›CREENING OF GJB2 INVOLVED IN NON-SYNDROMIC AUTOSOMAL RECESSIVE DEAFNESS IN SELECTED FAMILIES

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(2013-2015)

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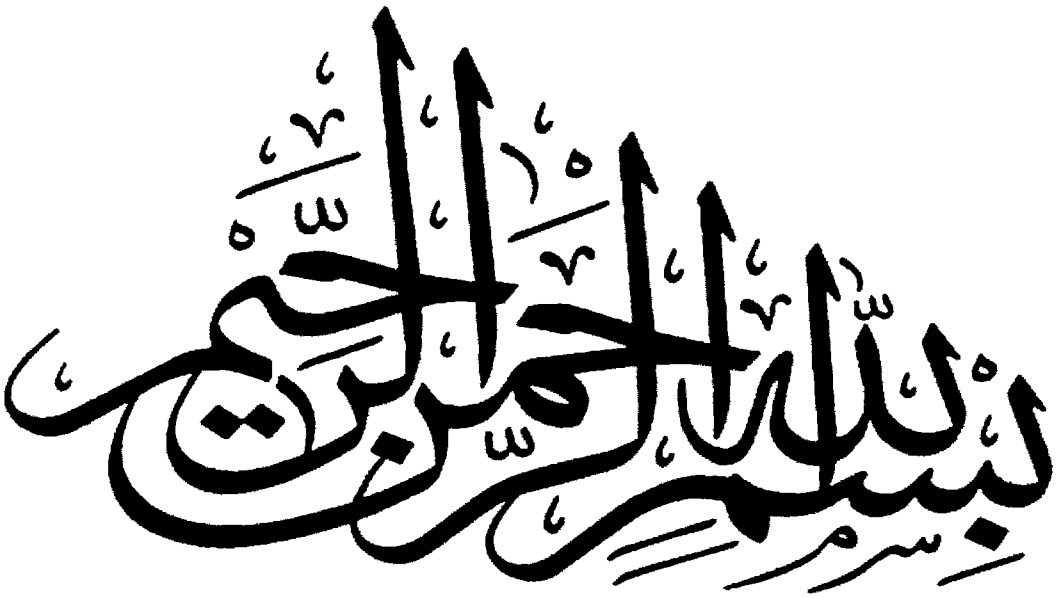
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FINAL APPROVAL

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Dedicated to My Respected Parents

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.

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LIST OF ABBREVIATIONS

3'UTR	3' Untranslated Region
35delG	35 Deletion Guanine
167delT	167 Deletion Thymine
235delC	235 Deletion Cytosine
377_378ins ATGCGGA	377_378 Insertion ATGCGGA
A	Adenine
ACD	Acid Citrate Dextrose
ARNSHL	Autosomal Recessive NonSyndromic Hearing Loss
Arg127His	Arginine 127 Histidine
BERA	Brain Stem Electric Response Audiometry
bp	Base Pair
C	Cytosine
CDH23	Cadherin 23
cm	Centi meter
Cx26	Connexin 26
dB	Decibel
delE120	Deletion Glutamic Acid 120
DFN	Deafness
DNA	Deoxyribonucleic acid
dNTPs	Deoxy Nucleotide Tri Phosphate
E	Extracellular
EDTA	Ethylene diamine tetraacetic acid
EVA	Enlarged vestibular duct
g	Gram
G	Guanine
GJB2	Gap Junction Protein Beta-2
GJB6	Gap Junction Protein Beta-6
Gln124X	Glutamine 124 Stop Codon

Glu147Lys	Glutamic Acid 147 Lysine
Glu47X	Glutamic Acid 47 Stop Codon
Gly200Arg	Glycine 200 Arginine
HCl	Hydrochloric Acid
IHCs	Inner Hair Cells
OHCs	Outer Hair cells
kb	Kilo Base
kDa	Kilo Dalton
$MgCl_2$	Magnesium Chloride
ml	Milli Litre
mM	Milli molar
mV	Milli volts
M34T	Methionine 34 Threonine
MRI	Magnetic Resonance imaging
mRNA	Messenger Ribonucleic acid
MYO15A	Myosin 15A
nm	Nano Meter
NSHL	Non syndromic hearing loss
OAE	Otoacoustic emissions
OTOF	Otoferlin
PCR	Polymerase Chain Reaction
PWDs	Persons with disabilities
R32H	Arginine 32 Histidine
RPM	Revolutions per Minute
SCL26A4	Solute Carrier Family 26 Member A4
SDS	Sodium Dodecyl Sulphate
SNHL	Sensorineural Hearing Loss
SNP	Single Nucleotide Polymorphism
STE	Sodium Chloride Tris EDTA
T	Thymine
Thr8Met	Threonine 8 Methionine

TM	Transmembrane
TMC1	Transmembrane channel 1
TAE	Tris Acetate Ethylene diamine tetraacetic acid
µl	Micro Litre
UV	Ultraviolet
UCSC	University of California, Santa Cruz
Val153Ile	Valine 153 Isoleucine
WHO	World Health Organization
W24X	Trptophan 24 Stop Codon
W77X	Trptophan 77 Stop Codon

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ABSTRACT

The profound bilateral loss of ARNSHL is observed in 1.6 out of 1000 neonates in Pakistan. Among the genetic causes, *GJB2* gene has gained interest because it is the foremost reason of causing ARNSHL in multiple populations with a worldwide prevalence of 21.3% for GJB2 associated ARNSHL and 53% of autosomal recessively inherited hearing loss cases within the Punjab province of Pakistan show mutations in this gene. MYO15A gene is found as the second most common cause with 13% prevalence in the Punjab province. Literature review focuses on GJB2 prevalence in different regions of world and within Pakistan. Exploring the deafness causing gene and mutations in the particular region will facilitate to develop screening plans to identify carriers, before birth and neonatal diagnostic ways well suited for that region providing early treatment for children and genetic counseling strategies for affected families. There is little data on frequencies of GJB2 gene mutations for the hearing impaired from the Northern region of Pakistan. In the current study, initial screening of GJB2 mutations was performed by Sanger sequencing in 8 ARNSHL families belonging to the Northern region of Pakistan. No recurrent or novel mutation was identified in this cohort of ARNSHL families. The families need to be analyzed further for any other causative gene. For the genetic diagnosis GJB2 (Connexin 26) mutations are the first choice to be screened in populations where its prevalence is very high, such as in Punjab and Sindh. But this report confirms that connexin 26 screening is not the ideal for genetic diagnosis in Pathan population.

Chapter 1

INTRODUCTION

Deafness is the partial or complete non-functionality of the sense of hearing for ordinary purposes of life and delay in its detection can influence and cause serious impact on development of speech, language skills acquisition and cognitive development that can lead to negative effects on emotional and social quality of life. Hearing loss is causing problems globally expressing at multiple ages with different severity and types as according to WHO estimates, more than 5% of the world population suffer from hearing impairment. Hearing loss is categorized into syndromic and non syndromic types on the basis of association with other phenotypic characters. Syndromic form is characterized by hearing loss along with additional physical findings or abnormalities in one or several organs. Non syndromic deafness is characterized by lack of hearing with no other associated clinical features (Irshad *et al.*, 2012). Hearing loss can be due to genetic as well as environmental factors or a combination of both. Among the different forms of hearing loss, autosomal recessive non syndromic hearing loss (ARNSHL) is the most common with about 80% prevalence (Shafique et al., 2014). *GJB2* gene has gained interest because it is the foremost reason of causing ARNSHL in multiple populations with a worldwide prevalence of 21.3% for GJB2 associated ARNSHL and 53% of autosomal recessively inherited hearing loss cases within the Punjab province of Pakistan show mutations in this gene (Chan *et al.*, 2014., Shafique *et al.*, 2014).

An individual's ability to hear is reduced due to defect in the normal process of sound transduction that can impede language acquisition if left unrecognized or untreated and has far-reaching effects on the quality of life. Earlier detection can improve the prognosis of disease. The severity of problem differs from person to person and there lies a wide range of different degrees of hearing between "hearing well" and "hearing nothing" (Dhingra et al., 2011). The different degrees of severity of hearing loss are measured in decibels and each degree has different impact on the ability to understand speech. 'Pure Tone Audiometry' is used to confirm the degree of hearing loss. A general categorization system and the impact of each category is shown in table 1-1, however there are slight differences in this categorization from study to study:

Table 1-1: Hearing loss and impact on hearing speech (Adapted from Dhingra *et al.*, 2011)

Hearing threshold in better ear	Impairment degree	Speech understanding ability
0-25	Not significant	Faint speech is easily understood.
26-40	Mild	Faint speech is difficult to understand.
41-55	Moderate	Normal speech is frequently difficult to understand
56-70	Moderately severe	Loud speech is even frequently difficult to understand
71-91	Severe	Amplified speech is only understood
Above 91	Profound	Amplified speech is even not understood.

The different ways to classify the loss of hearing are dependent on the onset age, type of defect, association and frequency range. Among others, prelingual, postlingual, conductive,

sensorineural, mixed, non-syndromic and syndromic are some forms of loss of hearing (Dror *et al.*, 2010, Musani *et al.*, 2011).

The incidence of severe prelingual sensorineural hearing impairment is reported in about 1 of 1000 neonates worldwide (Shafique *et al.*, 2014), which makes it the most widespread congenital neurosensory disorder requiring great improvement in diagnosis and treatment. The frequency of hearing loss increases to 2.7 per 1000 during adolescence and 3.5 per 1000 before the age of 5 (Morton and Nance, 2006). Moreover, 10% of people aged 60 and 50% of people aged 80 are victims of hearing loss called presbycusis, the hearing loss in the old age (Davis, 1989). The profound bilateral loss of hearing is observed in 1.6 out of 1000 neonates in Pakistan (Shafique *et al.*, 2014). ARNSHL is more prevalent in countries where consanguineous marriages are common such as it is estimated in Pakistan that the prevalence of the disease is near about 10% of the population (Masuni *et al.*, 2011, Sajid *et al.*, 2008) and 70% newborn cases are arising in consanguineous families (Elahi *et al.*, 1998) that are two third of the marriages in Pakistan (Riaz *et al.*, 2012).

The different causes for hearing loss can be prenatal, perinatal or postnatal. The etiology of hearing loss varies from country to country, however, it is generally considered that 40-50% of hearing loss is due to environmental factors while atleast 50-60% cases of early and delayed onset hearing loss including presbyacusis are genetically determined (Irshad *et al.*, 2012, Morton and Nance 2006, Teek *et al.*, 2010) that pose greater challenges.

Among the newborns with severe prelingual sensorineural hearing loss, genetic origin is shown in nearly 50% of cases (Birkenhager *et al.*, 2014, Du *et al.*, 2014). The segregation of deafness is as a monogenic trait but there are also some studies suggesting a digenic pattern of inheritance (Friedman and Griffith, 2003) like *GJB6* mutation was detected in trans with *GJB2* mutation in 26% of Malaysian hearing loss individuals (Asma *et al.*, 2011). The segregation forms followed by monogenic deafness are autosomal recessive, autosomal dominant, X-linked and Mitochondrial. ARNSHL is observed as most frequent hearing loss pattern and is usually congenital, stable with little progression, more severe than the other forms and cochlear defects

are determined as the exclusive cause for this form (Morton and Nance 2006, Petersen and Willems 2006).

The genes most frequently associated with ARNSHL are GJB2, SCL26A4, OTOF, MYO15A, TMC1 and CDH23. The gene that is considered most important for ARNSHL in most populations is *GJB2* since it accounts for up to 50% cases of ARNSHL in multiple populations worldwide (Birkenhager *et al.*, 2014, Battellino *et al.*, 2011, Matos *et al.*, 2013, Mustapha *et al.*, 2001, Sebeih *et al.*, 2013, Teek *et al.*, 2010).

GJB2 encodes "Connexin 26" that is a protein expressed highly in human cochlear cells and involved in ion homeostasis in inner ear. *GJB2* gene show variable prevalence in different populations throughout world and exhibits race specific mutations contributing to hearing loss i.e., actual mutations of *GJB2* contributing to hearing loss differs between specific ethnic groups (Chan *et al.*, 2014).

