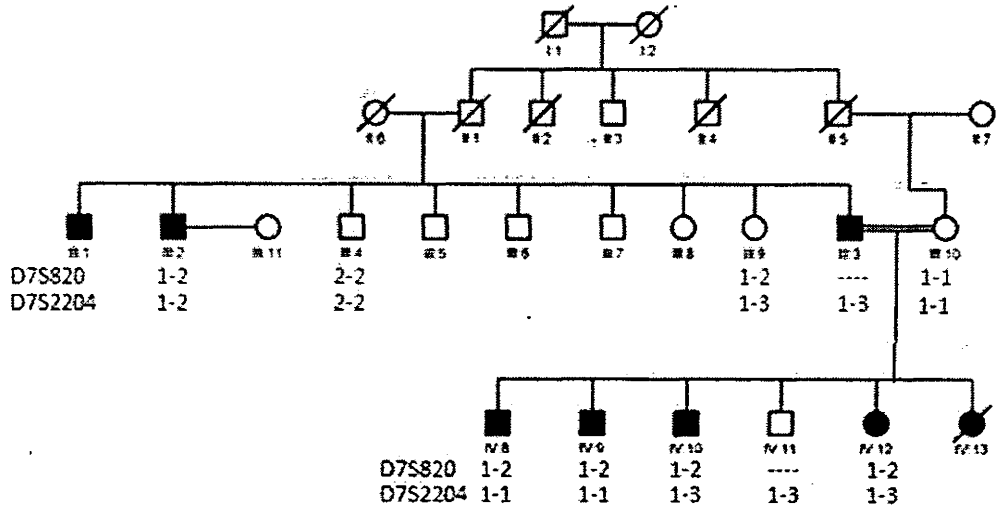
For marker D7S2204 four normal (III-09, III-20,IV-08 and IV-05) were heterozygous and one (III-21) was homozygous while only one patient (IV-12) was heterozygous and rest of the three (IV-09,IV-03,IV-07) were homozygous.

(2) Family DFN13:



By applying the same markers to family DFN13 in results all (III-24 III-19, III-06 , III-21,IV-02,IV-03,IV-04 and IV-05) the normal and patients were found to be homozygous for marker D7S820 while for marker D7S2204 among normal two(III-06 and III-21) were homozygous and three(III-24,IV-04 and IV-05) were heterozygous. All the patients (III-19,IV-02and IV-03) were heterozygous .

(3) Family DFN10:

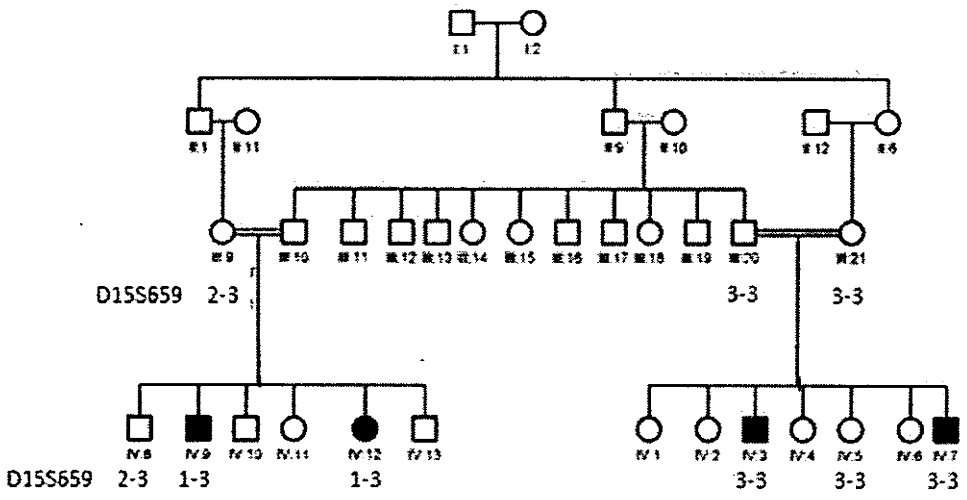


By applying marker D7S820 to family DFN10, one patients (III-03) was not amplified while rest of the five(II-02,IV-08,IV-09,IV-10 and IV-12) were heterozygous, In normal individuals two (III-04 and III-10)were homozygous, one (III-09) was heterozygous and one (IV-11) showed no result. For marker D7S2204 four patients (III-02, III-03,IV-10 and IV-12) were heterozygous and two (IV-08 and IV-09) were homozygous while among normal two (III-09 and IV-11) were heterozygous and two (III-04 and III-10) were homozygous.

Results: No linkage was observed at any family therefore these markers were excluded.

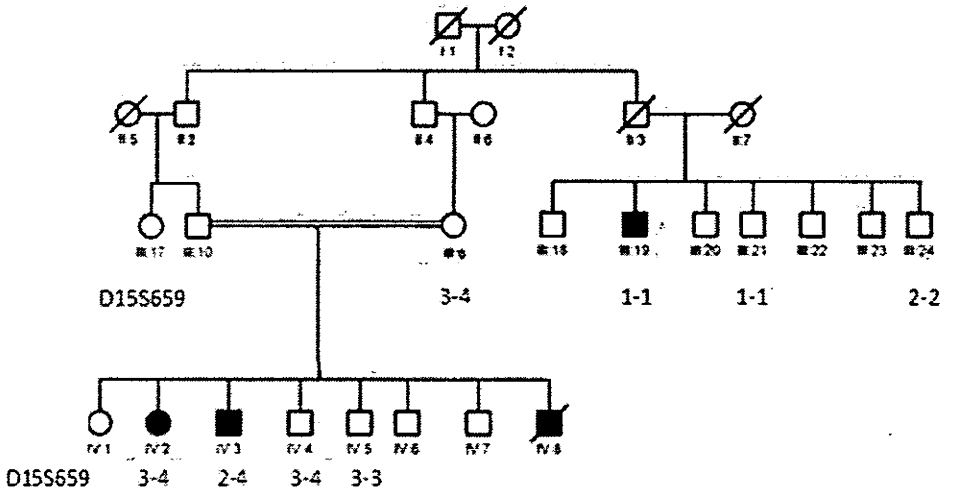
DFNB 16 (D15S659)

(1) Family DFN17:



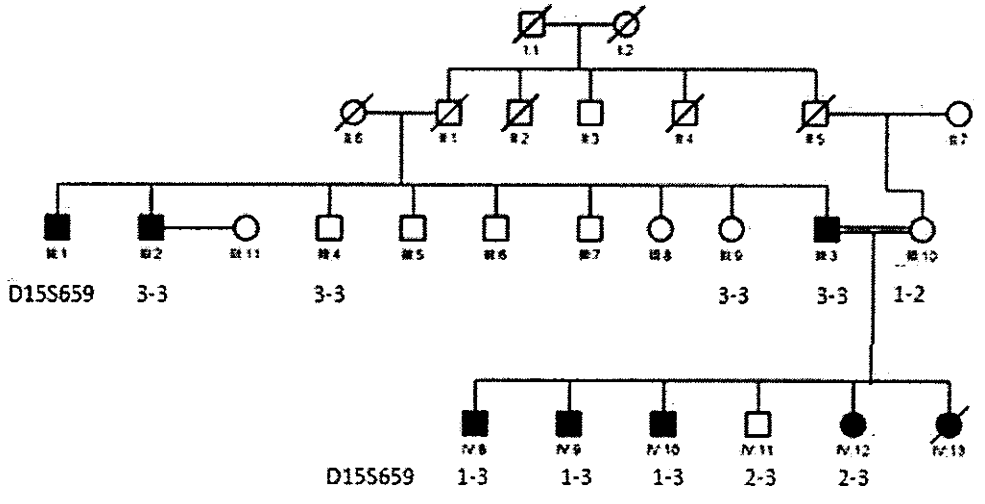
The marker D15S659 was applied on five normal and four effected individuals for screening DFNB16 locus to find linkage. Among normal individuals two (III-09 and IV-08) were found to be heterozygous and rests of the three (III-20, III-21 and IV-05) were homozygous. Similarly among patients two (IV-03 and IV-07) were homozygous and two (IV-09 and IV-12) were heterozygous.

(2) Family DFN13:



By applying the same markers to family DFN13, in results two (III-06 and IV-04) of the normal individuals were found heterozygous, three normal (III-21, III-24 and IV-05) were homozygous. Two patients (IV-02 and IV-03) were found to be heterozygous while one patient (III-19) was homozygous.

(3) Family DFN10:

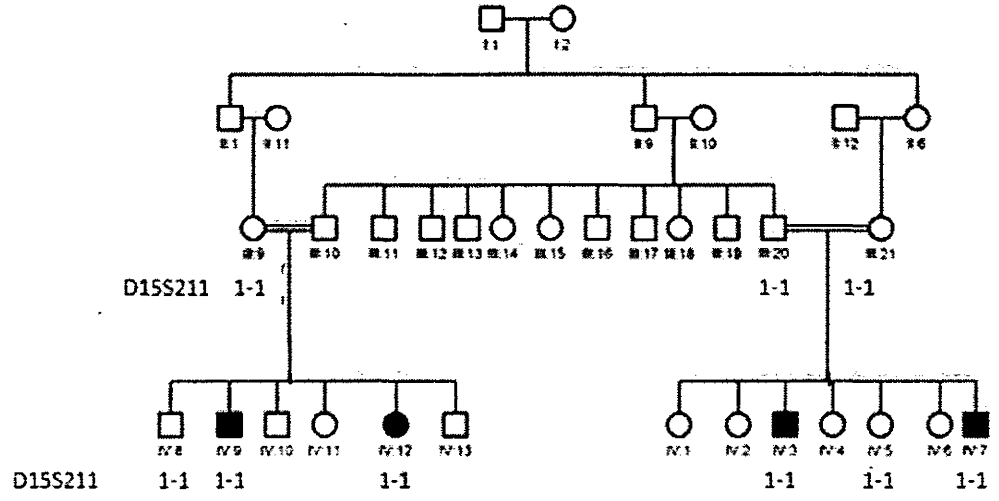


By applying marker D15S659 to family DFN10, four patients (IV-08, IV-09, IV-10 and IV-12) were heterozygous and two (III-02 and III-03) were homozygous. In normal individuals two (III-04 and III-09) were homozygous, and two (III-10 and IV-11) were heterozygous.

Results: No linkage was observed at any family; therefore this marker was excluded from the study.