The ARNSHL molecular diagnosis is complicated by extreme genetic heterogeneity as different mutations in one gene can cause hearing loss of different forms that can be autosomal dominant and recessive, syndromic and non-syndromic but an encouraging find inspite of this heterogeneity of non-syndromic deafness is the confirmation of high prevalence of single gene and single highly prevalent mutation responsible for the majority of cases of Sensineural Hearing Loss (SNHL) in specific populations having direct implications for population screening and patient management. For example, within the Indian subcontinent populations, it may seem reasonable to screen for the W24X mutation where it is very common (Ramshankar *et al.*, 2003) however within European population, it will not have much importance as 35delG is frequent mutation there but not W24X (Popova *et al.*, 2012). These studies help in designing screening strategies by establishing the prevalence of genes and their race specific mutations. Less data available on the prevalence of *GJB2* gene from the Northern region of Pakistani population led us to investigate the prevalence and identification of GJB2 mutations in Northern region of Pakistan.

The Northern area of Pakistan, a region of 72,496 sq km includes Dir, Swat, Chitral, Hazara, Gilgit Baltistan and comprises of app 970347 people, characteristic with high frequency

(60%) of consanguinity (Sajid *et al.*, 2008) favoring the incidence of autosomal recessive disorders. According to PWDs report 2012 (Persons with disabilites (PWDs) statistics in Pakistan 2012) deaf are 7.7% of KhyberPakhtoonkhaw population, however specifically for Northern region, no statistics are available. Sajid and group in 2008 found parental consanguinity in 86.4% children with sensorineural hearing loss from the KhyberPakhtoonkhaw province.

The current study is based on the need to know more about genetic constitution of the Pakistani population in correspondence with hearing loss, a deeper scrutiny of the prevalence of GJB2 gene for hearing loss in particular populations of Pakistan; hence it involves the screening of GJB2 gene in hearing loss families of a particular area. Exploring the deafness causing gene and mutations in the particular region will facilitate to develop screening plans to identify carriers, before birth and neonatal diagnostic ways well suited for that region providing early treatment for children and genetic counseling strategies for affected families that are at risk.

There is little data on frequencies of GJB2 gene mutations for the hearing impaired from the Northern region of Pakistan. There is no diagnostic genetic test available in this region for loss of hearing because of an incomplete genotypic picture and lack of any well-known molecular cause for ARNSHL in Pakistani families of this region. This study is part of comprehensive genetic analysis of deaf families from Northern region of Pakistan to determine the genetic factor causing deafness in these families. The aims and objectives of the present study are to:

Ascertain the GJB2 gene prevalence in Northern area families of Pakistan affected with ARNSHL.

Chapter 2

LITERATURE REVIEW

Loss of hearing is caused due to defect in the normal mechanism of sound transduction. The anatomy of human ear and normal process of hearing is described below:

2.1 Anatomy of Human Ear and Normal Hearing Mechanism

The different parts of the ear function as a single unit (Fig 2-1). Collection of sound is done by outer ear, amplification by middle ear and conversion to nerve impulse is done by the inner ear which is then transmitted to the brain and interpreted as sound.

2.1.1 The External Ear

The external ear comprises of the:

i. Pinna,
ii. Auditory canal,
iii. The tympanic membrane or eardrum (Fig 2-1).

The pinna after collecting sound waves from the environment directs them into the auditory canal. The sound travelling through the auditory canal reaches the tympanic membrane i.e: thin partition separating outer ear from the middle ear and vibrates the tympanic membrane. The vibration of the ear drum transmits sound from the air to the middle ear ossicles (Fig 2-1).

2.1.2 The Middle Ear

The middle ear comprises of the:

i. Tympanum cavity,
ii. Eustachian tube,
iii. The three auditory ossicles (Fig 2-1).

Tympanum cavity is a narrow cavity filled with air. Tympanic membrane separates it from external ear and a bony partition separates it from inner ear. Eustachian tube is a small tube connecting middle ear to the nasopharnx and is responsible for filling fresh air in the middle

ear cavity that ensures air pressure is equalized on both sides of the tympanic membrane. The three middle ear bones are the important structures of the middle ear involved in sound transduction to the cochlea in the inner ear. The Malleus receives vibrational energy created by the sound striking the ear drum and transmits this energy via incus to the stapes that has its attachment with the oval window present on the bony partition separating air-filled tympanic cavity from the fluid filled membranous labyrinth. As the third ossicle stapes vibrates, it displaces the oval window (*fenestra vestibule*) that acts as opening between middle and inner ear and the vibrational energy is transmitted to the cochlear fluid and basilar membrane in the inner ear (Friedman and Griffith, 2003). The round window acts as a piston in this process. The relative differences in size between the pinna, tympanic membrane and the ossicles cause the amplification of sound during this period.

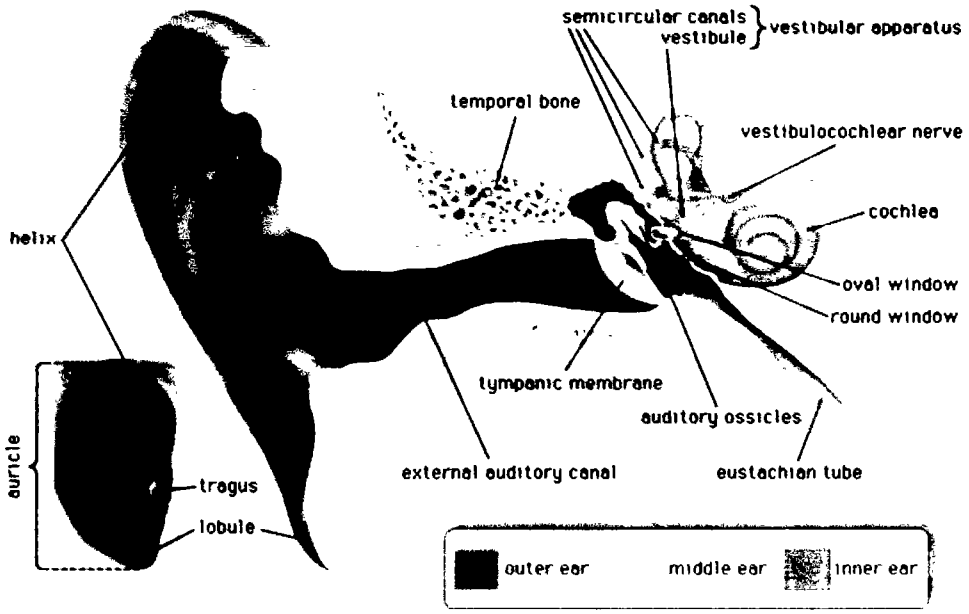


Fig 2-1 Structure of human ear showing Outer, Middle and inner ear (Retrieved from http://www.britannica.com/EBchecked/media/530/Structure-of-the-human-ear?topicId=175622)

2.1.3 The Inner Ear

The inner ear or labyrinth is responsible for controlling two sensory systems: cochlear system for sound transduction and the vestibular system for maintaining balance. Inner ear is comprised of the cochlea, the cochlear duct, vestibule, the semicircular canals and ducts, the endolymphatic duct and sac and the utricle and saccule. Of all these structures, the cochlear system is considered to be the most important in the hearing process. The cochlea is a small coiled tube of 35mm length and 10mm width forming 2.5 turns to 2.75 turns. Three fluid-filled

compartments: scala vestibuli, scala tympani and scala media comprises the cochlea (Friedman and Griffith, 2003). Scala tympani and scala vestibuli are interconnected through helicotrema and perilymph fills them that is a fliud with ionic composition similar to other extracellular fluids: i.e, being low in potassium ions (~5mM) and high in sodium ions (~145mM). Endolymph fills the Scala media and endolymph is of different composition to that of perilymph i.e, being high in potassium ions (~150mM) and low in sodium ions (1.3mM) (Jagger *et al.*, 2010). Scala media is about 80mV more positive than the Scala tympani and Scala vestibule resulting in a difference called as endocochlear potential (Purves *et al.*, 2001). Cochlear duct consists of basilar membrane, the Reissner's membrane and the stria vascularis which secretes endolymph (Fig: 2-2)

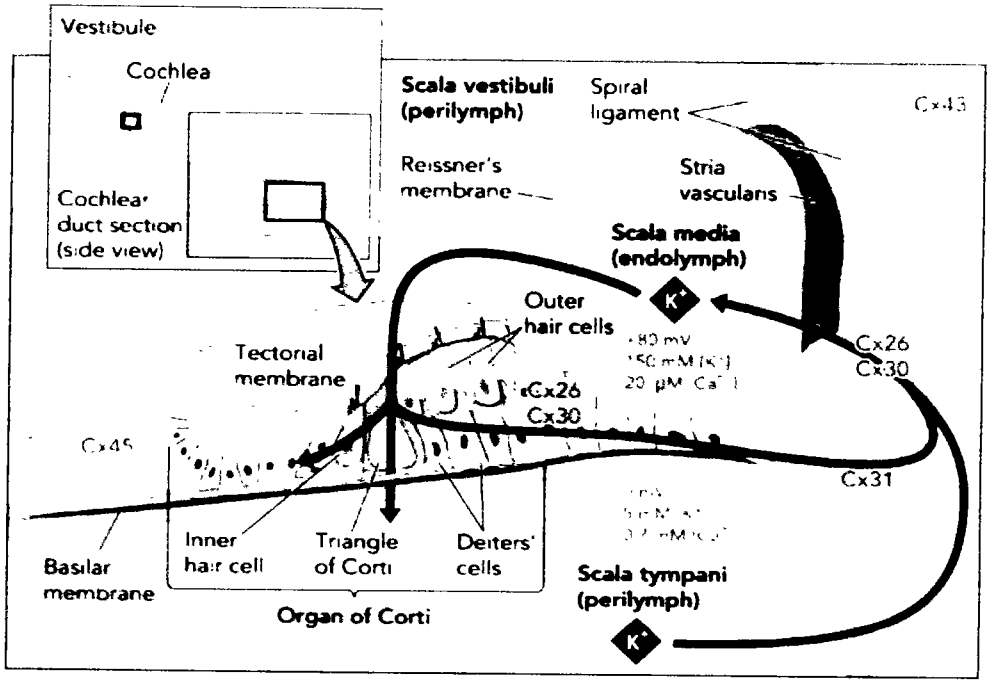


Fig. 2-2 Cochlear duct showing basilar membrane, Organ of Corti, Hair cells, Supporting cells, Reissner's membrane, Stria vascularis, Scala vestibule, Scala media and Scala tympani (Retrieved from http://physiologyonline.physiology.org/content/22/2/131).

Organ of Corti converts the mechanical vibration in the fluid of the cochlear duct or scala media into electrical impulses so it is the sensory transduction system of the ear. Organ of Corti is composed of several cell types including supporting cells (e.g. inner and outer pillar cells, Claudius', Hensen's and Deiters' cells), outer and inner hair cells, a specialized basement membrane (the basilar membrane), nerve endings and the tectorial membrane (Fig: 2-2).

The actual sound transducing cells are the inner hair cells. Outer hair cells are playing role in amplification of the low-level sound that enters the cochlea. The mechanical wave in the cochlear fluid moves the basilar membrane in the scala media deflecting the stereocilia of hair cells which opens ion channels of the hair cells and influx of potassium ions to the hair cells depolarizes the hair cells, this change in action potential opens the calcium channels in the hair cell membrane and movement of calcium ions inside the hair cells through calcium channels trigger neurotransmitter release onto the endings of the auditory nerve and ultimately nerve impulses are generated. (Purves *et al.*, 2001). These nerve impulses traveling through the auditory nerve reaches the brain where they are interpreted as sound.

Relative to perilymph, the resting potential of hair cell is between -45 and -60mV which means that the perilymph is about 45mV more positive than the hair cell inside and endolymph is 125 mV more positive than the hair cell inside, the gradient produced as a result of this across the stereocilia membrane (about 125mV) moves K^+ through open ion channels to the inside of the hair cell (Purves *et al.*, 2001). High concentration of potassium ions in the endolymph and low concentration in the perilymph is necessary for maintaining this positive endocochlear potential that is required for generating positive current in the hair cells of inner type. The outside movement of potassium ions from the basal surface of the hair cells occurs due to low concentration of potassium ions in the perilymph that bathes the basal end of hair cells. The gap junction channels extending from the plasma membrane of epithelial supporting cells under the hair cells to the spiral ligament and to the stria vascularis reset the mecahnoelectrical transduction system of ear by allowing recirculation of the potassium ions from the perilymph to the stria vascularis and the endolymph. This ion homeostasis is requirement of normal auditory function and any disturbance in it can lead to hearing impairment (Wei *et al.*, 2013).