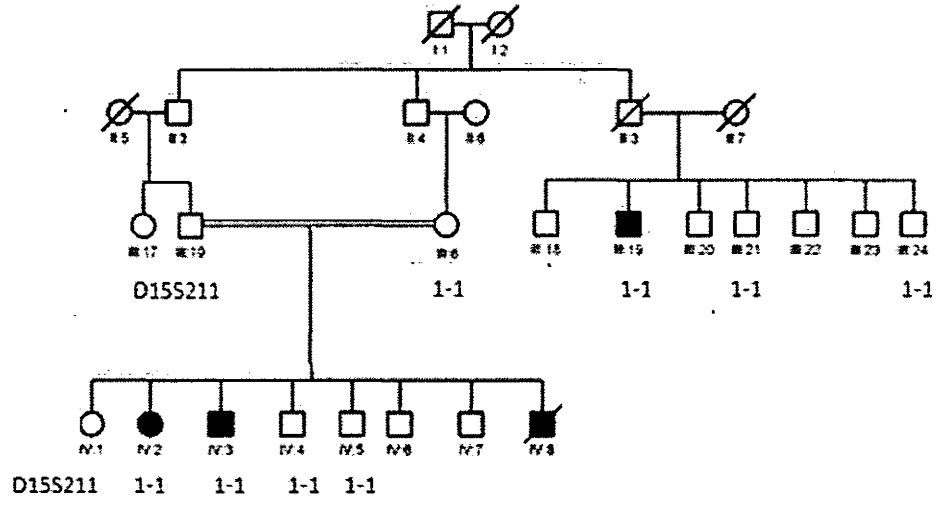
DFNB48 (D15S211)

(1) Family DFN17:



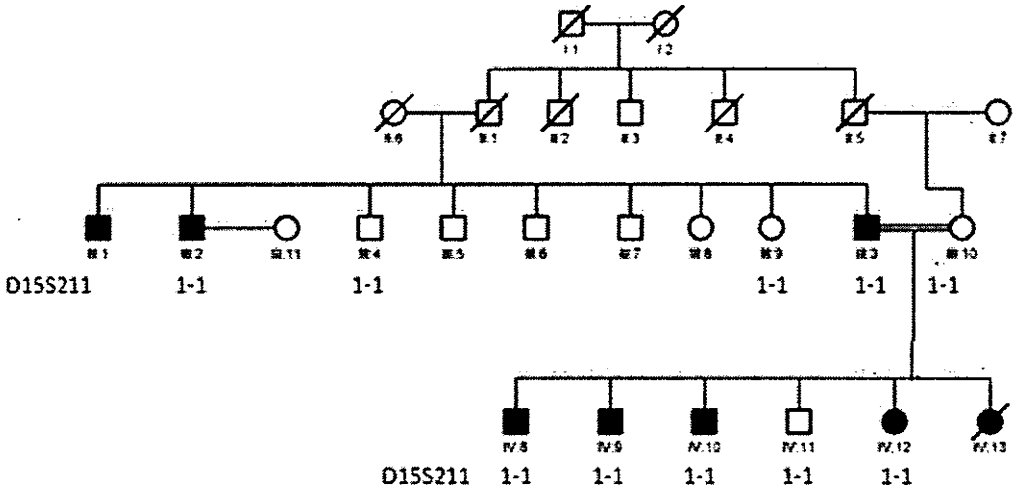
The marker D15S211 was applied on five normal and four effected individuals for screening DFNB48 locus to find linkage. All normal and patients were found homozygous.

(2) Family DFN13:



By applying the same markers to family DFN13, in results all the normal and patients were found homozygous for D15S211.

(3) Family DFN10:

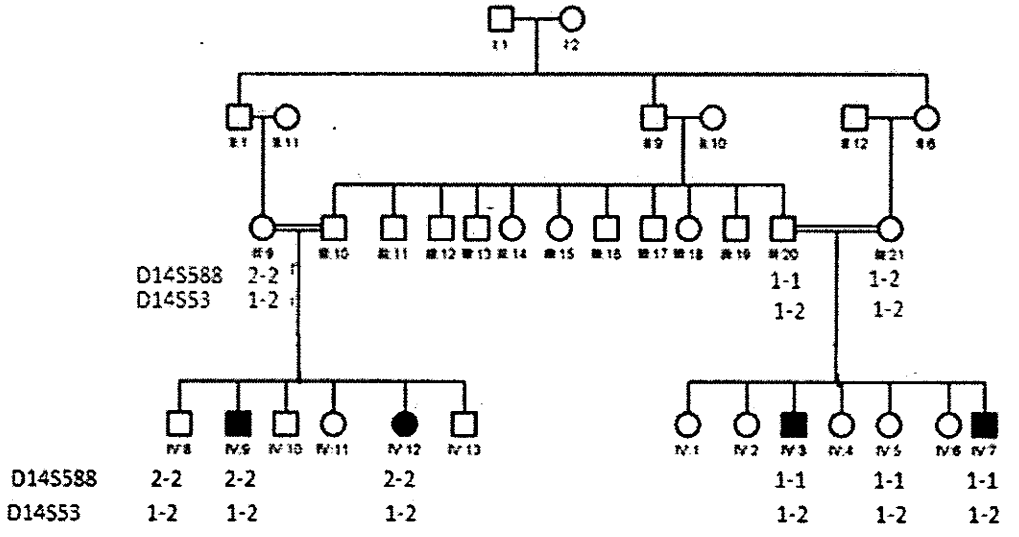


By applying marker D15S211 to family DFN10, all the individuals both normal and effected were homozygous.

Results: No linkage was observed at any family therefore this marker was excluded.

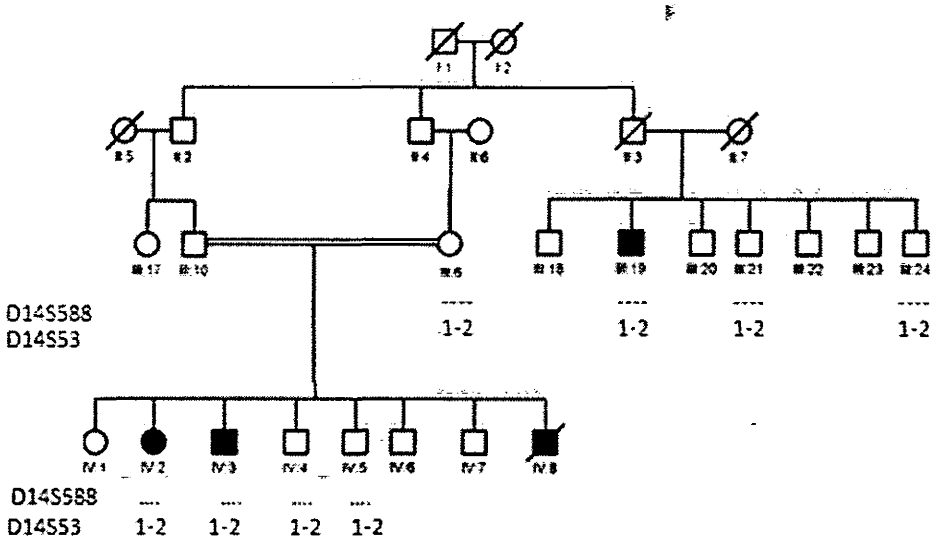
DFNB35 (D14S588 and D14S53)

(1) Family DFN17:



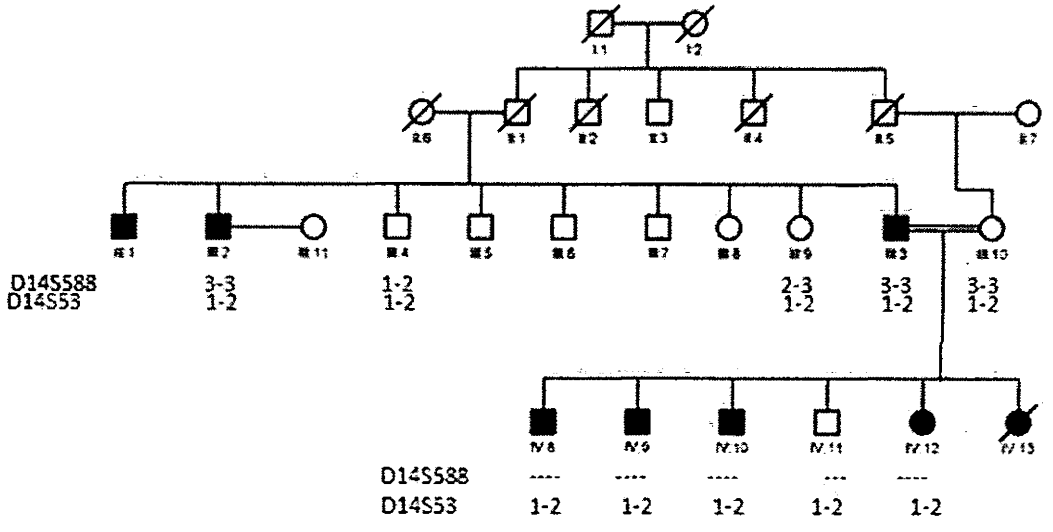
The markers D14S588 and D14S53 were applied on five normal and four effected individuals for screening DFNB35 locus to find linkage. For D14S588 among normal individuals four (III-09, III-20, IV-08 and IV-05) were found homozygous and one (III-21) was heterozygous .while all individuals were heterozygous for D14S53.

(2) Family DFN13



By applying the same markers to family DFN13 in results all the normal and patients were not amplified for marker D14S588 while for marker D14S53 all the members were heterozygous.

(3) Family DFN10:

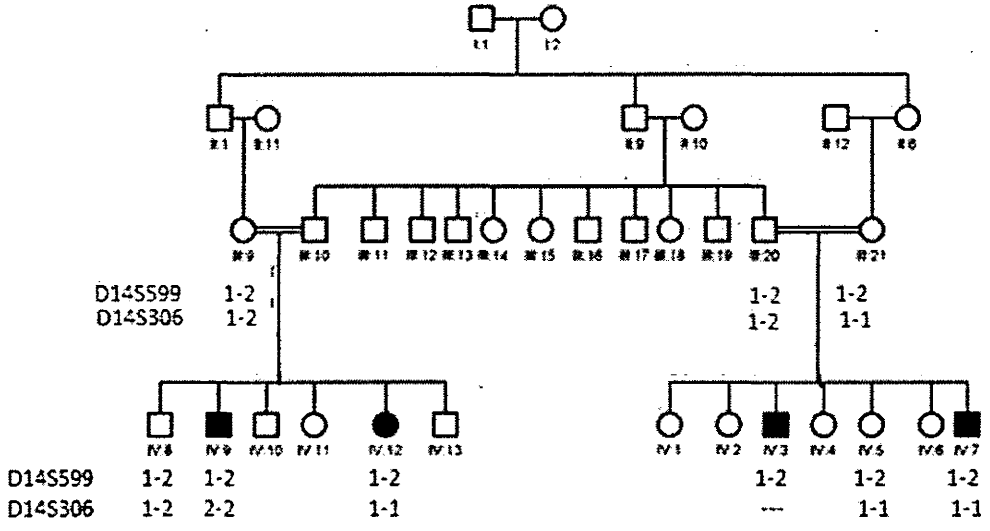


By applying D14S588 to family DFN10, only two patients and two normal individuals were amplified, out of which the patients (III-02 and III-03) were homozygous while two (III-04 and III-09) normal were found to be heterozygous while rest of the members were not given the result. Similarly for the second marker D14S53 the total members of this family were heterozygous.

Results: No linkage was observed at any family therefore these markers were also excluded.

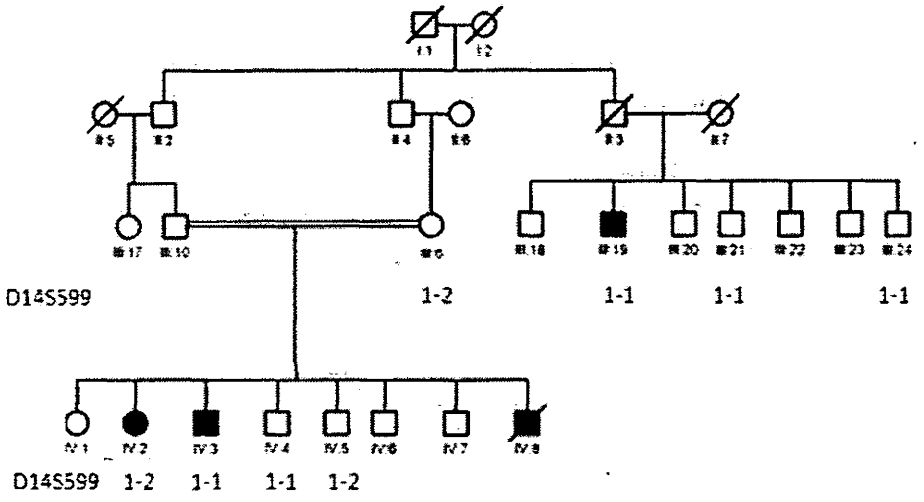
DFNB5 (D14S599 and D14S306)

(1) Family DFN17:



By using marker D14S599 for family DFN17 both normal and patients were found heterozygous with non informative pattern. While the second marker used showed three (III-09,III-20 and IV-08) heterozygous and two (III-21 and IV-05) homozygous in normal individuals, while in patients one (IV-03) member was not given the result and rest of the three (IV-08,IV-12 and IV-07) were found to be homozygous.

(2) Family DFN13:



D14S599 AND D14S306 were applied to Family DFN13 but unfortunately the second marker was not amplified while the first marker D14S599 was shown the results in which two patients (III-19 and IV-03) were homozygous and one (IV-02) was heterozygous. Among the normal individuals two (III-06 and IV-05) were heterozygous and three (III-21,III-24 and-IV-04) were homozygous.

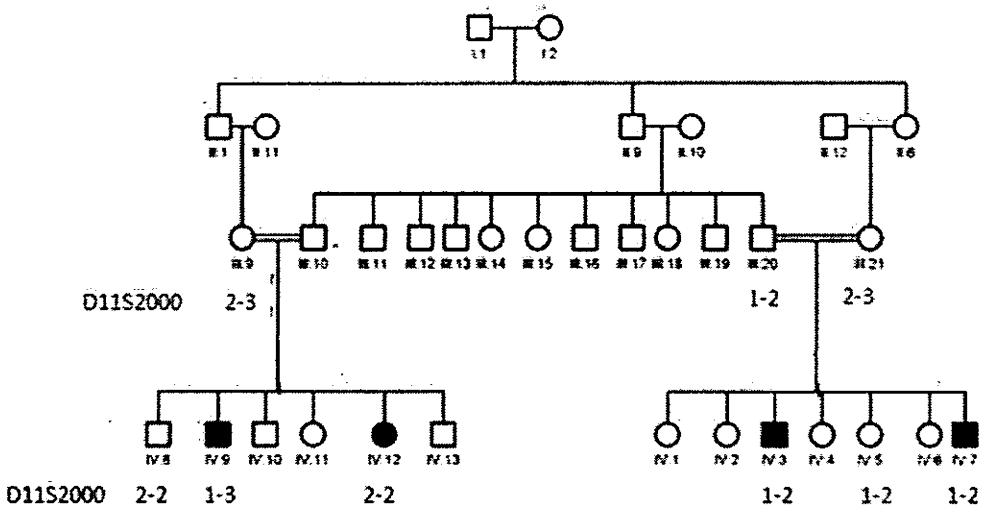
(3) Family DFN10:

Family DFN10 was not amplified for both markers D14S306 and D14S599.

Results: No linkage was observed at any family therefore this marker was excluded.

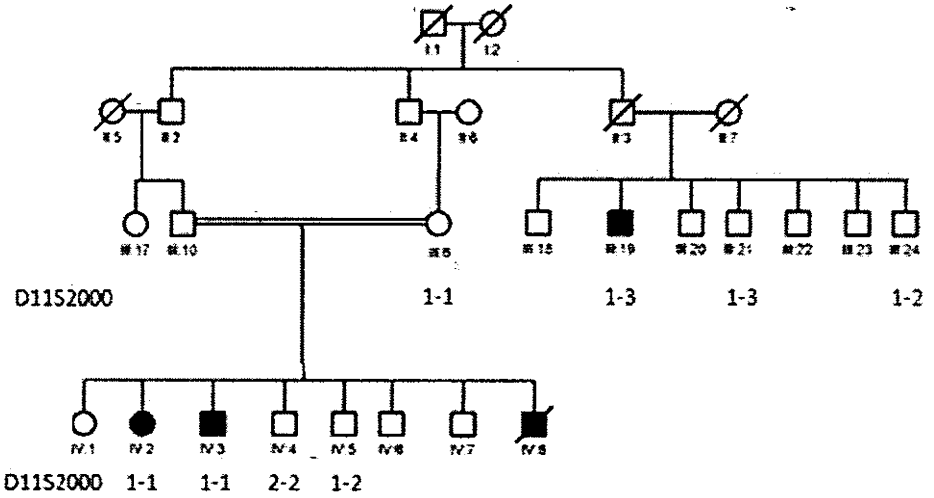
DFNB24 (D11S2000)

(1) Family DFN17:



The marker D11S2000 was applied on five normal and four effected individuals for screening DFNB24 locus to find linkage. Among normal individuals four (III-09,III-20,III-21 and IV-05) were found heterozygous and only one (IV-08) was homozygous. Similarly among patients, one (IV-12) was homozygous and three (IV-12,IV-03 and IV-07) were heterozygous.

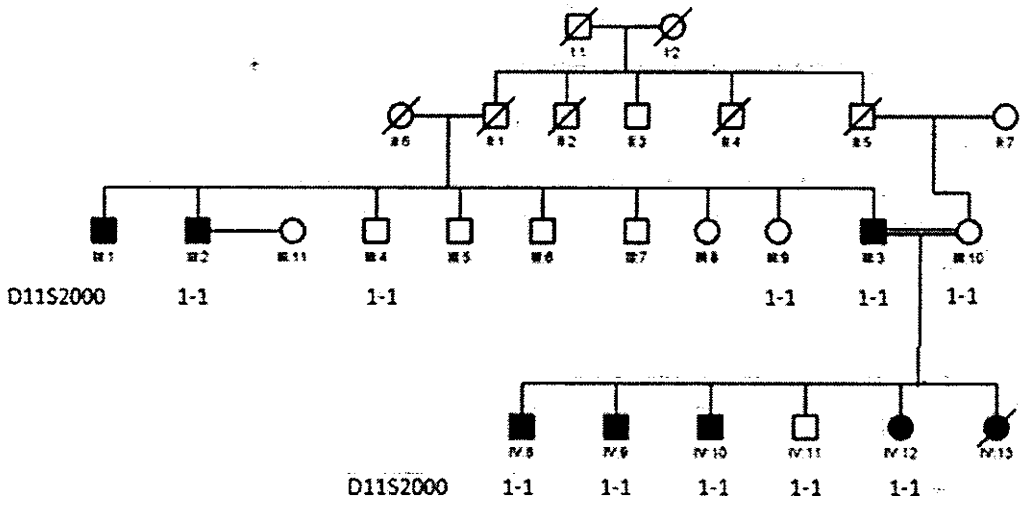
(2) Family DFN13:



By applying the same markers to family DFN13, in results the three (III-21,III-24 and IV-05) normal were heterozygous and two (III-06 and IV-04) were homozygous, In patients two (IV-

02 and IV-03) were found to be homozygous and one (III-19) was heterozygous for marker D11S2000.

(3) Family DFN10:

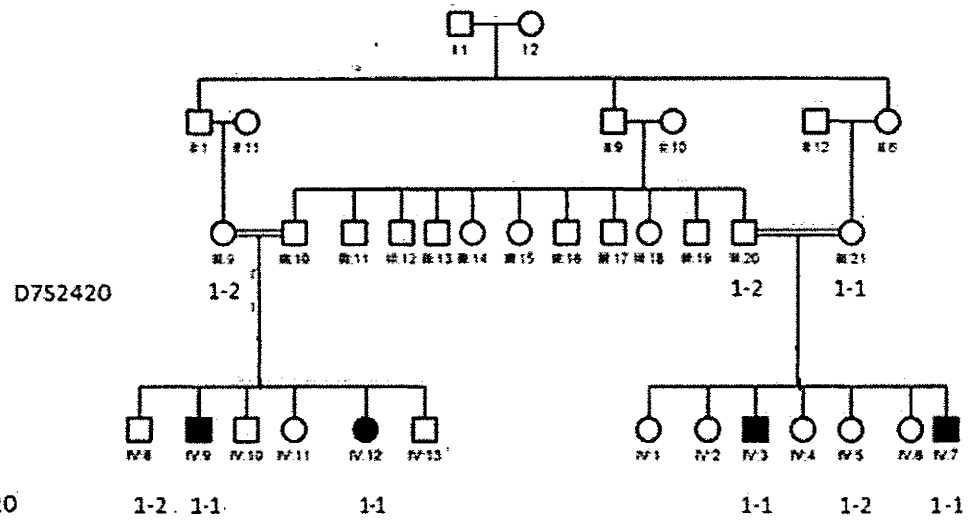


By applying marker D11S2000 to family DFN10, both normal and patients were found homozygous with non informative result.

Results: No linkage was observed at any family therefore this marker was excluded.

DFNB4 (D7S2420)

(1) Family DFN17:



The marker D7S2420 was applied on five normal and four effected individuals for screening DFNB4 locus to find linkage. among normal individuals four (III-09,III-20,IV-08 and IV-05) were found to be heterozygous and one (III-21) was homozygous while all the patients (IV-09,IV-12,IV-03 and IV-07) were homozygous for the used marker.

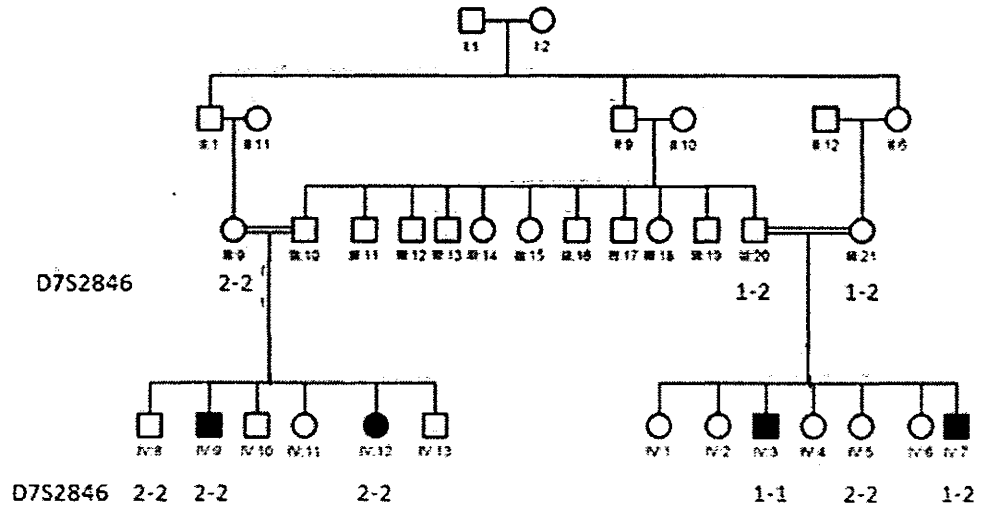
(2) Family DFN13 Showed linkage with DFNB4 locus as discussed with detail at the end of this chapter.

(3) Family DFN10:

Family DFN10 was not amplified by Marker D7S2420.