2.2 Hearing Loss Classification

There are different ways to classify the hearing loss. Sometimes, it is classified on the type of onset age into prelingual that develops before the acquiring of language skills and post lingual that develops after the acquiring of language skills (Irshad *et al.*, 2012).

Based on the defective part of the ear, hearing loss is classified into conductive, sensorineural and mixed types. Conductive hearing loss disturbs the movement of sound to the inner ear from the outer due to faulty outer or middle ear. Sensorineural deafness is due to problem in the pathway through which sound waves reach the auditory nerve and brain and

sound is not transmitted to the brain by the nerves or sensory cells. Sensorineural and conductive hearing loss both in combination is called hearing loss of mixed type (Masuni *et al.*, 2011., Irshad *et al.*, 2012).

Hearing loss is categorized into Syndromic and Non-Syndromic types on the basis of association with other phenotypic characters. Syndromic form is characterized by hearing loss along with additional physical findings or abnormalities in one or several organs. Non-Syndromic deafness is characterized by lack of hearing with no other associated clinical features (Irshad *et al.*, 2012).

2.3 Hearing Assessment Tests

The negative effects of hearing loss can be reduced by earliest possible detection and treatment of the problem otherwise, it will be detected later on when the individual is bearing its effects. There are different tests available for assessment of hearing loss. In neonates, Otoacoustic Emissions (OAE) measurement is the standard and primary method for detection of hearing loss whereas Brain stem electric response audiometry (BERA) is done as a next step (Dhingra *et al.*, 2007). These tests are based on detection of a response at a particular threshold. Hearing loss therapy and further investigations initation is based on these objective measurements. The different tests available for assessment of hearing in adults include Finger friction test, Speech tests, Watch test and tuning fork test Pure Tone Audiometry, Speech Audiometry, Threshold Tone Decay Test, Evoked Response Audiometry, Otoacoustic Emissions and Central Auditory Tests (Dhingra et al., 2007). A complete physical examination is required to rule out the syndromic form and acquired cases of hearing loss. Hearing loss with a genetic basis is diagnosed by physical and audiological examination excluding the acquired causes, family history and DNA based testing. The importance of genetic analysis can not be denied when screening newborns as genetic testing will help in making a definite and speedy diagnosis of the underlying cause of inherited non syndromic hearing loss preventing further unnecessary investigations, providing prognostic information, optimal management and allowing subsequent genetic counseling.

2.4 Aetiology of Hearing Loss

2.4.1 Non-Genetic Causes

Non genetic causes include prenatal infections (Toxoplasmosis, Cytomegaloviruses, Rubella, Syphilis, Herpes type 1 and 2) and postnatal infections (measles, mumps, varicella, influenza, meningitis, encephalitis), ototoxic medications, radiation to mother in first trimester, neonatal anoxia, prematurity and low-birth weight i.e., less than 1500g, birth injuries, neonatal jaundice, secretory otitus media, trauma, noise exposure and some other factors (Saunders *et al.*, 2007). Ototoxic drugs either given during pregnancy like streptomycin, gentamycin, tobramycin, amikacin and quinine cross the placental membrane and damage cochlea or may be given latter in life and cause damage like drugs used for neonatal meningitis (Yorgason *et al.*, 2006). Birth injuries that may include forceps delivery leading to extravasation of blood into the inner ear is one of the causes of hearing loss (Dhingra *et al.*, 2011). Secretory otitus media is due to damage to the Eustachian tube accumulating the fluid in middle ear and sound transduction is hindered. Sudden loud noise can result in Acoustic trauma leading to temporary and/or permanent hearing loss, on the other hand continuous exposure to high intensity sound for prolonged periods also poses high risk for auditory function (Dror *et al.*, 2011). Malnutrition, diabetes, thyroid deficiency and alcoholism of mother may affect the development of auditory system of foetus (Dhingra *et al.*, 2011). Reduction or prevention of environmental factors is possible by raising awareness for appropriate protection.

2.4.2 Genetic Causes and Inheritance Pattern of Deafness

The two categories of genetically determined deafness or hearing impairment are syndromic and non syndromic. Syndromic deafness accounts for 30% cases of genetically determined deafness (Shafique *et al.*, 2014). Syndromic deafness can be either Dominant (Treacher-Collins syndrome, Crouzon's syndrome and Apert's syndrome etc.), Recessive (Usher syndrome, Klippel-Feil syndrome and Pendred syndrome etc.), or X-linked (Wildervanch syndrome, Alport syndrome etc.) (Dhingra *et al.*, 2011). Non-syndromic accounts for the remaining 70% of cases (Van Camp *et al.*, 1997). The extraordinarily genetic heterogeneity for non-syndromic hearing impairment is continuously being explored with the progress of molecular genetics and up to now about 160 genetic loci and 90 genes responsible

for NSHL have been explored (http://hereditaryhearingloss.org/, updated in May 2015). ARNSHL is observed in 80% of cases, autosomal dominant (18%), X-linked (1-3%) and mitochondrial<1% (Bayazit *et al.*, 2006). Autosomal dominant mode of inheritance designated as DFNA is followed by 58 of the identified loci (32 genes), autosomal recessive referred as DFNB is followed by 86 loci (60 genes), X-linked mode of inheritance named as DFN is followed by six loci (four genes) and there has been also report of two mitochondrial genes (Hereditary hearing loss homepage: http://hereditaryhearingloss.org).

2.5 Frequent Genes for ARNSHL

The genes most frequently observed in ARNSHL are shown in the table 2.1:

Table 2-1: Genes most frequently observed in ARNSHL

Locus	Cytogenetic location	Gene	Reference (MIM#)	Number of mutations worldwide	Exon	Protein	Role in hearing process
DFNB1A	13q11-q12	*GJB2*	121011	>220	2	Connexin 26	Homeostasis of ions
DFNB4	7q31	*SLC26A4*	605646	60	21	Solute carrier family 26 (anion exchanger) member 4	Homeostasis of ions
DFNB3	17p11.2	*MYO15A*	602666	28	66	Myosin XVA	Development and maintenance of stereocilia of hair cells, motor protein
DFNB9	2p23-p22	*OTOF*	603681	26	48	Otoferlin	Neurotransmitter release
DFNB12	10q21-q22	*CDH23*	605516	21	69	Cadherin related 23	Organize Hair bundle, adhesion protein
DFNB7/11	9q13-q21	*TMC1*	606706	21	24	Trans membrane channel like 1	Ion channels, ion pumps or transporters

(This table is modified form of table taken from Hilgert *et al.*, 2009)

2.6 Connexins and *GJB2*

The 5.5kb long Gap junction beta-2 gene (*GJB2*) coding for 26kDa protein Connexin 26 (*Cx*26) of 226 amino acids was the first non-syndromic hearing loss gene identified by Kelsell and team in 1997. It is localized on chromosome 13q11-q12 (Fig: 2.3) at genetic locus DFNB1 in tandem orientation with the *GJB6* gene (MIM 604418) and is highly expressed in human cochlear cells apart from the other tissues (skin, liver and pancreas) (Kelsell *et al.*, 1997).

Fig 2-3: Chromosomal location of GJB2 gene. (Retrieved from http://www.genecards.org/cgi-bin/carddisp.pl?gene=GJB2)

GJB2 gene belongs to the Connexin gene family. Connexin gene family comprises of 21 members in humans that code for transmembrane proteins expressing in different tissues and forming clusters of intercellular channels that serve as gap junctions facilitating communication between the neighbouring cells (Sohl *et al.*, 2004). GJB2 is comprised of 2 exons, the first one remains untranslated while the 2nd one is coding producing an mRNA product of 2.4Kb. The exon 1 encoding 5'-untranslated region is separated by an intron of different length from the exon 2 comprising of open reading frame of 678 nucleotides and the 3'-UTR.

6 connexin (*Cx*) sub-units bind in groups around a central pore of 2-3nm to form a hexamer molecular complex called connexon that is localized in the cell membrane and enables the exchange of a number of compounds of molecular weight <1-2kDa including metabolites, secondary messengers, nutrients and charged ions such as potassium and calcium by associating with the corresponding connexon residing in the plasma membrane of neighbouring cells with covalent bonds and forming an intercellular canal. The clustering of these multiple canals in a specialized membrane region is termed as gap junction (Sohl *et al.*, 2004, Kumar *et al.*, 1996). (Fig 2-4.A).

Connexin sub-units are comprised of four domains spanning the membrane (TM1-TM4), two extracellular domains (E1, E2), a cytoplasmic loop and cytoplasmic amino and carboxy termini. The three conserved cysteine residues in extracellular loops are suggested to stabilize the extracellular region of the connexin structure by forming three intramolecular disulphide bridges between the conserved cysteine residuals in connexon hemichannels (Birkenhager *et al.*, 2014).

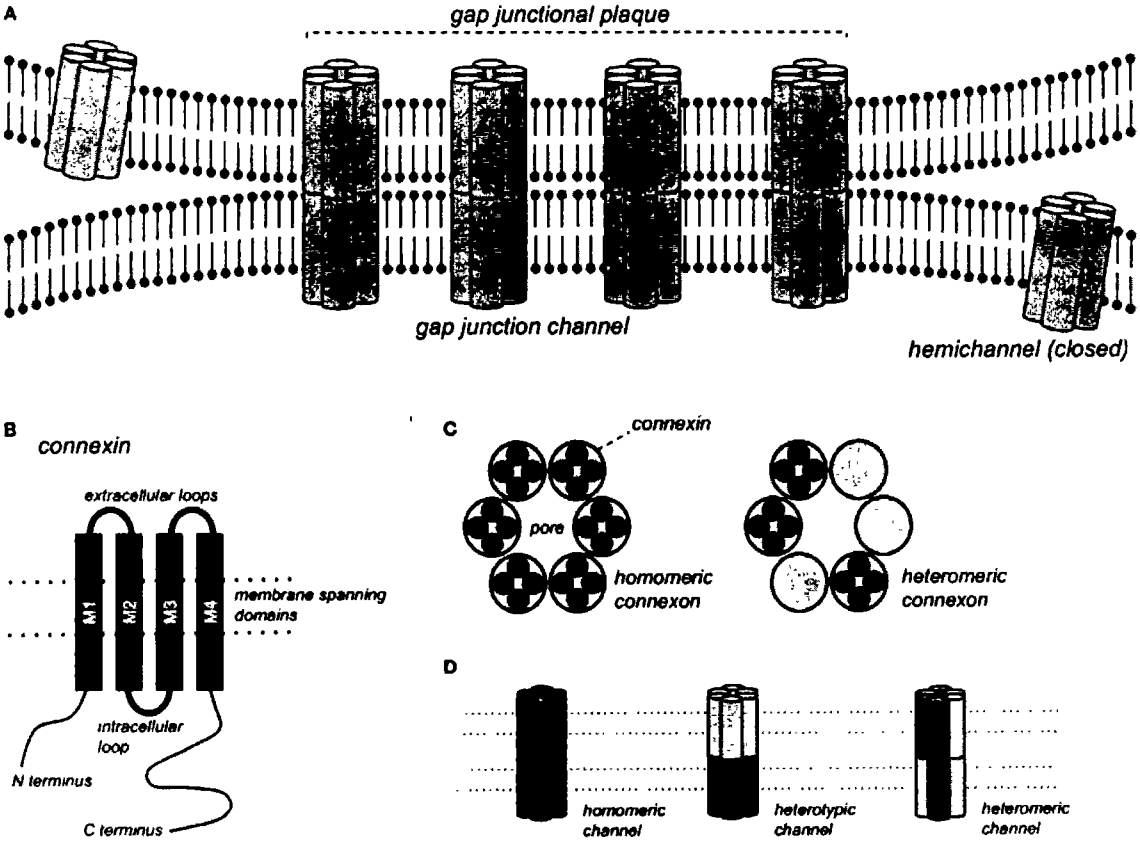


Fig. 2-4 A: Gap junction channel, B: Structure of Connexin, C: Homomeric and hetromeric connexons, D: Homomeric, hetrotypic and heteromeric channel (Retrived from http://www.frontiersin.org/files/Articles/53434/fphar-04-00081-HTML/image_m/fphar-04-00081-g001.jpg)

Connexons can be formed by combination of one type of connexins (homomeric) or by combination of different type of connexins (heteromeric) (Fig 2-4.C). Each Connexin protein perform distinct physiological activities with difference in ionic selectivity and gating mechanisms. The gap junctions formed by connexin 26 in the inner ear are responsible for maintaining potassium ions homeostasis during the process of sound transduction (Birkenhager *et al.*, 2014). Disturbance in the electrolyte exchange system in the cochlea leads to hearing impairment by changing the electrical potential of cochlea that is required for transmission of sound signals to the brain (Wei *et al.*, 2013).

2.7 Global Prevalence and Mutation Spectrum of *GJB2* Gene for Hearing Loss

The research started with the identification of DFNB1 locus on 13q11 as the first ARNSHL locus in consanguineous families from Tunisia (Guilford *et al.*, 1994). *GJB2* gene was first identified by Kelsell and colleagues (1997) by studying a Caucasian pedigree in which both autosomal dominant palmoplantar keratoderma and congenital sensorineural deafness were segregating. The mutation M34T identified in two affected individuals from this pedigree segregated with profound deafness but not the skin disorder suggesting that *GJB2* is the genetic basis of the autosomal dominant nonsyndromic sensorineural deafness. As the recessive deafness locus DFNB1 also maps to the same region on chromosome 13 as *GJB2*, further study by Kelsell and team (1997) on consanguineous ARNSHL families belonging to Pakistan linked to DFNB1 identified this gene to be associated with ARNSHL by detecting p.W77X and p.W24X mutations in these pedigrees segregating with hearing loss.

The small size, simple organization and high mutation spectrum in multiple populations make GJB2 gene an important and relatively easy target for mutation screening. Research found *GJB2* to be leading cause for ARNSHL with about 200 recessive mutations identified worldwide (Birkenhager *et al.*, 2014). These include missense, frameshift, nonsense, deletions, splice site and mutation that results in no protein production. These mutations can be expressed in homozygous or compound heterozygous form. (Connexin Deafness Homepage: http://davinci.crg.es/deafness/).

Since the indication of GJB2 gene for ARNSHL, multiple studies had been conducted worldwide for determining its prevalence. The GJB2 prevalence for ARNSHL and the common mutation found in some of the European countries is shown in the table 2-2:

Table 2-2: Prevalence of GJB2 gene in European countries

Location	Study subjects	GJB2 prevalence	Common mutation found	Reference
Austria	24	61.9%	35delG	Ramsebner *et al.*, 2007
Belarus	391	54%	35delG	Danilenko *et al.*, 2012
Bulgaria	51	41%	35delG	Popova *et al.*, 2012
Cyprus	30 families	33%	35delG	Neocleous *et al.*, 2006
Czech Republic	156	37.8%	35delG	Seeman *et al.*, 2004
Estonia	233	49%	35delG	Teek *et al.*, 2010
France	140	33%	35delG	Denoyelle *et al.*, 1999
Germany	335	20.59%	35delG	Bartsch *et al.*, 2010
Italy and Spain	193	46%	35delG	Rabionet *et al.*, 2000
Macedonia	80	12.5%	35delG	Stefanovska *et al.*, 2009
Poland	102	40.19%	35delG	Wiszniewski *et al.*, 2001
Portugal	264	20.8%	35delG	Matos *et al.*, 2013
Slovenia	208	26.6%	35delG	Batellino *et al.*, 2011
Sweden	79	33%	35delG	Carlsson *et al.*, 2012
Turkey	151	19.2%	35delG	Yalmiz *et al.*, 2010

Among these populations, the most frequent cause of ARNSHL identified from these studies is 35delG point mutation in GJB2 gene with prevalence rates of about 50-91% of all GJB2 hearing impairment alleles (Papova *et al.*, 2012, Manzoli *et al.*, 2013, Matos *et al.*, 2013). 35delG is deletion of one guanine among the stretch of six guanines present from 30 to 35 position in the GJB2 gene coding region. As a result, a truncated protein of 12 codons is produced that obstructs the normal circulation of potassium ions within the inner ear (Cifuentes *et al.*, 2013).

Within African populations, LucaTrotta (2011), Lasisi (2014), Gasmelseed (2004), Saunders (2007) with their respective teams found not a single case of hearing loss associated with GJB2 mutations among Cameeron, Nigerian, Kenyan and Nicaraguan hearing loss individuals respectively. Al-Achkar and team (2011) found biallelic GJB2 mutations in 52% of Syrian hearing loss families. In Ghana, Hamelmann and colleagues (2001) found biallelic

GJB2 mutations in 15.61% of hearing loss individuals and R143W mutation is found in 90% of affected chromosomes. Abidi and team (2007) determined GJB2 prevalence of 43.20% in Moroccon hearing loss individuals.

Within Saudi Arabia and Iran, 10% GJB2 prevalence is determined (Al-Qahtani *et al.*, 2010, Hashemi *et al.*, 2012). In 2012, Alkowari and colleagues reported biallelic GJB2 mutations in 9.1% of Qatari and hearing loss population. A high GJB2 prevalence of 33% is found in Lebanon while low prevalence of 6% is determined in Kuwait (Mustapha *et al.*, 2001, Sebeih *et al.*, 2013).

Within East Asian populations, Abe and team (2000) found GJB2 prevalence of 31.4% in Japanese hearing loss individuals with 73% of mutant alleles being 235delC. Wang and colleagues (2002) found GJB2 prevalence of 7.1% in Taiwan with 76% of mutant alleles being 235delC. Wei and team (2013) found GJB2 prevalence of 14.28% in Chinese hearing loss individual and 59% of the mutant alleles are 235delC.

A few other alleles with moderate frequency and some rare mutations reported in only one or two pedigrees are also observed (Hilgert et al., 2009).

2.8 *GJB2* Prevalence and Mutation Spectrum in the Indian Subcontinent and Different Regions of Pakistan

Multiple studies have been conducted in the Indian-Subcontinent for determining the prevalence of GJB2 gene for ARNSHL. In a study in 2003 by Ramshankar and team on ARNSHL Indian families the prevalence of GJB2 gene was found 18.18% with W24X present in all of the families and is 94% of the mutated alleles. Ramchander and colleagues (2005) found that W24X accounted for 86% of the pathogenic mutations identified and a GJB2 prevalence of 7.5% for hearing impairment in Indian population. Godbole and colleagues (2010) reported biallelic GJB2 alterations in 18.8% of hearing loss individuals in the Indian population with W24X and W77X found as the most common mutations. Bhalla and team in 2009 found homozygous GJB2 mutations in only 2.8% of North Indian hearing loss individuals. In 2008, Bajaj and team found GJB2 prevalence of 26% in British Bengali hearing loss individuals and W24X being the most prevalent is seen in 57% of patients.

Studies have shown a variable prevalence for GJB2 gene in different regions of Pakistan and p.(W24X) is found as a common mutation in Pakistan. The p.W24X mutation is due to substitution of G to A at nucleotide 71 resulting in a stop codon at codon 24 and forming a protein that is lengthwise one tenth of the original protein (Kelsell *et al.*, 1997). The low prevalence or absence of c.35delG, c.167delT and c.235delC and finding of p.W24X mutation to be the most frequent in the Indian sub-continent contributes further to the concept of race specific mutations observed worldwide.

The studies on Pakistani ARNSHL cases with relevance to the GJB2 gene are illustrated in Table 2-3 below:

Table 2-3: Prevalence of GJB2 gene in different regions of Pakistan

Location	Study Subjects	GJB2 Prevalence	GJB2 mutations	Reference
Pakistan	196 families	6.1%	p.W77X, p.W24X, c.167delT, p.R32H, p.delE120	Santos *et al.*, 2005
	20 families	50%	p.W24X, p.W77X, c.35delG, p.Gln124X, p.Thr8Met, p.Val153Ile	Anjum *et al.*, 2014
British Pakistani	79 patients	6.9%	p.W24X, c.35delG, p.Glu47X	Yoong *et al.*, 2011
Hazara	70 patients	4.28%	c.35delG, p.W24X	Bukhari *et al.*, 2013
Punjab	30 families	53%	p.W24X, p.W77X, c.35delG, p.Glu147Lys, p.Arg127His, c.377_378insATGCGGA, p.Gly200Arg	Shafique *et al.*, 2014

Chapter 3

METHODOLOGY

3.1 Study Approval

Committee of International Islamic University Islamabad gave the approval for this study. The family members were informed about the genetic research and a signed informed consent was obtained from the head of each family.

3.2 Collection of Family Information and Blood Samples

All the families presented here were collected from different regions of Northern area of Pakistan. Families with atleast two deaf children and no other physical abnormality associated with hearing loss were included in the study.

Blood samples were collected and histories were obtained by interviewing the families. About 5-6 ml blood was drawn from the available affected and unaffected individuals, ACD vaccutainers at kept at 4°C till the DNA extraction.

3.3 Pedigree Drawing on Computer Software

Information was taken from the family members and pedigrees were constructed. According to the standard method described by Bennet and colleagues (1995) for drawing pedigree, a square is used to represent male and a circle is used to represent female. Affected individual is represented by filled box while unaffected individual is represented by unfilled box. Slanting line over individual's box show deceased individual. The computer software i.e. Cyrillic 2.1, is used to draw all pedigrees.

3.4 DNA Extraction

Phenol chloroform method described by Sambrook *et al.*, 1989 was used for extracting genomic DNA from intravenous blood samples. Each blood sample (5ml) collected in the acid citrate dextrose (ACD) vaccutainer was shifted to falcon tubes and lysis buffer (Appendix A) was added to each sample (three times the volume of blood) and then for 30 minutes, the samples were placed on ice. Samples were centrifuged at 1200rpm for 10 minutes at 4°C, the supernatant formed was discarded and pellet was resuspended in minimum volume of the cell

lysis buffer. The pellet was washed twice by 10ml of cell lysis buffer, then treated with 4.75ml of STE buffer (Appendix A), 250µl of 10 % sodium dodecyl sulphate (SDS) (Appendix A) and 10µl of proteinase K (20mg/ml) and incubated in shaking water bath at 55°C for overnight. Next day, the samples were extracted with equal volume of equilibrated phenol, shaken for 10 min, kept on ice for 10 min and then centrifuged at 3200rpm for 30min at 4°C. After centrifugation, aqueous layer was carefully removed with cut tips into separate labelled tubes and chilled choroform-isoamyl alcohol (24:1) was added in equal volume, mixed and kept on ice for 10 min.

Sample tubes were centrifuged at 3200rpm for 30min at 4°C and aqueous layer was again carefully removed into separate labeled tubes using cut tips. 10ul of RNase (10 mg/ml) was added into the samples and incubated in water bath shaker at 37°C for 2 hours. The samples were taken out from water bath and further treated with 250ul of 10% SDS and 10ul of proteinase K (20mg/ml) and incubated in water bath shaker at 55°C for 1 hour. Phenol and chloroform iso-amylalcohol (24:1) was added in the samples for re-extraction of remaining proteins. Aqueous layer was obtained and treated with 500ul of 10 M ammonium acetate (Appendix A) and 5ml of chilled isopropanol (or 10ml of chilled absolute ethanol) and shaken until DNA precipitates as white visible threads. Samples were placed at -20°C for overnight and next day the sample tubes were centrifuged at 3200 rpm for 60min at 4°C. Supernatant was discarded, pellets were dried and DNA samples were re suspended in 10mM Tris HCl (pH 8.0) (Appendix A).

3.5 Optical Density Measurement

The optical density of DNA samples is determined using Spectrophotometer (Thermo Scientific Nano drop 2000C) that can measure the concentration of the DNA. The DNA samples were transferred to 1.5ml Eppendorf tubes and stored at -20°C until further use. 50ng/ul dilution of stock DNA was prepared for PCR amplification and stored at -20°C.

3.6 Designing of Primer

UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway) was used for designing primers for the coding sequence of *GJB2* gene. In-Silico PCR utility (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start) was used to check the virtual performance of designed primers against human genome. The sequence of forward primer is 5′ TCT TTT CCA GAG CAA ACC GC 3′ and sequence of reverse primer is 3′ ACT CGT GCC CAA CGG AGT AG 5′.