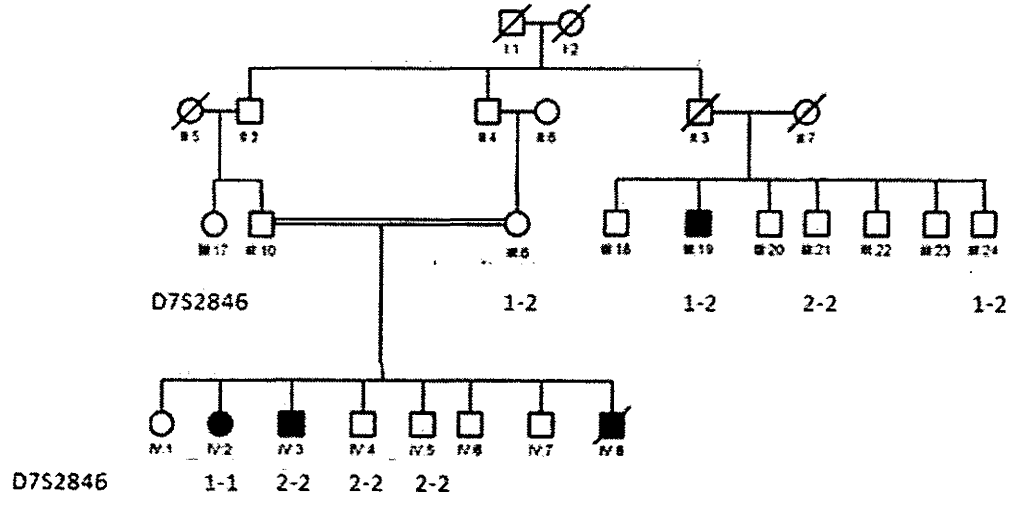
DFNB44 (D7S2846)

(1) Family DFN17:



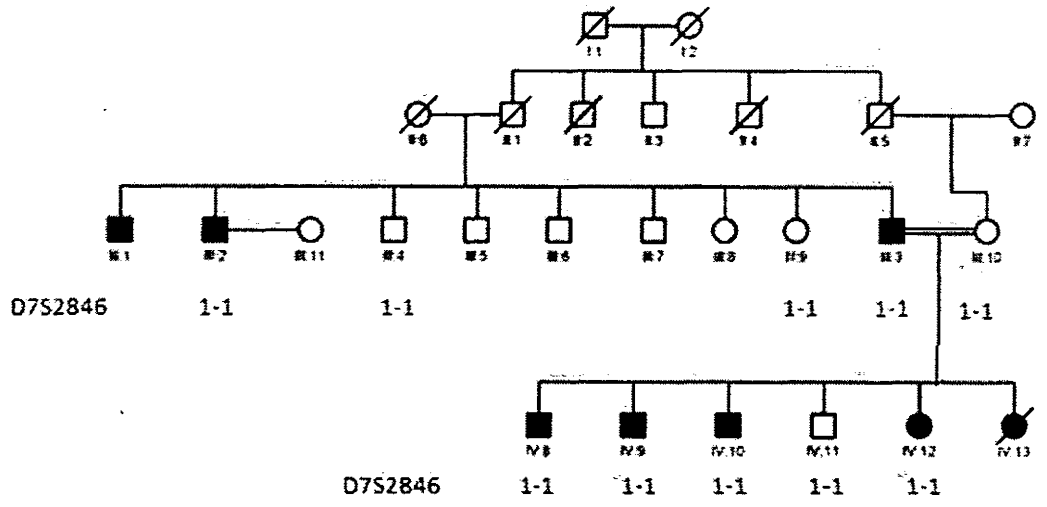
The marker D7S2846 was applied on five normal and four effected individuals for screening DFNB44 locus to find linkage. Among normal individuals two (III-20 and III-21) were found heterozygous and rests of the three (III-09,IV-05 and IV-08) were homozygous. Similarly among patients IV-09, IV-12 and IV-03 were homozygous and IV-07 was found to be heterozygous.

(2)Family DFN13:



By applying the same markers to family DFN13 in among the normal two (III-06 and III-24) were heterozygous and III-21,IV-04 and IV-05 were found to be homozygous. Similarly in patients IV-02 and IV-03 were homozygous while only III-19 was found to be heterozygous.

(2) Family DFN10:

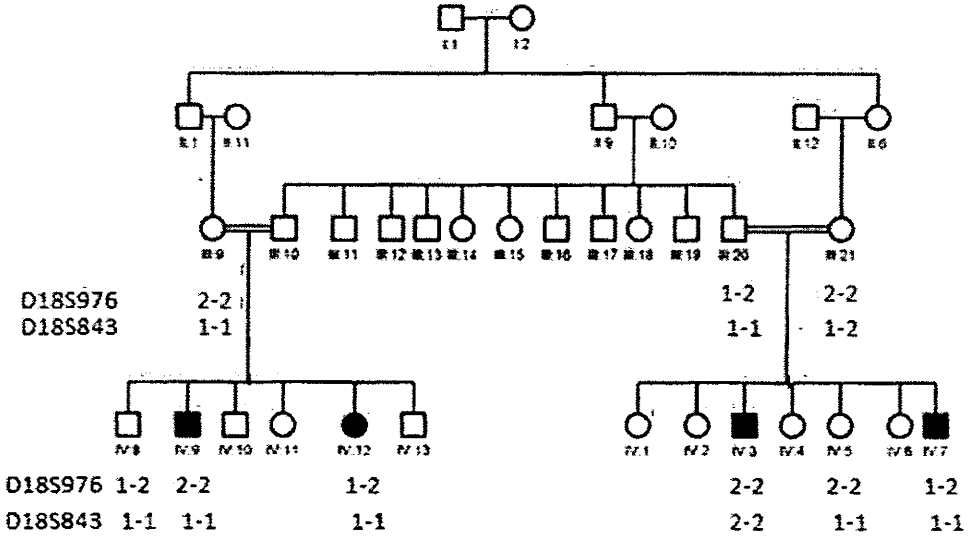


By applying marker D7S2846 to family DFN10, all patients (IV-08, III-02, III-03IV-09,IV-10 and IV-12) were homozygous. In normal individuals all (III-10,IV-11, III-04 and III-09) were also homozygous.

Results: No linkage was observed at any family; therefore this marker was excluded from the study.

DFNB19 (D18S976, D18S843)

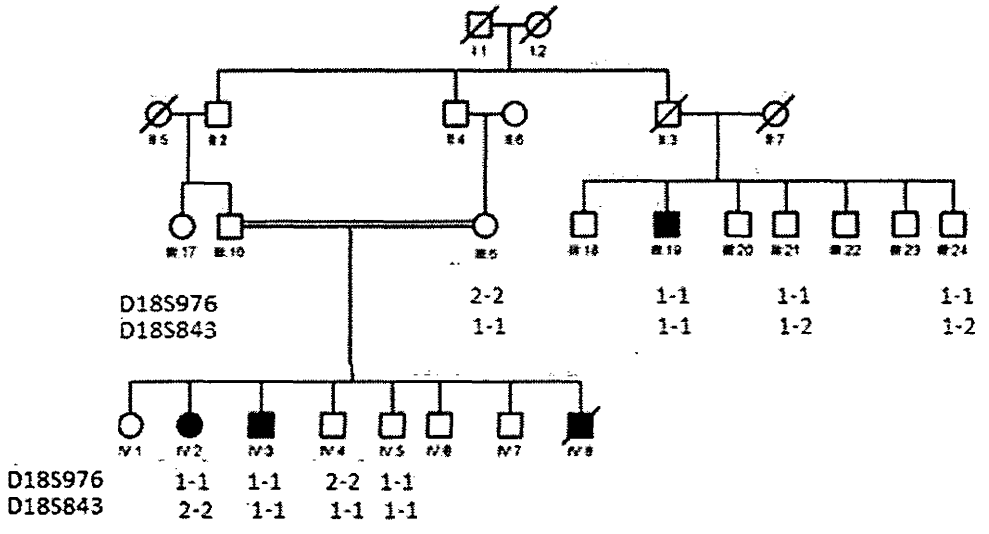
(1) Family DFN17



The markers D18S976 and D18S843 were applied on five normal and four effected individuals for screening DFNB19 locus to find linkage. For D18S976 among normal individuals III-20 and IV-08 were found heterozygous and rests of three (III-09,III-21 and IV-05) were homozygous. Similarly among patients two (IV-09 and IV-03) were homozygous while two (IV-12 and IV-07) were heterozygous.

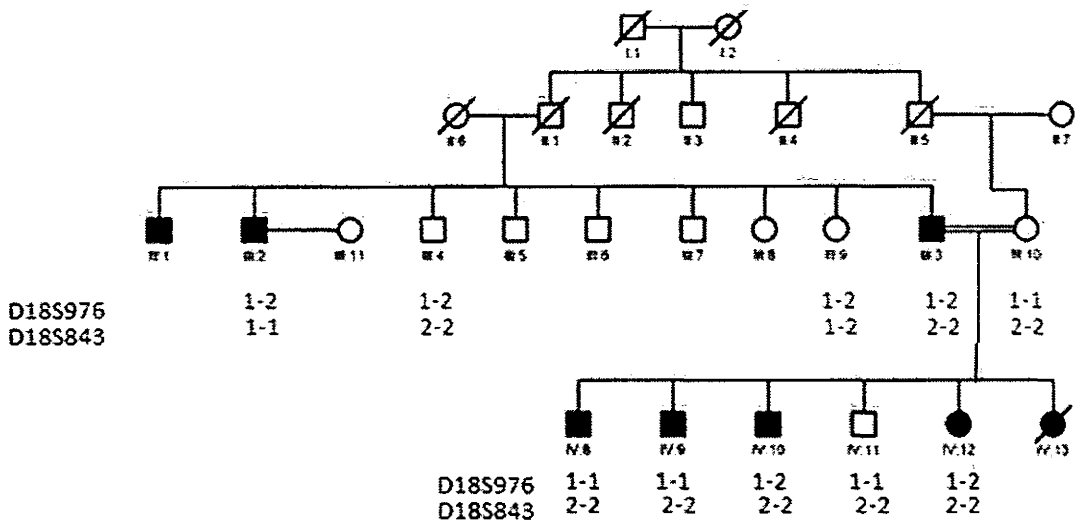
For marker D18S843 among normal four (III-09, III-20, IV-08 and IV-05) were homozygous while only III-21 was heterozygous.

(2) Family DFN13



By applying the same markers to family DFN13 , the results of marker D18S976 were, all the members including normal and patients were homozygous. while the results of the D18S843 are that among normal three (III-06,IV-04 and IV-05) were homozygous and two (III-21 and III-24) were heterozygous .Similarly all the patients were homozygous.