3.7 Polymerase chain reaction

The reaction comprising of 25µl reaction mixture with 50ng genomic DNA was performed in 0.2 ml tubes (Axygen, USA). Apart from that, reaction mixture contains 4µl 10x PCR buffer, 2µl 25mM $MgCl_2$, 1µl 25mM dNTPs, 1 unit of Taq DNA Polymerase, 1µl 0.5µM (5 picomol) forward and 1µl 0.5µM (5 picomol) reverse primer. Required volume is achieved by adding distilled water. After careful centrifugation of the reaction mixture for few seconds, the samples were placed in the thermal cycler. The conditions were initial denaturation at 95°C for 3mins, denaturation at 95°C for 0.45seconds, annealing at 62°C for 0.45seconds, extension for 1 min and final hold at 72°C for 10 minutes. The PCR was performed by using BIO RAD T100 ™ thermal cycler for 37 cycles. PCR amplified product is then resolved on ethidium bromide stained gel and were observed by visual inspection.

3.8 Preparation of 2% Agarose Gel

7gms of powered agarose was mixed with 350ml of 1x TAE buffer (Appendix B) and heated in microwave till a clear solution was formed. 5µl ethidium bromide (50mg/ml) (Appendix B) was added. For 25-30 minutes, gel solution was incubated in water bath at 55°C for 25-30. For pouring the gel, gel apparatus was arranged and 200cm gel plates were used with 20 to 40 wells comb. The solution of gel was poured and allowed to set for half an hour to about an hour. Comb was taken out of the gel after polymerization and gel was placed in the TAE buffer. Before loading the samples into the wells, in 7µl of sample, 5µl loading dye was added. For running the gel, the optimized conditions are 120 volts for 35minutes. After completing gel running process, the bands were visualized under the UV light using the gel documentation apparatus.

3.9 Sequencing PCR

Required segment of the DNA was amplified using specific forward and reverse primers at the optimized temperature in final volume of 50µl. 10µl of the PCR product was used to run on 2% agarose gel to check the quality and quantity of amplified products. For sequencing, 40µl PCR product was used.

3.9.1 Purification of the PCR Products

40 µL of PCR product, 4µL ammonium acetate (10M) in the 1:10 ratios and 80µL absolute ethanol were mixed and for 20 minutes kept on ice. For 10mins, the mixture was centrifuged at 14000rpm. Supernatant was discarded and pellet was washed with 200µL 70 % ethanol. Again, the samples were centrifuged at 14000rpm for 10minutes and air dried. Pellet was then resuspended in 15µl of deionized water.

3.9.2 Sequencing Reaction

2.0µL autoclaved deionized water, 4.0µL Big dye (*ABI* part no.402079), 3.0µL purified PCR product and 1µL of forward or reverse primer making up a total volume of 10µL were used for carrying out the sequencing reaction. The sequencing reaction was carried out as 25 cycles with 96°C for 1sec, 50°C for 5sec, and 60°C for 4min and final hold at 4°C.

3.9.3 Purification of Sequencing Reaction

10µL sequencing reaction product, 2.5µl 125mM EDTA and 30µl of absolute ethanol were mixed and for 15mins, left at room temperature as a part of the procedure to purify the sequencing reaction product. Then for 20mins the samples were centrifuged at 14000rpm. Supernatant was discarded and pellet was washed with 60 µl chilled 70 % ethanol. Again, the mix was centrifuged at 14000rpm for 10mins. Pellet was air dried after discarding the supernatant. Samples were resuspended was done with 10µl HiDi formamide (*ABI* part no. 4311320). Sequencing was carried out in Applied Biosystems (AB) 3130 Genetic Analyzer and performance optimization polymer 6 (*ABI* part no. 4363785) is used as separation matrix. Sequencing analyzer software was used to analyze the collected sequence data.

3.9.4 Analysis of Sequencing Results

The sequence data obtained for the *GJB2* gene was analyzed by comparing it with the reference sequence of wild type *GJB2* (GenBank No. NG_008358.1).

Chapter 4

RESULTS

4.1 Families for *GJB2* Sequence Analysis

Eight recessive families with individuals suffering from non syndromic hearing loss were analyzed in the current study. The families were collected from different regions of Northern area of Pakistan (Table 4-1). Families having at least two deaf children and no other physical abnormality associated with hearing loss were included in the study.

Histories were obtained by interviewing the families. After having written informed consent, about 5-6 ml blood was drawn from the available affected and unaffected individuals and kept at 4°C. These blood samples were used for the DNA extraction by phenol-chloroform extraction method. Sanger sequence analysis was used to determine the GJB2 prevalence in these families.

Table 4-1: Number of Families and their Region

No.	Pedigree no.	Region
1	DFN 42	Nowshera
2	DFN 43	Mardan
3	DFN 44	Mardan
4	DFN 45	Mardan
5	DFN 46	Mardan
6	DFN 47	Swat
7	DFN 48	Swat
8	DFN 49	Swat

4.1.1 DFN 42

Twelve members of family DFN 42 (Fig: 4-1) were sampled (DFN 4205, DFN 4210, DFN 4212: affected, DFN 4201, DFN 4202, DFN 4203, DFN 4204, DFN 4206, DFN 4207, DFN 4208, DFN 4209, DFN 4211: unaffected) from Nowshera, Pakistan. Samples were collected after written informed consent. The family is having non syndromic autosomal recessive deafness at an early age of presentation. All the affected individuals were having prelingual, severe to profound hearing loss. The normal individuals were also collected for segregation analysis.

4.1.2 DFN 43

The family DFN 43 (Fig: 4-2) was collected from Mardan, Pakistan. Ten members of the family were samples (DFN 4301, DFN 4306, DFN 407, DFN 4308, DFN 4310: affected, DFN 4302, DFN 403, DFN 4309: unaffected) after written informed consent. The pedigree structure of DFN 43 in figure 4-3 is showing autosomal recessive inheritance pattern. All the affected individuals were having prelingual, severe to profound hearing loss.

4.1.3 DFN 44

The family DFN 44 (Fig: 4-3) was ascertained from Mardan, Pakistan. Seven members were sampled (DFN 4405, DFN 4406, DFN 4407: affected, DFN 4401, DFN 4402, DFN 4403, DFN 4404: unaffected) after written informed consent. The pedigree structure in figure 4-3 is showing autosomal recessive inheritance pattern. The family had non syndromic hearing loss at an early age of presentation.

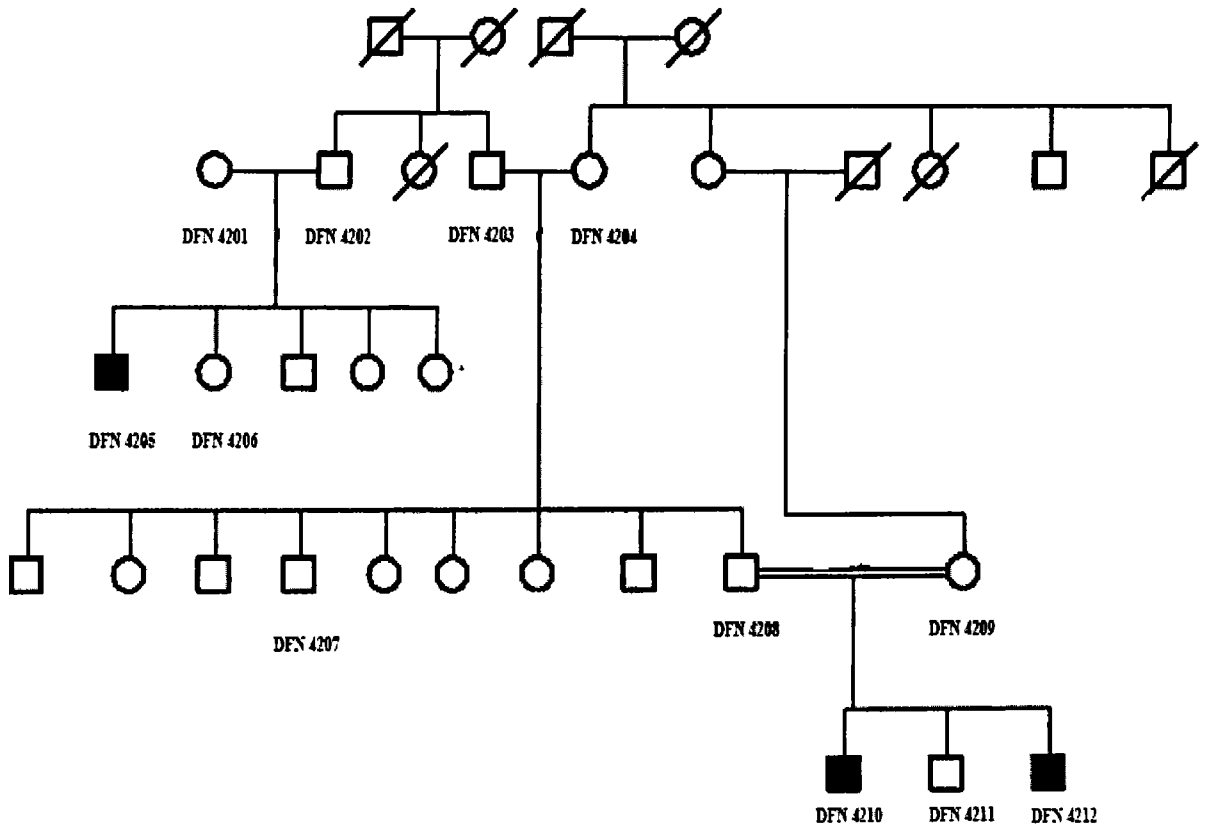


Figure 4-1: Pedigree of Family DFN 42. The females are represented by circles, squares are for males while filled squares are for affected males. The diagonal lines across the symbols represent the expired family members. Sampled individuals are represented by individual numbers (DFN 4201- 4212). Consanguinity is represented by double line in the pedigrees.

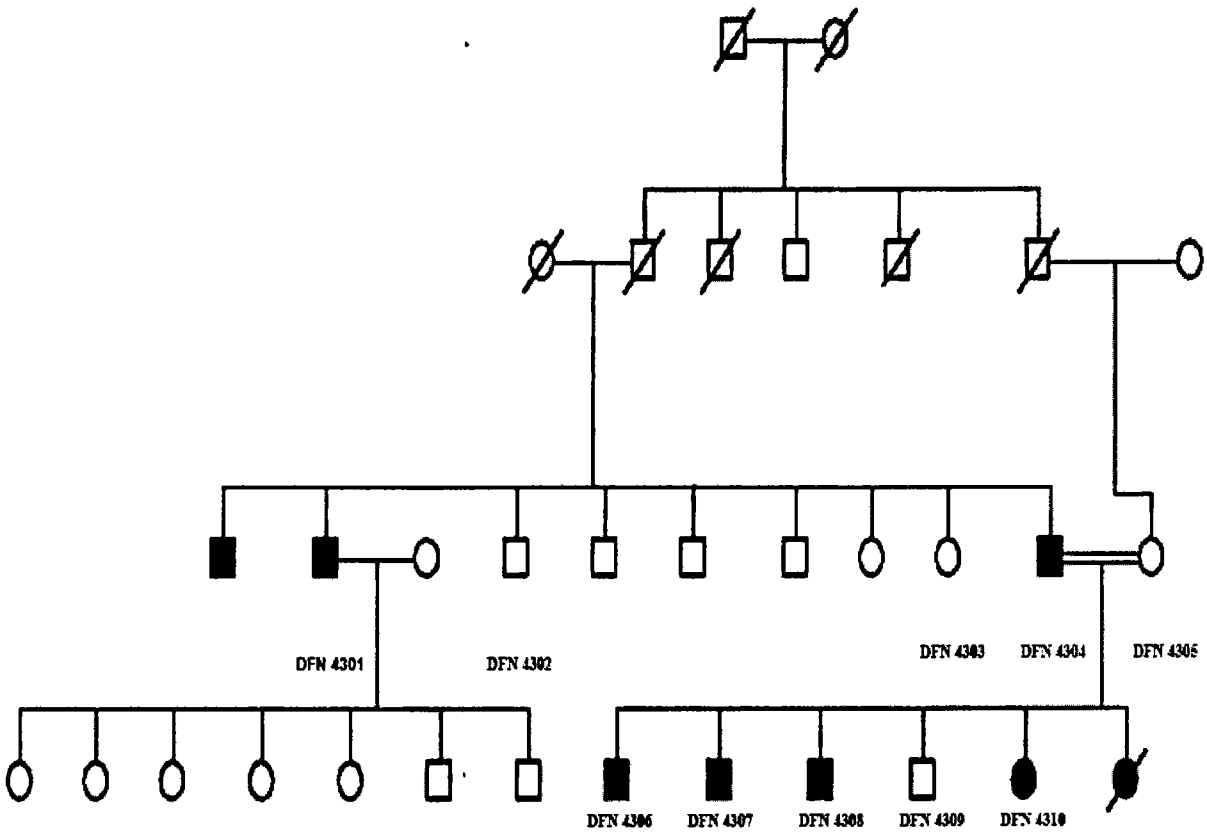


Figure 4-2: Pedigree of Family DFN 43. In the pedigree, females are represented by circles, squares are for males. While filled squares are for affected males and filled circles are for affected females. The diagonal lines across the symbols represent the expired family members. Sampled individuals are represented by individual numbers (DFN 4301- 4310). Consanguinity is represented by double line in the pedigree.

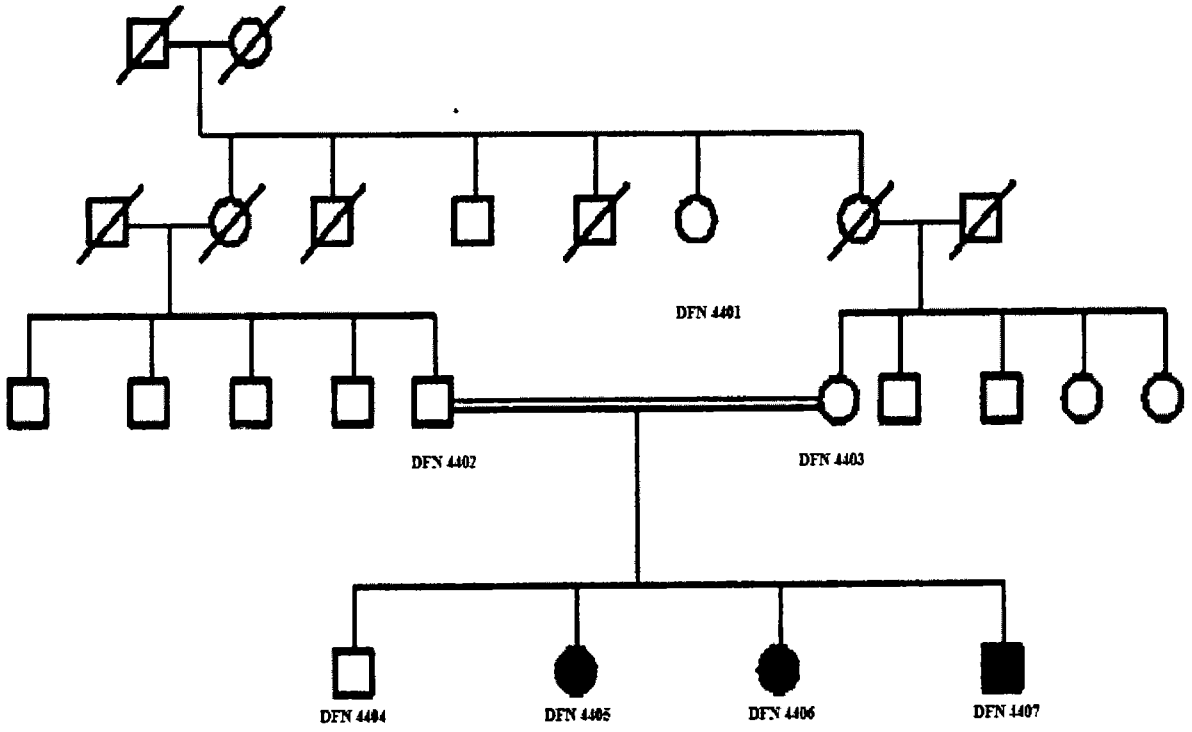


Figure 4-3: Pedigree of DFN 44. The circles represent females while squares are for males. The filled circles indicate affected females and filled squares represent affected males. The diagonal lines across the symbols indicate the deceased individuals. The double line in the pedigree shows the consanguineous marriage. Sampled individuals are represented by individual numbers (DFN 4401- DFN 4407).

4.1.4 DFN 45

This family belonged to Mardan, Pakistan. The family had three affected individuals DFN 4506, DFN 4507 and DFN 4509. They were sampled after written informed consent. Seven normal individuals (DFN 4501, DFN 4502, DFN 4503, DFN 4504, DFN 4505, DFN 4508, DFN 4510) were sampled. Autosomal recessive inheritance pattern is followed by DFN 45 (Fig: 4-4). There were two affected females and one affected male in DFN 45. All the affected individuals were having prelingual, severe to profound hearing loss.

4.1.5 DFN 46

The family DFN 46 was sampled from Mardan, Pakistan. The sampled members were (DFN 4601, DFN 4602, DFN 4603, DFN 4604, DFN 4608: unaffected and DFN 4605, DFN 4606, DFN 4607: affected). Samples were collected after written informed consent. The family is having non syndromic autosomal recessive deafness at an early age of presentation. All the affected individuals were having prelingual, severe to profound hearing loss.

4.1.6 DFN 47

The family DFN 47 is a consanguineous Pakistani family (Fig: 4-6) from Swat, Pakistan. The pedigree structure in figure 4-6 is showing autosomal recessive inheritance pattern. All the affected individuals were having prelingual, severe to profound hearing loss. After having written informed consent, in total nine samples were collected from this family. Out of these nine samples, five affected (DFN 4705, DFN 4706, DFN 4707, DFN 4708) and four were normal (DFN 4701, DFN 4703, DFN 4709).

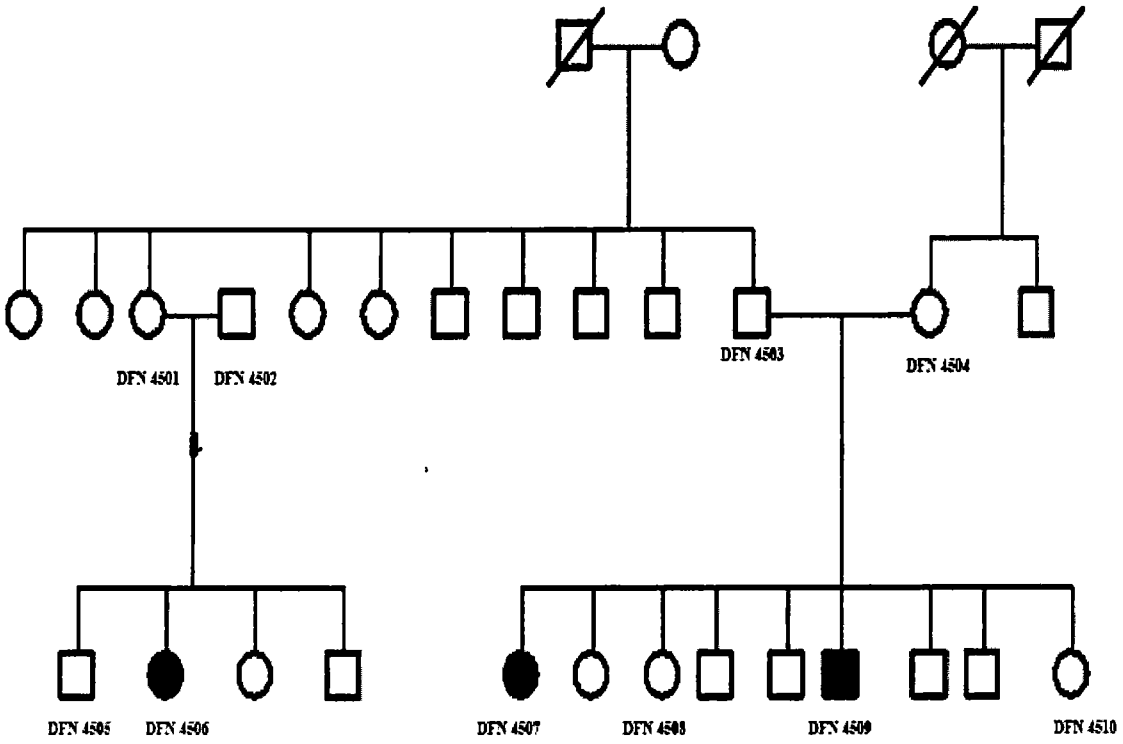


Figure 4-4: Pedigree of DFN 45. In the pedigree, females are represented by circles, squares are for males. While filled squares represent affected males and affected females are represented by filled circles. The diagonal lines across the symbols are for deceased individuals. Sampled individuals are represented by individual numbers (DFN 4501- 4510). Consanguinity is represented by double line in the pedigree

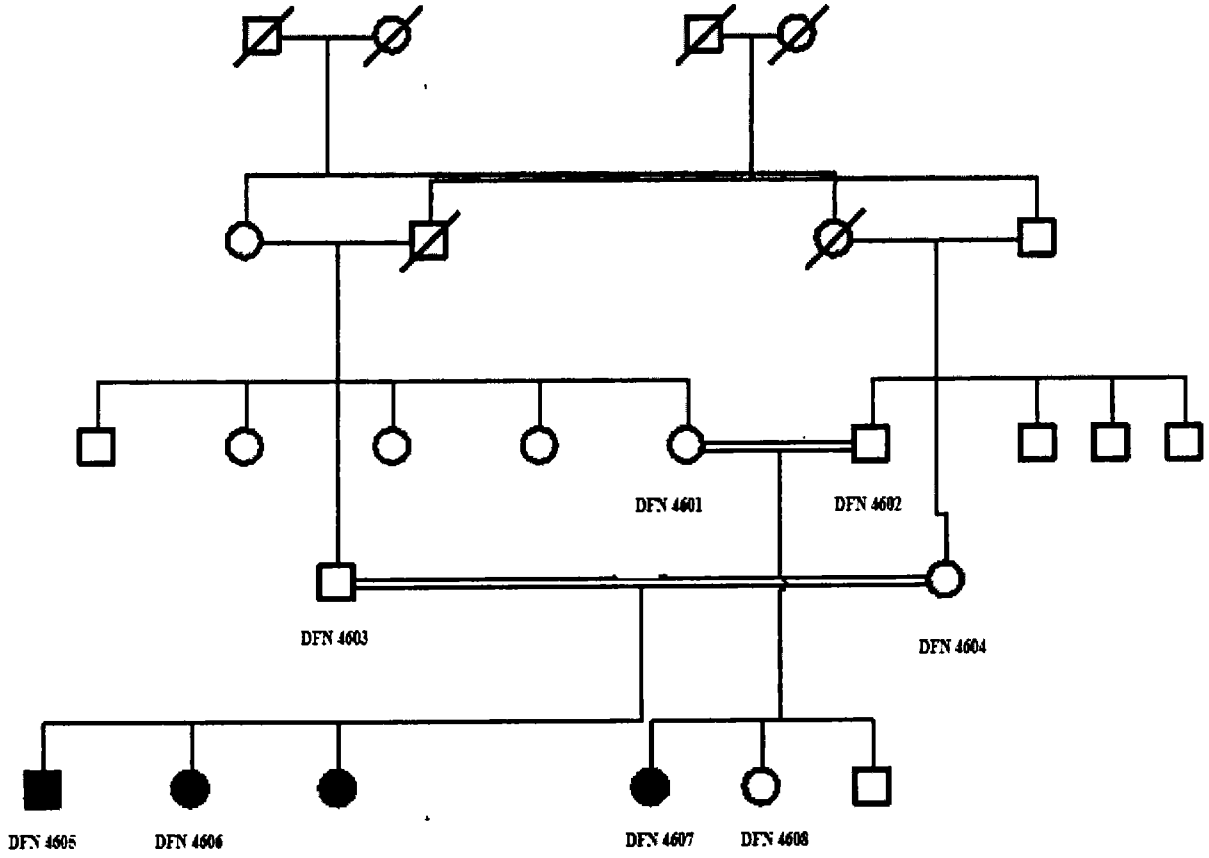


Figure 4-5: Pedigree of DFN 46. In the pedigree, females are represented by circles, squares are for males. While filled circles are for affected females and filled square represents affected male. The diagonal lines across the symbols are for deceased individuals. Consanguinity is represented by double line in the pedigree. Sampled individuals are represented by individual numbers (DFN 4601- 4608).