(2) Family DFN10

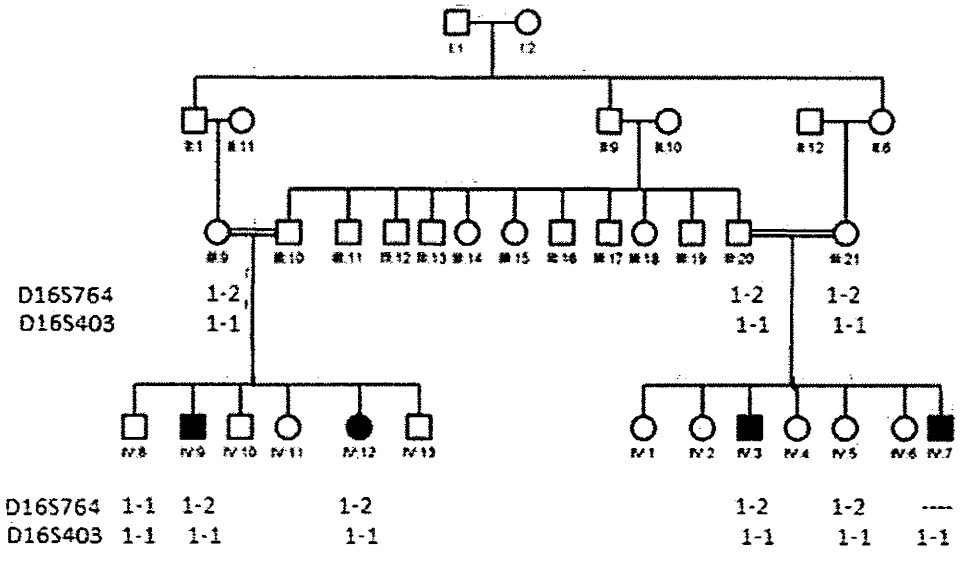


D18S976: Among normal individuals III-04 and III-09 were heterozygous and III-10 and IV-11 were homozygous. While in the patients II-02, III-03,IV-10 and IV-12 were heterozygous and IV-08 and IV-09 are homozygous.

D18S843: among normal individuals III-04, III-10 and IV-11 were homozygous and III-09 was heterozygous. Similarly in patients all were found to be homozygous

DFNB22 (D16S764, D16S403)

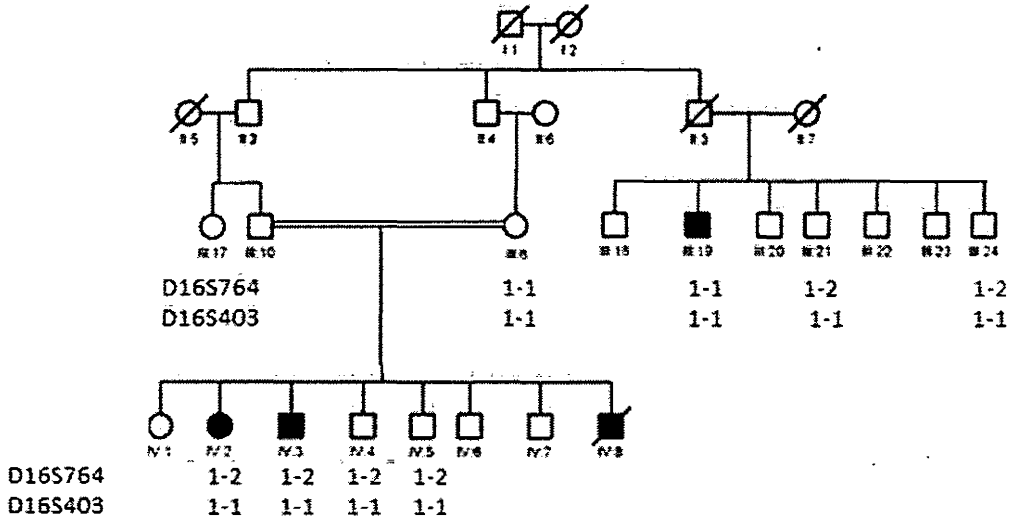
(1) Family DFN17



The markers D16S764 and D16S203 were applied on five normal and four effected individuals for screening DFNB22 locus to find linkage. For D16S764 among normal individuals III-09, III-20 and III-21 were heterozygous and IV-08 was homozygous. In patients IV-09,IV12 and IV-IV-03 were heterozygous while one patient (IV-07) was not amplified

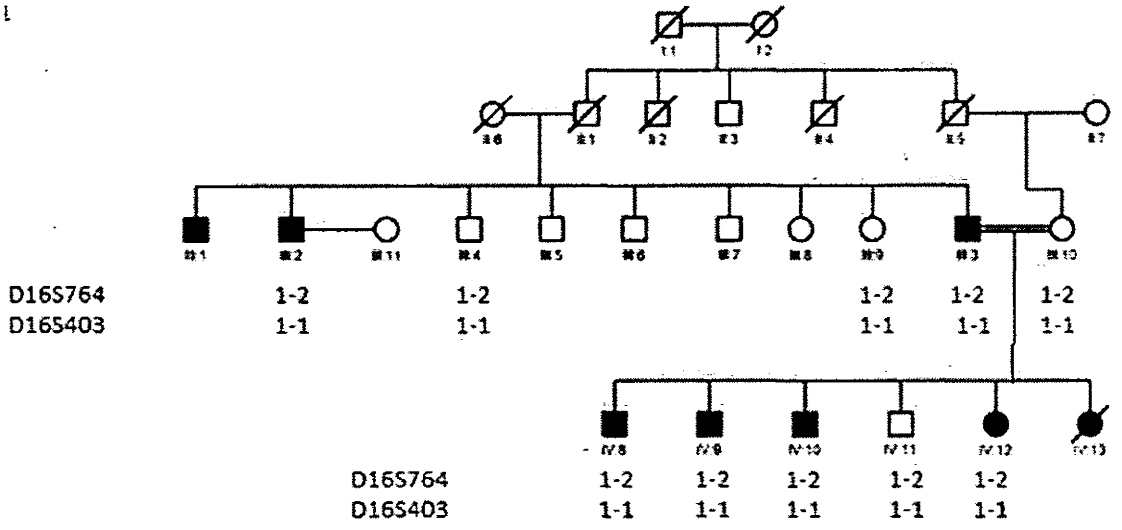
For marker D16S403 all members were homozygous with non informative results.

(2) Family DFN13



By applying the same markers to family DFN13, for D16S764 in results all the normal (III-21,III-24 ,IV-04 and IV-05) were found heterozygous, Two patients (IV-02 and IV-03) were found to be heterozygous while one patient (III-19) was homozygous. Similarly for D16S403 all the normal and patients were shown as homozygous.

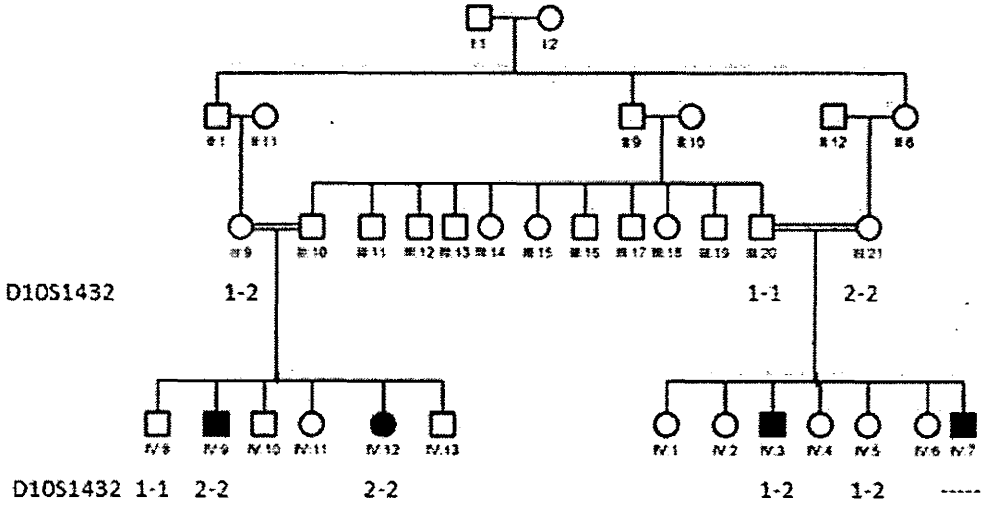
(2) Family DFN10:



The same markers were used to searched for a linkage in DFN10 family. But the results were non informative in studying both markers. For D16S764 all the members both normal and patients were found to be homozygous. while the results of the secondly used marker D16S403 were also no informative because all were in homozygous status.

DFNB12 (D10S1432)

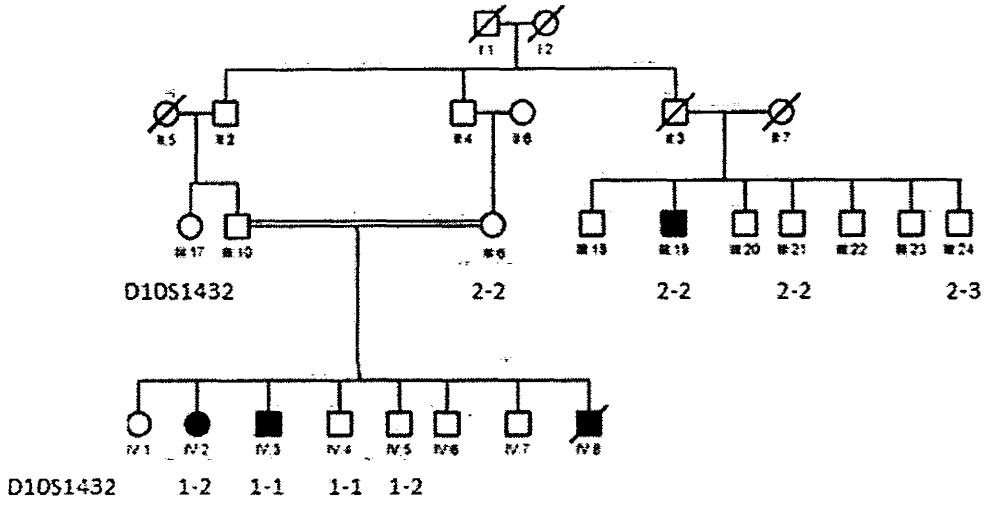
(1) Family DFN17



For the search of DFNB12 locus marker D10S1432 was used.in the result among the normal III-09 and IV-05 were found heterozygous while rest of the three III-20,III-21 and IV-08 were

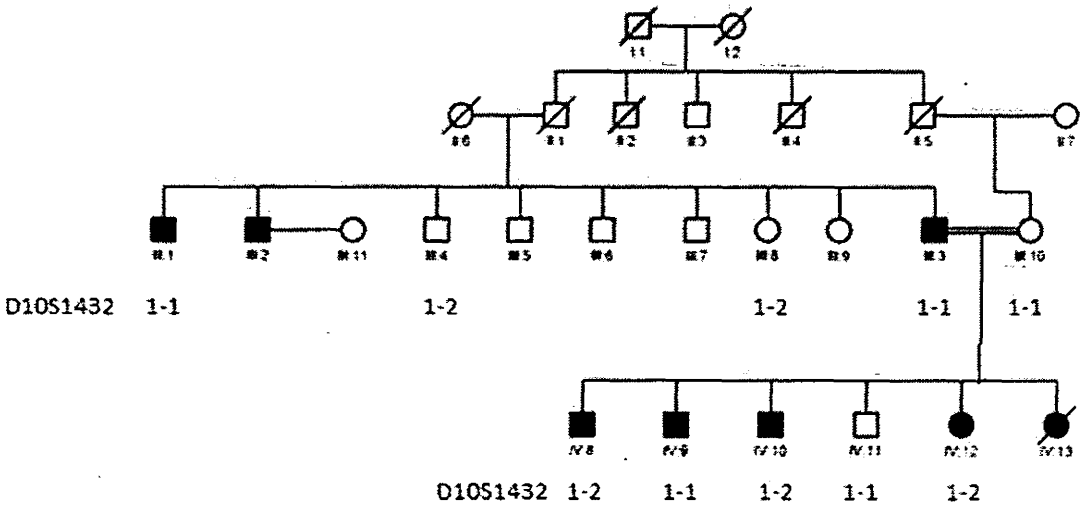
homozygous for D10S 1432.among the patients IV-03 was heterozygous ,IV-07 was not amplified and IV-09 and IV-12 were homozygous.

(2) Family DFN13



The same marker showed a differ result in the DFN13 family in which out of total normal III-06 ,III-21 and IV-04 were homozygous while III-24 and IV-05 were heterozygous for D10S1432. Similarly in patients III-19 and IV-03 were homozygous and IV-02 was heterozygous.

(3) Family DFN10

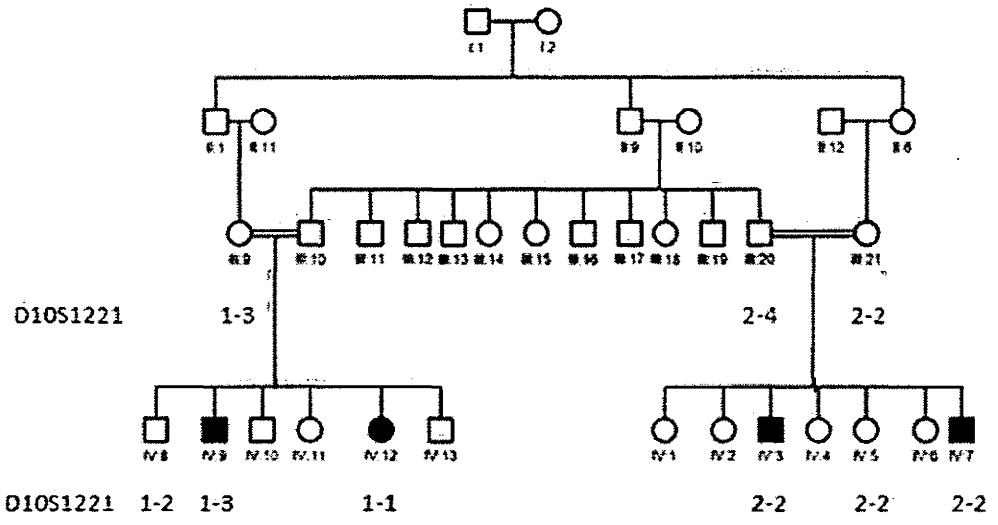


Marker D10S 1432 was also used for DFN10 family in which IV-08IV-10 and IV-12 were heterozygous and III-01,III-03 and IV-09 were homozygous among the patients. On the other

side among the normal individuals III-04 and III-08 were heterozygous and III-10 and IV-11 were found to be homozygous.

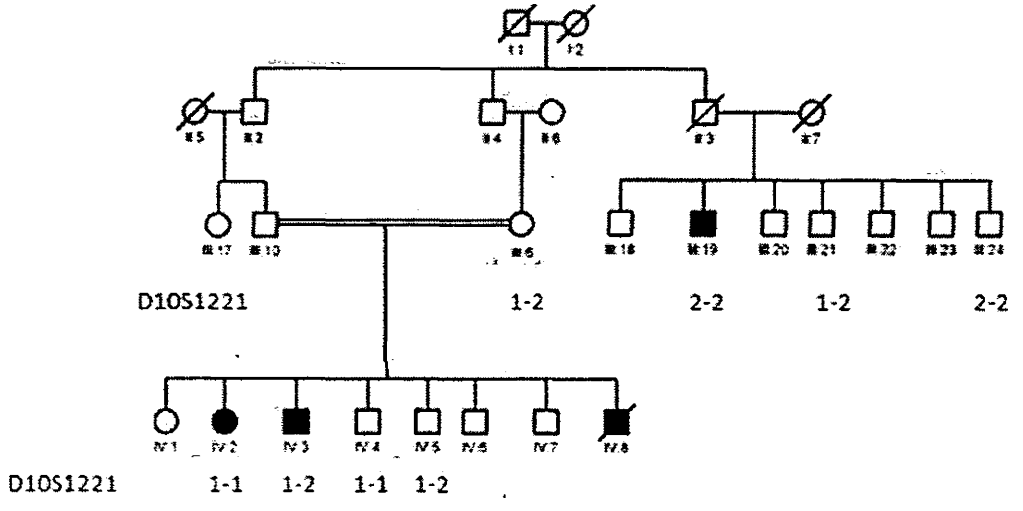
DFNB23 (D10S1221)

(1) Family DFN17:



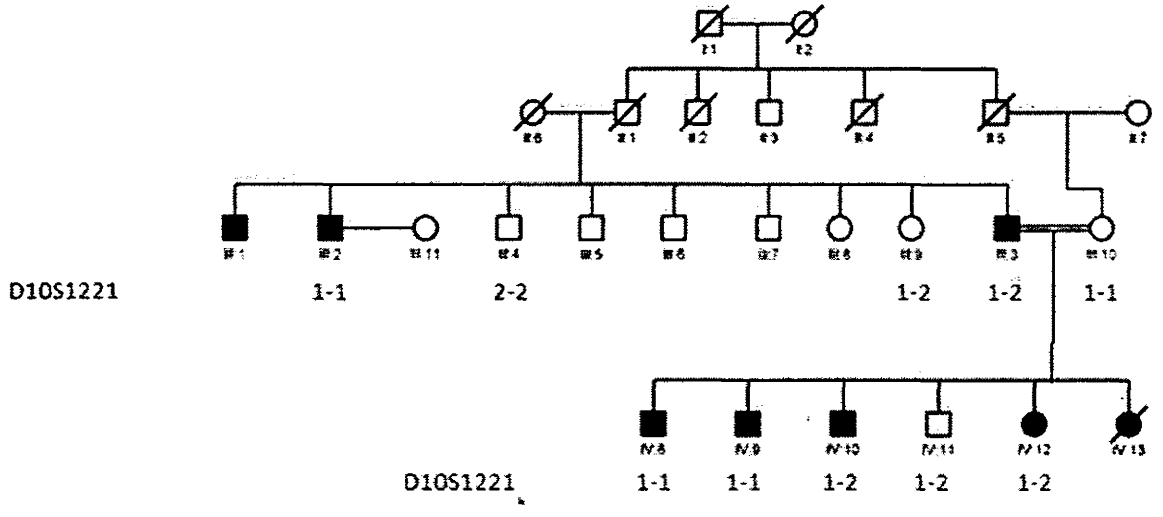
To search out DFNB23 in the selected three families a marker D10S1221 was used .in which the normal III-09,III-20 and IV-08 were heterozygous while III-21 and IV-05 were homozygous . In patients IV-09 was heterozygous and rests of the three were homozygous.

(2) Family DFN13



By using the same marker for the same locus as used in the previous family for DFN13 the results were as: among the patients III-19 and IV-02 were homozygous and one (IV-03) was heterozygous. Among the normal individuals III-06, III-21 and IV-05 were found to be heterozygous while III-24 and IV-04 were homozygous for DFNB23.

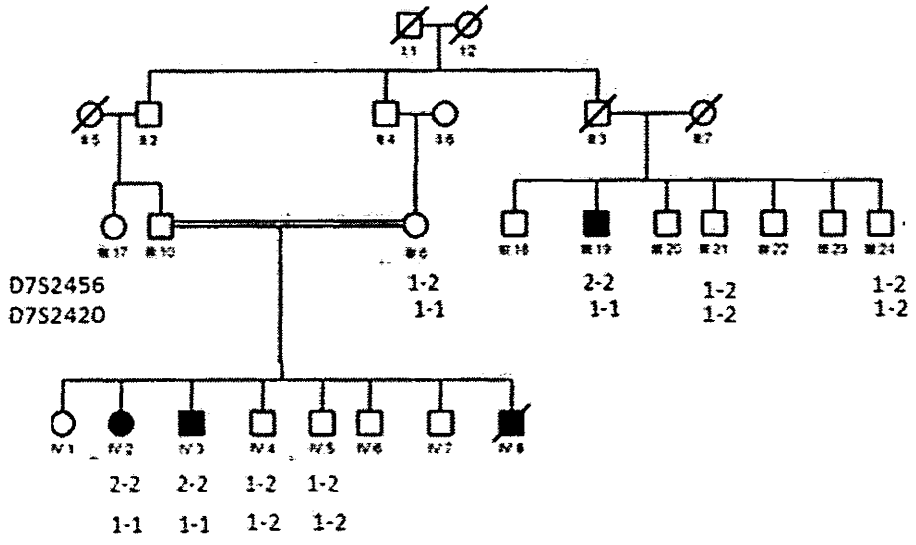
(3) Family DFN10



applying marker D10S1221 to family DFN10 to search DFNB23. Among the patients III-02,IV-08 and IV-09 were homozygous and III-03,IV-10 and IV-12 were heterozygous. in the normal individuals III-09 and IV-11 were heterozygous and III-04 and III-10 were found to be homozygous for the used marker.

Results: No linkage was found.

4.3. Linkage of Family DFN13 on DFNB4 (SLC26A4):



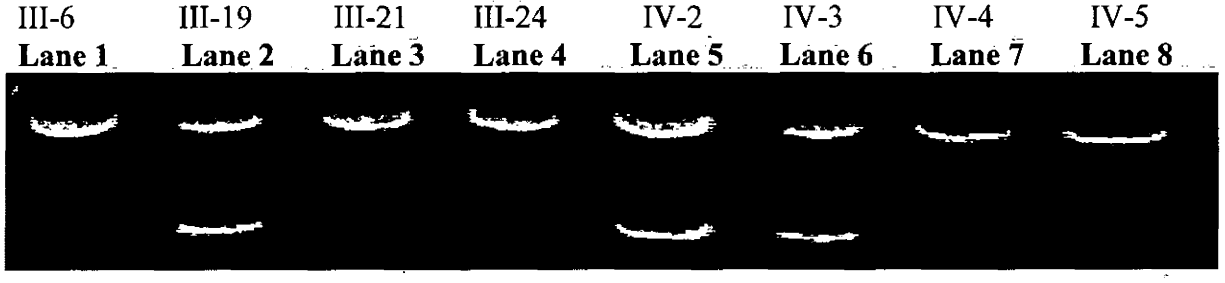


Figure 4.1: Gel Image of D7S2456 (Family DFN13)

The haplotype of the entire family DFN13 was found to be linked to *DFNB4* locus at chromosome 7q31.The affected individuals (III-19,IV-02 and IV-03) were homozygous while normal three (III-06,IV-04, III-21, III-24 and IV-05) were heterozygous for marker D7S2456.By using the second nearest marker D7S2420 the linkage was confirmed.

This small family was collected from Thoro Mardan, Khyber Pakhtunkhwa, consisting of four affected individuals. At the time of enrollment only three patients and five normal individuals were available and were included in the study .The only phenotype in the affected individuals was the hearing impairment and there was no other associated problem observed on detail history

and examination. Haplotype analysis of the family clearly showed linkage to DFNB4 locus. A two point LOD score of 1.2 at θ=0 was observed for marker D7S2456.

Table 4.1: LOD score calculation of linked markers

S.NO	MARKERS	Chromosome	Position cM	Family	LOD _Max	θ at Max
1	D7S2456	7	120.61	DFN13	1.2154	0.0000
2	D7S2420	7	119.81	DFN13	1.0815	0.0000

4.4. Mutation Screening in SLC24A4 gene (Family DFN13):

We used several markers for finding linkage with different loci. Out of three studied families only one family (DFN13) was found to be linked on DFNB4 locus (7q31). Our next objective was to screen the SLC26A4 gene (locus DFNB4) for mutation. Markers were designed for all 21 exons.