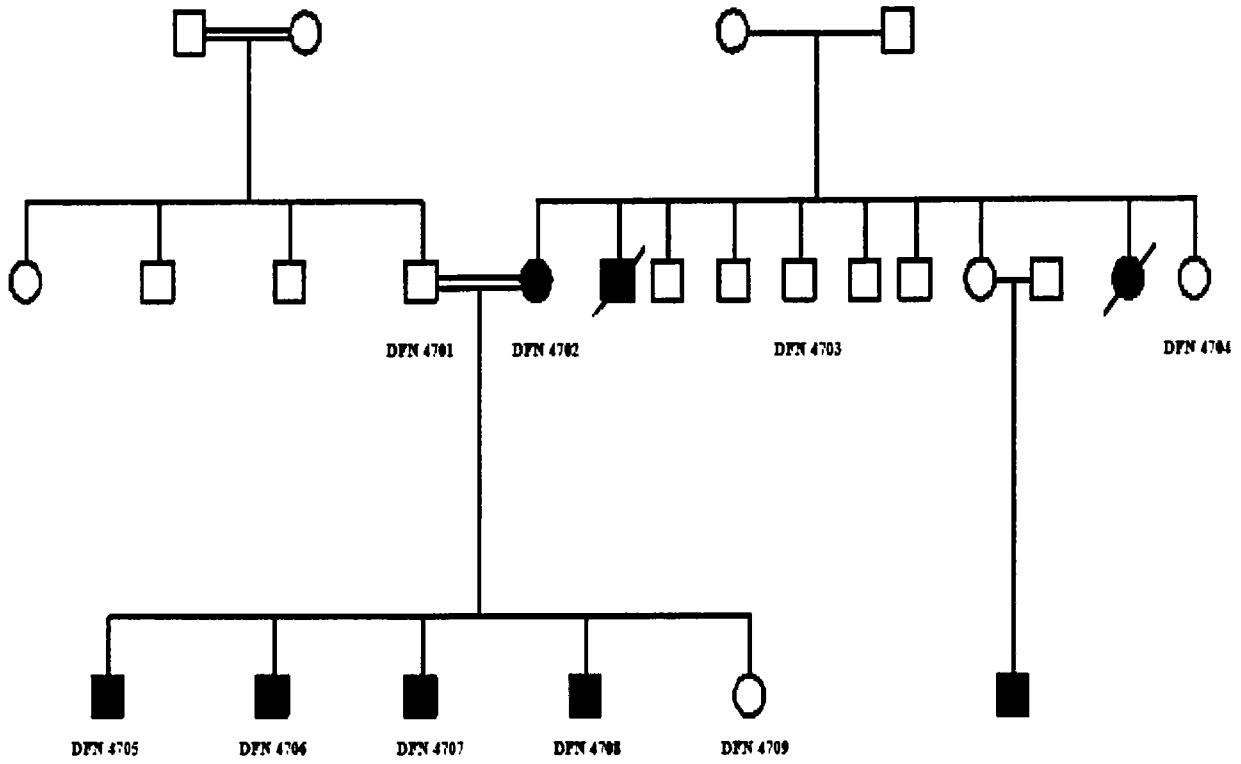


Figure 4-6: Pedigree of DFN 47. In the pedigree, females are represented by circles, squares are for males. Filled squares represent affected males while affected females are represented by filled circles. The diagonal lines across the symbols are for deceased individuals. Consanguinity is represented by double lines in the pedigree. Sampled individuals are represented by individual numbers from DFN 4701- 4709.

4.1.7 DFN 48

DFN 48 was collected from Swat, Pakistan. Nine members of the family were sampled (DFN 4801, DFN 4802, DFN 4803, DFN 4804, DFN 4808: unaffected, DFN 4805, DFN 4806, DFN, 4807, DFN 4809: affected) after written informed consent. The family is having non syndromic autosomal recessive deafness at an early age of presentation. All the affected individuals were having prelingual, severe to profound hearing loss.

4.1.8 DFN 49

Six members of family DFN 49 (fig: 4-8) were sampled (DFN 4904, DFN 4905: affected, DFN 4901, DFN 4902, DFN 4903, DFN 4906: unaffected) from Swat, Pakistan. Samples were collected after written informed consent. The pedigree structure of DFN 49 in figure 4-8 is showing autosomal recessive inheritance pattern. All the affected individuals were having prelingual, severe to prefound hearing loss.

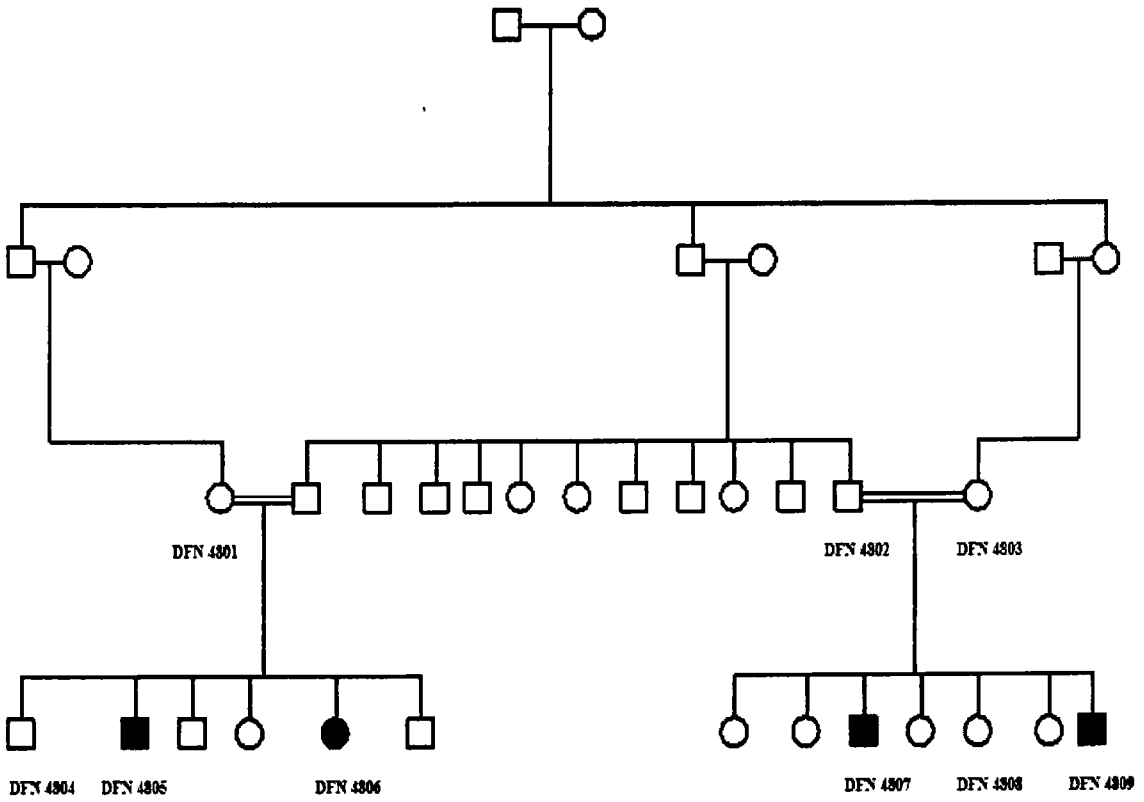


Figure 4-7: Pedigree of DFN 48. In the pedigree, the females are represented by circles, squares are for males. While filled circles are for affected females and filled squares represent males. While affected females are represented by filled circles. Consanguinity is represented by double lines in the pedigree. Sampled individuals are represented by individual numbers from DFN 4801- 4809.

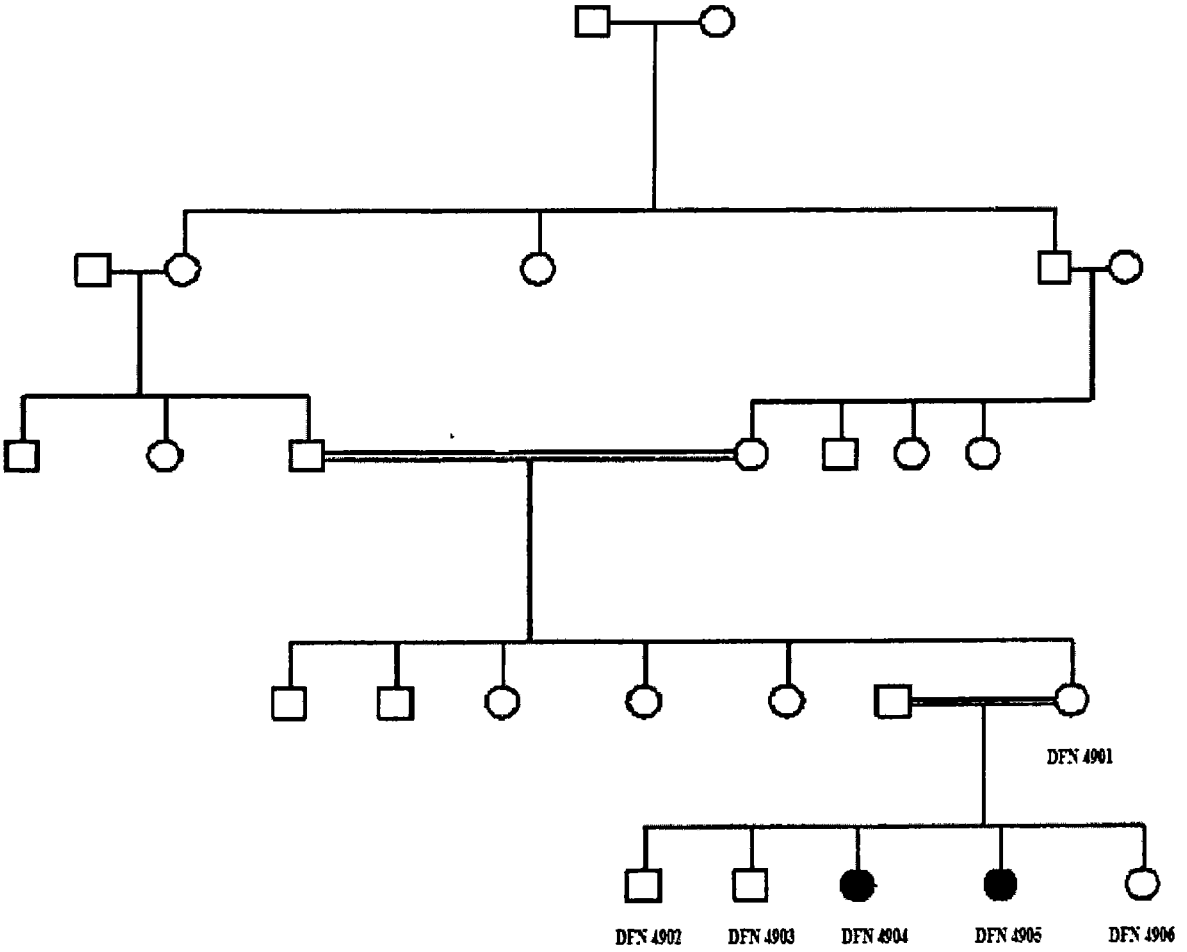


Figure 4-8: Pedigree of DFN 49. In the pedigree, the females are represented by circles, squares are for males. While filled circles are for affected females. Consanguinity is represented by double line in the pedigree. Sampled individuals were represented by individual numbers from DFN 4901-4906).