To determine the contribution of *SLC26A4*mutations to recessive deafness in a family (DFN13) of Khyber Pakhtunkhwa, 21 exons of SLC26A4 gene were screened for sequence analysis. We did analyse all *SLC26A4* exons in two normal and two patients of DFN13 family. The two genomic substitution mutations at exon 2, One was at +2 splice site (T>G) and the second was at +5 intronic region (C>G) were detected by PCR amplification and sequencing with primers 5-TCTTCCCCTCCGATCGTCCT -3 and 5- CCTCCCCAAGGCGTGGAC -3.Figure 4.1 shows the sequencing results of a patient (IV-02) with two point mutations while the second image (figure 4.2) demonstrate the results of sequencing in a normal individual (III-21) of DFN13 family.

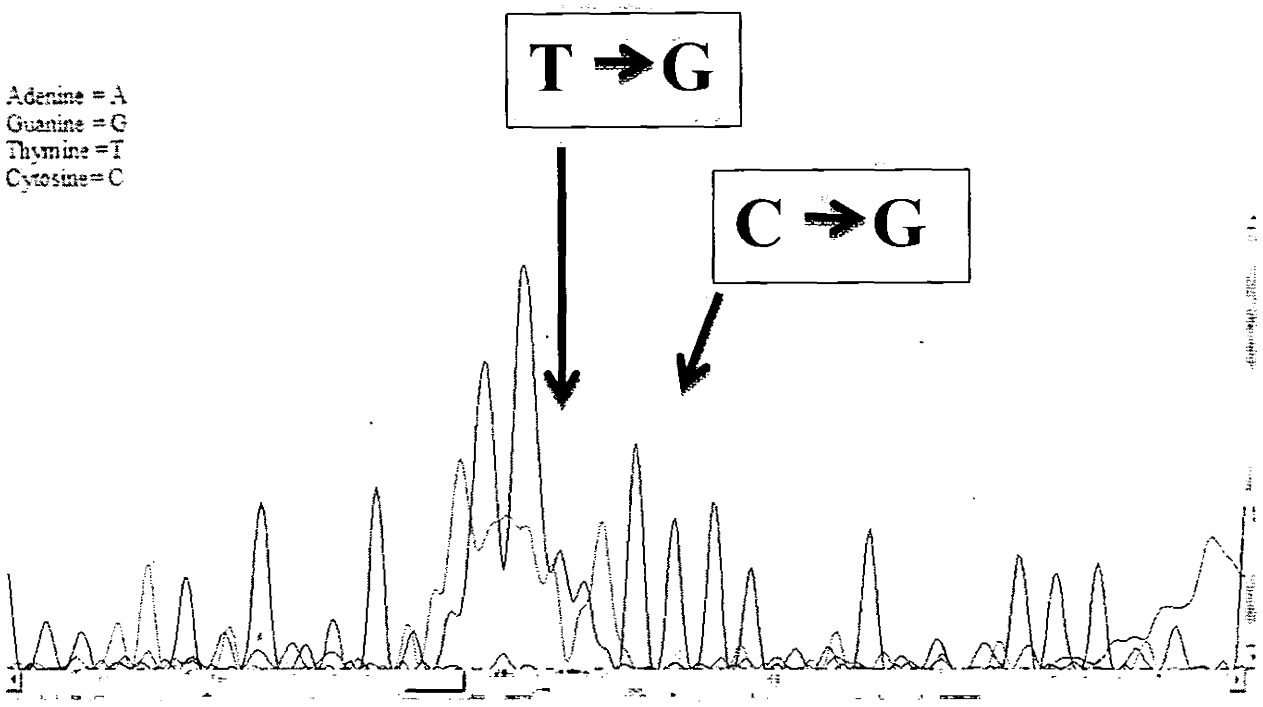


Figure 4.4: Patient IV-02, sequencer results of exon2 (mutated region) SLC26A4 gene.

TCTTCCCCTCCGATCGTCCTCGCTTACCGCGTGTCCTCCCTCCTCGCTGTCCTCTGGCTCGCAGGTCATGGCAGCGC
CAGGCGGCAGGTCGGAGCCGCCGCAGCTCCCCGAGTACAGCTGCAGCTACATGGTGTCGCGGCCGGTCTACAGCGAG
CTCGCTTTCCAGCAACAGCACGAGCGGCGCCTGCAGGAGCGCAAGACGCTGCGGGAGAGCCTGGCCAAGTGCTGCAG
G**G**AG**G**GGCCGCGCGGGCCTGCGTAGAGAGAAGCGGAGCGGGGCGTCCACGCCTTGGGGAGGG

Mutated nucleotides sequence of exon2.

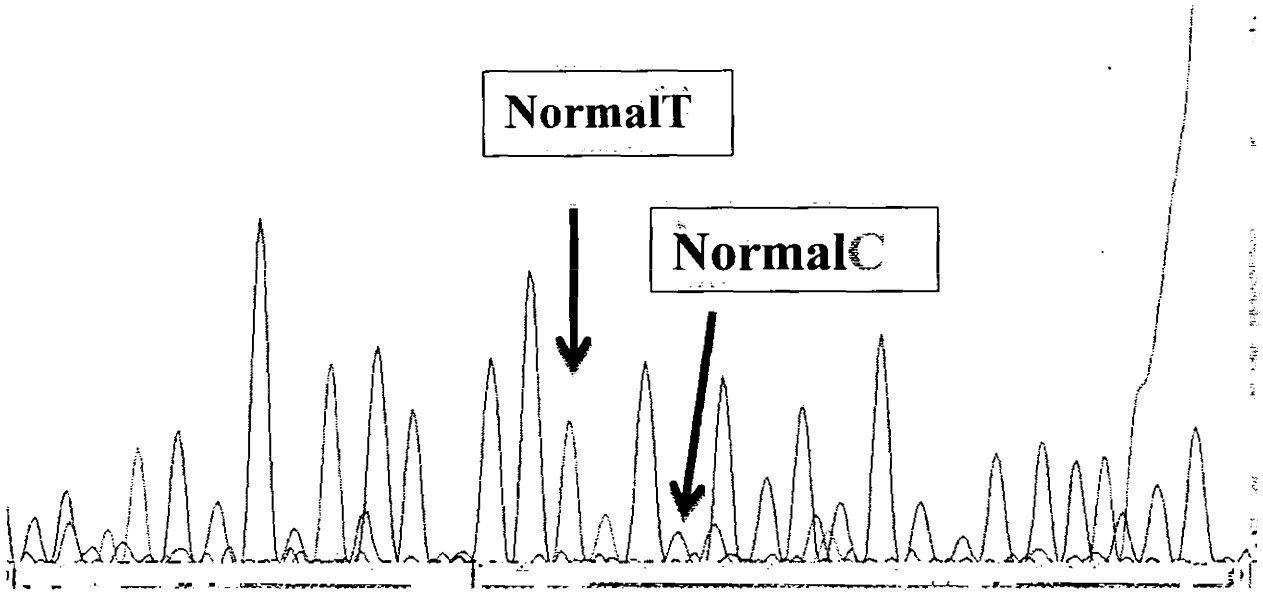


Figure 4.4: Normal individual (III-21). Sequencer results of exon2 (SLC26A4 gene).

TCTTCCCCTCCGATCGTCCTCGCTTACCGCGTGTCCTCCCTCCTCGCTGTCCTCTGGCTCGCAGGTCATGGCAGCGC
CAGGCGGCAGGTCGGAGCCGCCGCAGCTCCCCGAGTACAGCTGCAGCTACATGGTGTCGCGGCCGGTCTACAGCGAG
CTCGCTTTCCAGCAACAGCACGAGCGGCGCCTGCAGGAGCGCAAGACGCTGCGGGAGAGCCTGGCCAAGTGCTGCAG
GTAGCGGCCGCGCGGGCCTGCGTAGAGAGAAGCGGAGCGGGGCGTCCACGCCTTGGGGAGGG

Normal nucleotides sequence of exon2.

In the above results the normal individual has the normal T and C nucleotides, while in the patient T is replaced by G and C is replaced by G.

CHAPTER 5

DISCUSSION

Discussion

Deafness is found to the most prevalent disease affecting both developed and developing countries. It arises either due to environmental (e.g. bacterial, viral, toxicity or acoustic trauma) and genetic factors. The onset period varies (e.g. from birth to adult hood). 1 per 1000 population in developed countries is suffered with hearing loss, while this ratio is much higher in developing countries, which is 1.6 per 1000. 50% cases arise due to genetic involvement. The incidence of hearing loss in male is higher than female. As discussed earlier that out of genetic incidence 75% are found to e Autosomal recessive, 15% are having Autosomal dominant pattern of inheritance while 10% are estimated as X-Linked and mitochondrial or due to chromosomal aberration (Robertson *et al.*,1999, Mortan, 1991, and Readron W, 1992). The genetic hearing impairments that are either congenital or prelingual are mostly found to be inherited through Autosomal recessive pattern. While Autosomal dominant mode of inheritance is estimated to be mostly involved in post lingual hearing defects. Clinical categories of deafness are based on sensorineural versus conductive versus mixed hearing loss, as well as severity, age of onset, environmental influences, audiological profile and associated symptoms in other regions of the body. For the genetic hearing impairment a variety of tools are used to identify and map the involved mutated genes. A variety of markers associated with deafness Loci are used to narrow down region for the search of a mutated gene. Through Linkage analysis of markers and Loci it is brought in front that which part of the chromosome is involved mutation. In genetic deafness, 70% are non syndromic and 30% are syndromic hundreds of associated symptoms with hearing impairment have been observed (Teas, 1989). In non syndromic hearing loss only the hearing system is disturbed (e.g. conductive, sensorineural or mixed hearing loss). On the other hand in syndromic deafness other abnormalities are seems to be present along with hearing loss. In non syndromic deafness ~15% are Autosomal dominant (DFNA1-DFNA39, ~80% are Autosomal recessive (DFNB1-DFNB30) and ~ 2% are mitochondrial all the data regarding to hearing loss e.g. locus for each disorder, markers, and appropriate references can be found on the hereditary hearing loss Home Page. Within the past few years several genes involved in non syndromic hearing loss are identified (Earnest et al 2000). Examples are, the human homoloy of drosophila diaphanous gene (DIAPH) in DFNA1, the gap junction protein conexin 26 (GJB2) in DFNB1 and DFNA3. The tectorin membrane protein tectorin (TECTA) in DFNA 8/12, the POU4F3 transcription factor in DFNA15, the putative sulfate transporter. PDS in both Pendred syndrome

and DFNB4, the novel cochlea gene (COCH) in DFNA9, the unconventional myosin (MYO15) in human DFNB3 and mouse shaker 2, a gene (USH2A) with homologies to laminin epidermal growth factor and fibronectin in USHE Syndrome Type 11 and a gene with very little homology to any known protein, all of these gene have shown to be expressed in cochlea, demonstrating how knowledge of gene expression in the membranous labyrinth is critical for further understanding of hearing and deafness, majority of the reported deaf individuals belonging to those families where consanguineous marriages are common because in the same family a mutant genes flow from generation to generation. The custom of consanguineous marriages with high rate is found mostly in Asian countries. However congenital deafness is relatively common in geographical remote areas with high consanguinity. This simplifies genetic linkage analysis using homozygosis mapping in consanguineous families. homozygosis mapping is based on the assumption that a rare mutation is inherited from a common ancestor through both parents, so that affected siblings are homozygous by descent, for polymorphic marker close to the disease homozygosis mapping is used to elucidate the gene defect in a family (Garrad 1908). In the light of past studies currently a new project was designed to identify type of mutated genes involved in three families with congenital deafness in Autosomal recessive pedigrees of Khyber Pakhtunkhwa Pakistan. All the families were affected with congenital, non syndromic hearing loss. From all pedigrees it was concluded that the pattern of inheritance was Autosomal recessive. Families were given their own ID,s e.g. DFN10, DFNB13 and DFNB17 respectively. These numbering were given to follow the working laboratory catalog.

Among these affected families samples were collected from patients and some normal individual as well. In family DFN10 five normal individual (III-09, III-20, III-21 IV-08and IV-05) and four patients (IV-09, IV-12, IV-03 and IV-07) we genotyped with microsatellite markers, for each locus two three markers were used for genotyping of the subjects. Similarly normal and effected individuals of family-DFN13 were also screened for linkage by using all available microsatellite markers, three patients (III-19, IV-02 and IV-3) and five normal (III-20, III-21, III-24, IV-04 and IV-05) were genotyped. While the normal members of family DFN17 were III-20, III-21 III-09, IV-08, IV-12 and IV-05, the patients of this family were IV-09, IV-12, IV3 and IV-07.

The most common deafness loci among the Pakistani population are DFNB1, DFNB2, DFNB3, DFNB4, DFNB8/10, DFNB7/11, DFNB12, DFNB18, DFNB23, DFNB39, and DFNB48. In the

present study all of these loci and other reported loci from the rest of the world were search in three families of Khyber Pakhtunkhwa Pakistan. In which only one family (DFN13) was shown Linkage with DFNB4, while rests of the two families (DFN10 and DFN17) were unfortunately not linked with any marker used. By mutational screening of SLC26A4 which is a gene located on DFNB4 and involved in deafness, a novel splice site mutation was screened at +2 T>G and another mutation was found on +5 C>G.BOU THE second mutation was at intronic region, so is not considered in deafness but the splice site mutation has affect on the phenotype.

DFNB1, the first discovered non syndromic Autosomal deafness locus, (Guilford *et al.,* 1994) encodes a gene GJB2 (Kelsell *et al.,* 2000). From the reports of different research projects it is clear that in 20% to 50% cases DFNB4 is found to be involved in non syndromic Autosomal recessive deafness. In our study we search three families with congenital deafness for this locus but no linkage was found in any family.

DFNB4 is a locus responsible for pendred syndrome (Eeverett *et al.,* 1997), and enlarged vestibular aqueduct syndrome (Usmani *et al.,* 1999). SLC26A4 gene is located on this locus and is causative agent for hearing loss. From various reports of East Asia DFNB4 is involved in 5% cases of prelingual deafness similarly in 5% at South Asia with recessive deafness (Park *et al.,* 2003). In our project one family DFN13 was found linked with DFNB4. By screening SLC26A4 a novel mutation was found at splice site +2 T>G. as two types of hearing loss have been found to be caused due to mutation at this gene, one is pendred syndrome and other one is non syndromic hearing loss. Both can be differentiated by appearance or absence of goiter which is actually present in pendred syndrome (Reardon et al., 1999). Usually the developmental age of the goiter is first 10 years after birth (Reardon et al., 1999). But in our studied family individuals ages ranges from 2 years to 32 years but no goiter with any patient was observed. From the absence of goiter this family was considered as non syndromic DFNB4. Pendrin protein are encoded by SLC26A4 gene which are 780 amino acid chain functionally found to be anion transporters, in thyroid cells iodide are transported with the help of pendrin (Everett *et al.,* 1997). More than hundred mutant alleles have been identified. In which missense mutation are mostly common. As we have found a new mutation at this gene at splice site. To date thirty four mutations at splice site have been reported. but for the first time we reported mutation on +2 T>G. DFNB18 was found in an Indian large consanguineous family. The gene was localized

between D11S 1307 and D11S1308 in haplotype analysis. Jain *et al.,* 1997 stated that DFNB18 is al allelic. We also made an attempt to screen DFNB18, but no family was linked with DFNB18. DFNB21 was identified by Mustapha *et al.,* 1999 is found to be functional in hearing loss, which encodes alpha tectorin found in tectorial membrane in the inner ear, is responsible for recessive DFNB21 hearing loss. WE used D11S968 to locate DFNB21 but no linkage was found.

Similarly MY07A gene at DFNB2 was mapped to chromosome 11q13.5 with markers D11s911, D11S527 and D11S937 in the study of Guilford *et al.,* 1994. We used D11S937 in three families but no informative results were obtained. So this locus was excluded from the study.

In 1995, friedman *et al.* conducted a study and DFNB3 was found linked to chromosome 17p11, 2q12, with markers D17S122, D17S805 and D17S842. But this locus was not found is out families been searched for. Similarly in the study of Boone-Tammir *et al.,* 1996, DFNB 10 was mapped to chromosome 21q23.3 by markers D21S168 and 21S1259. Likewise DFNB12 (CDH23) was found to be present on 10q21-22 by the help of markers D10S168, D10S535 and D10S580 (chaib *et al.,* 1996) for DFNB12 we used D10S537 and D10S1432 with no results. Jain *et al.,* 1998 found DFNB18 by using D11S902 and D11S901. So USHIC gene was located on this locus. In our study D11S981 and D11S902 were used and were excluded from the study due to no linkage found. DFNB21 (TECTA gene) linked with marker D11S4111 and D11S925 (Mustapha *et al.,* 1999). Similarly DFNB6 (TMIE gene) was found and reported by Fukishima *et al.,* 1995, they used D3S1767 and D3S1289 markers. Likewise DFNB21 was hunted by using markers, D11S4464 and D11S1998 but same like our results no linkage was found (Fukhisima *et al.,* 1995b).

A study was designed by Jain *et al.,* 1995 they used D9S50, D9S301 and D9S301 and found a linkage on chromosome 9q 13-q21 as DFNB7/11 (TMCI gene). We used all of the above markers for finding the relevant Loci, but no linkage within any family was found. In the same field chaib *et al.,* 1996 searched and found DFNB9 (OTOF) by using D2S171 and D2S174 markers. In our study we used D2S165 marker for linkage analysis of DFNB9 but no linkage was found. So these markers were excluded. For DFNB30 we used a marker, D10s1423, in result no information about linkage was gain so was excluded but in the study of Walsh *et al.,* 2002 they used the same marker and got linkage in a family, we search DFNB 16 in three families but

were not successful to get any linkage in these families but Campbell *et al.*,1997 and Verpy *et al.*,2001 were successfully found linkage with DFNB30 (MYO3A).

DFNB 15 was fond linked on chromosome 3q 21-q25 with the help of D3 S1309 and D3S1593. (Chen *et al.*, 1997). While for this locus we used available markers D3S1569 and D3S1279. But unfortunately no linkage was found. For searching DFNB17 we used D7S3061 and D7S2847 while Grienwalel *et al.*, 1998 did find this locus in their study by using D7S655 and D7S780 so they got linkage to chromosome 7q31. Likewise D18S976 and D18S843 were used in our study to find linkage with DFNB19 but were unable to find. But Green *et al.*, 1998 found it in 18P11 chromosome by using same by using same markers.

Furthermore Pulley *et al.*, 2000 were successfully found DFNB27 by suing DS326 and D2S2257 while we used different markers D2S1776 and D2S326 and found no Linkage.

DFNB28 was found Linked to chromosome 22q13 with markers D22S283 and D22S423 (walsh *et al.*, 2000). In this search we use a single marker D22S283 but no linkage was observed. DFNB35 was linked to chromosome 14q24.1 – 24.3 with markers D14S57 and D14S59. (Ansar *et al.*, 2003), and we used D14S588, D14S258 and D14S53 for DFNB35 no linkage was screened in results 1995A, by applying markers D2S335 and D2S1391. We used D14S599, D143306 and D14S288 but unfortunately no linkage was found in 1998, Mustapha *et al.*, conducted a study on congenital deafness and used markers D7S661 and D7S498 and found DFNB13 Locus linked to chromosome 7q34-36. We used GATA104 and D7S 1805 for the same Locus, but no informative results were obtained. In the same year Mustapha *et al.* found a second linkage at DENB14 to chromosome 7q31 with markers D7S527 and D7S3074 and in the light of that study we applied D7S821, D7S515 and D7S1799 for DFNB14 but were no satisfactory results of linkage. For DFNB20, Moginnan *et al.*, 1999 used D11S969 and D11S439 and observed linkage at chromosome 11q25. Similarly we used D11S968 and found no hints for linkage. In our study another locus DFNB23 was searched by using D10S1221 and D10S1227 markers with no results this locus was excluded while Riazuddin *et al.*, 2000 found linkage of same locus in his study in a Pakistani family.

The study presented here, include linkage finding and a novel mutation at SLC26A4 gene on locus DFNB4 at chromosome 7q31 in a family (DFN13) been collected from village Thoro

District Mardan. We made first attempt to find linkage and successfully linkage was found than 21 exons of SLC26A4 gene were screened for mutation by using direct sequencing of each exon and finally a single point mutation was found at +2 position of exon 2 in which T was found to be replaced by G. Similarly another known mutation was also detected at intronic region +5, in which C was replaced with G. The first mentioned mutation is found as novel at splicing site. Total 34 mutations at splicing site on SLC26A4 are identified and discovered but all 34 mutations do not contain the type, which we discovered. The rests of the markers we used showed no linkage in other two families (DFN10 and DFN17).

Our recent finding of linkage analysis and discovery of the novel mutation will guide the investigators of future in the research of molecular studies of deafness. As SLC26A4 gene encodes pendrin protein. So the DFN13 family is explored to be involved in problem at pendrin protein. So we now have a better understanding of the genetic control of whole auditory system. In the light of already conducted studies and the present project, in future new genes and mutations will be identified and mouse models will be constructed to better understand the pathological processes involved in deafness. The positional cloning will be accelerated from the availability of sequences of genes involved in hereditary hearing loss. As Fetal cochlear cDNA Library is currently established which is helpful in studies conducted on hearing loss. Similarly EST database of human cochlear material have been made available, which has provided freely access to cochlear clone data. With the discovery of genes in mouse genome are sequenced which are almost near to completion will enable the scientists in the advent of gene chip technology. Gene discoveries and functional genomics in the auditory system are continuously improving at a rapid pace, from all other related studies and the project we conducted will carry the researchers to an easy diagnostic ways, pre-symptomic genetic testing, early inventions and disease based treatments. Identification of the loci in family DFN13 and characterization of the protein it codes will further increase our knowledge of the molecular process involved in the auditory system.

The appliance of molecular genetics methods to the study of hereditary hearing impairment has contributed appreciably to our understanding of the genetics of deafness. Still, there remain a lot to study about these mechanisms. The need is to characterize deafness at molecular level and identify genes that contribute the most to hearing loss in Pakistan. This will help to offer genetic

counseling to the families to reduce the incidence of hereditary deafness in our population thereby decreasing socio-economic burden. The efforts made toward the discovery of mutation in SLC26A4 gene ,its refinement and its characterization of the gene will significantly make a contribution to identification of a novel gene that is essential for the development and/or persistence of normal hearing and healthier understanding of molecular events involved in the course of hearing. One the mutation in the gene is consistent with DFNB4 is found, function of the gene and effect of the particular haplotype and associated mutation may provide insight to the associated clinical findings.

CHAPTER 6

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REFERENCES

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