4.2 Mutational Analysis of GJB2 in Selected Patients through Sequencing

After collection of the ARNSHL families, out of these 72 samples, 11 samples were selected for the GJB2 mutation analysis. The selected sample IDs, sex and area are mentioned in Table 4-2. The GJB2 coding region was analyzed by using Sanger sequence analysis. Sanger sequencing procedure using dideoxynucleotides was used. These patients were screened by using the reverse and forward primer for exon 2 of GJB2 gene. The sequence data obtained for the *GJB2* gene was analyzed by comparing it with the reference wild type *GJB2* sequence (GenBank No. NG_008358.1). There are above 200 mutations already identified in GJB2 gene worldwide, however we hadn't found any of the recurrent or novel mutation in our sequenced samples. The snapshots in Table 4-3 show the sequences or genetic codes of patients. In these snapshots, arrows are showing the most commonly reported mutations of Pakistan that were not found in our samples. The most common mutation reported in the Pakistani population is p.W24X (Shafique et al., 2014, Anjum et al., 2015). The p.W24X mutation results due to substitution of G to A at nucleotide 71 that generate stop codon at amino acid 24. The protein formed as a result of this mutation is just one tenth of the length of the original protein. Shafique 2014 found this mutation in 31% of ARNSHL Punjabi families. Similarly Anjum 2015 found it to be in 20% of ARNSHL Pakistani families. The p.W77X mutation is due to substitution of G to A at nucleotide 231. This results in a stop codon at codon 77. The protein produced as a result can't form functional gap junctions. Shafique 2014 found this mutation in 25% of ARNSHL Punjabi families.

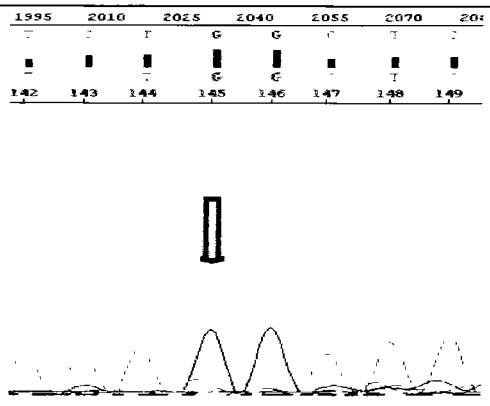
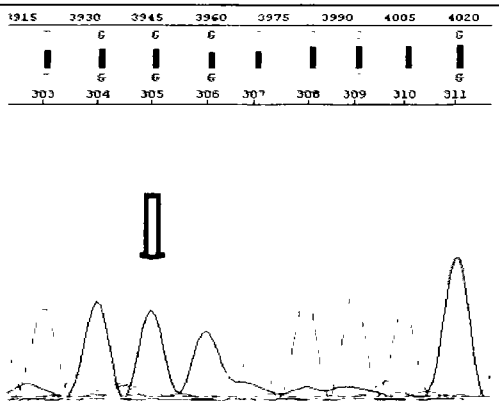
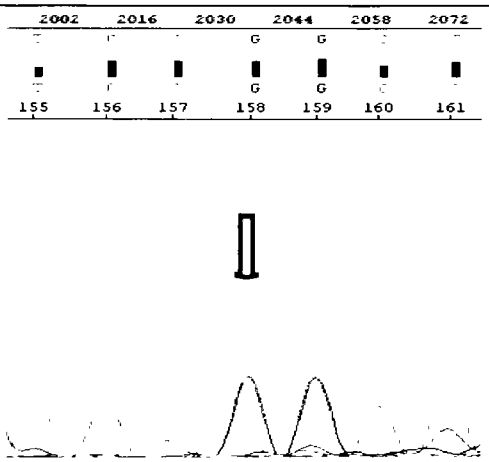
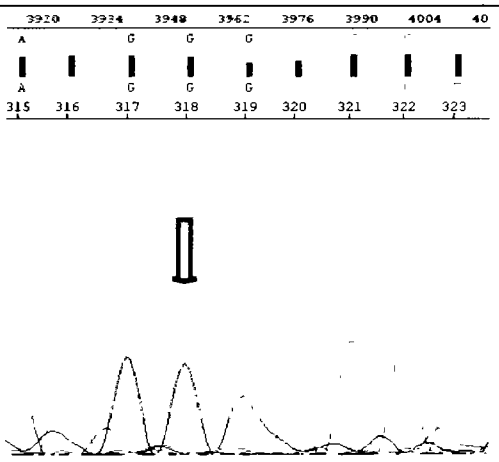
The results obtained after Sanger sequencing of the selected individuals were interpreted by different peaks showing different nucleotides like guanine is shown by the black peak, thymine is shown by red peak, blue peaks shows cytosine and adenine is shown by green peak.

8 ARNSHL families were screened by sequencing the exon 2 of GJB2 gene. Overall, we have not found GJB2 mutations in this cohort of deafness families from Northern area. There could be involvement of other genes. So, these samples should further be analyzed for the other genes involved in the ARNSHL.

Table 4-2: Samples selected for sequencing

Sample ID	Sex	Area
DFN 4205	Male	Nowshera
DFN 4210	Male	Nowshera
DFN 4307	Male	Mardan
DFN 4406	Female	Mardan
DFN 4506	Female	Mardan
DFN 4507	Female	Mardan
DFN 4607	Female	Mardan
DFN 4707	Male	Swat
DFN 4806	Female	Swat
DFN 4807	Male	Swat
DFN 4905	Female	Swat

Table 4-3: Snapshots of most common Pakistani mutations not found in our study

Sample ID	Snapshots of most common Pakistani mutations not found in our study	
	p. W24X **c. 71G→A**	**p. W77X** **c. 231G→A**
DFN 4205		
DFN 4210		

(accounting for three homozygous individuals) affected with GJB2 pathogenic alterations including (p.W24X), c.104 T → G, and c.35delG, which were not found in the current study from Northern area (Bukhari *et al.*, 2013). While, Shafique (2014) found GJB2 prevalence of 53% in Punjab population with cohort of 30 ARNSHL families. Similarly, Anjum 2014 found a GJB2 prevalence of 50% in 20 consanguineous Pakistani families.

Some other populations like Cameroon (LuccaTrotta et al., 2011), Uganda (Javidnia *et al.*, 2014), Nigeria (Lasisi *et al.*, 2014), and Nicaragua (Saunders *et al.*, 2007) also have low GJB2 prevalence for hearing loss.

Overall, the obtained molecular data from current study suggest that GJB2 do not have a predominant role in the genetics of deafness in the Northern area population. The GJB2 prevalence found from the Northern region is entirely different from the high prevalence in other Pakistani population; this could be because of different origin of the various ethnic groups indicating separate GJB2 prevalence studies are required from each province to have better idea about the percentage contribution of various deafness genes.

5.1 Conclusion

It is concluded from the current study that the role of GJB2 gene for ARNSHL is less prevalent in the Northern area of Pakistan. According to these results, GJB2 mutation screening in the northern area populations might not be clinically warranted. However, before making a stronger statement, more subjects should be tested. The cause of genetic deafness in these families is suspected to be another mutated deafness gene which needs to be explored.

5.2 Future Work

As hearing loss can affect a person badly emotionally and socially, there is need to reduce the problem through proper genetic diagnosis and counseling. For that, it is needed to have a firm idea about genetic basis of ARNSHL. In our case, all the families need to be further analyzed for mutations in any other gene. The association of second most prevalent gene in Pakistan for ARNSHL that is Myo15A gene can be analyzed. Further, SNP array and Next generation sequencing to analyze the genetic cause of hearing loss in these families and confirm the particular gene and mutations present can be done.

Chapter 6

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Electronic Sources

Connexin Deafness Homepage: http://davinci.crg.es/deafness/

http://physiologyonline.physiology.org/content/22/2/131

http://www.britannica.com/EBchecked/media/530/Structure-of-the-human ear?topicId=175622

http://www.frontiersin.org/files/Articles/53434/fphar-04-00081-HTML/image_m/fphar-04-00081-g001.jpg)

http://www.genecards.org/cgi-bin/carddisp.pl?gene=GJB2

NCBI Pubmed: http://www.ncbi.nlm.nih.gov/pubmed/

NCBI: http://www.ncbi.nlm.nih.gov/gene/2706

OMIM: http://www.omim.org/entry/121011

USCS: http://genome.ucsc.edu/cgi-bin/hgPcr?command=start

ANNEXURE A

Lysis buffer (pH: 7.4)

NH_4Cl : 8.29 g

$KHCO_3$: 1g

0.5M di sodium EDTA : 20ml

Dissolve these in 800ml of d. H_2O, adjust the pH with conc HCl or NaOH and raise the volume to 1000ml.

STE buffer (Saline + Tris + EDTA)

3M NaCl: 175.5g of NaCL in 1000ml of d. H_2O.

1M Tris HCl (pH: 8)

0.5M disodium EDTA (pH: 8): Dissolve 46.5g of disodium EDTA in 50ml deionized water by placing it on magnetic stirrer. Start adjusting the pH by adding 4N NaOH pellet. Once the pH is adjusted to 8, adjust the volume upto 250ml by adding deionized water.

For 1000ml STE Buffer:

Add 33.3ml of 3MNaCl, 50ml of 1MTris and 2ml of 0.5M EDTA in a cylinder. Make the volume upto 1000ml with deionized water. Mix the solution thoroughly and store in 1000ml bottle at room temperature.

10% SDS

Weigh 100 g of SDS (Sodium Dodecyl Sulfate) and add about 600 ml of deionized H_2O. Mix the solution through with magnetic stirrer. Mix the volume upto 1000ml with deionized water. Filter the solution using filteration assembly. Store at room temperature.

Chloroform Isoamyl Alcohol

For the measurement of these chemicals, a glass cylinder should be used. Chloroform and isoamyl alcohol are mixed in the ratio of 24:1. To prepare a total volume of 500ml of chloroform isoamyl alcohol, add 480ml of choloform and 20ml of isoamyalcohol. Mix the two chemicals thoroughly and store them at 4°C in glass bottles wrapped with aluminium foil.

10M Ammonium acetate (1L or 1000ml)

Dissolve 771g ammonium acetate in 800ml of d. H2O. Then adjust volume to 1000ml. Sterilize the solution by passing it through a 0.22um filter. Store the solution in tightly sealed bottles at

4°C or at room temperature. Ammonium acetate decompose in hot water and should not be autoclaved.

Isopropanol

It is just poured from the stock bottle and stored at 4°C in bottles wrapped with aluminium foil.

Proteinase K

Dissolve 25mg of proteinase K in 1.25ml of deionized autoclaved water and store at -20°C.

RNAase A

For 15ml RNAase A

RNAase A = 150mg or 0.15g

10mM Tris = pH 7.5 = 15ul

Adjust the pH of 10mM Tris at 7.5. Weigh 0.15g of powered RNAase A and dissolve in 15ml of 10mM Tris (pH =7.5). Shake well till it dissolves thoroughly. Transfer in 1.5ml eppendorf tubes. Store at -20°C.

10mM Tris HCl (pH: 8)

121.1g Trizma base in app 800 ml of d. H2O. Adjust the pH to 8 with conc HCl. Adjust to 1L by adding d. H_2O. Filter through 0.4um filter paper. Store at room temperature. 0.1M and 10mM Tris are prepared from this stock.

Tris EDTA (TE Buffer) 500ml

IM Tris: 5ml

0.5 M EDTA: 1ml

Mix and raise the volume to 500ml with d. H2O. Store at room temperature.

ANNEXURE B

10x TAE buffer (5000ml)

Tris base = 242grams

Glacial Acetic acid = 57ml

0.5M EDTA= 100ml

Weigh out 242g of Tris base. Add 57ml of Glacial acetic acid. Add 100ml of 0.5 M EDTA. Mix it well by placing it on magnetic stirrer. Make up the volume to 5000ml by d. H_2O. Label and keep it at room temperature.

EtBr (50mg/ml)

Add 2 grams of Ethidium Bromide in 40ml of d. H2O.

4°C or at room temperature. Ammonium acetate decompose in hot water and should not be autoclaved.

Isopropanol

It is just poured from the stock bottle and stored at 4°C in bottles wrapped with aluminium foil.

Proteinase K

Dissolve 25mg of proteinase K in 1.25ml of deionized autoclaved water and store at -20°C.

RNAase A

For 15ml RNAase A

RNAase A = 150mg or 0.15g

10mM Tris = pH 7.5 = 15ul

Adjust the pH of 10mM Tris at 7.5, Weigh 0.15g of powered RNAase A and dissolve in 15ml of 10mM Tris (pH =7.5). Shake well till it dissolves thoroughly. Transfer in 1.5ml eppendorf tubes. Store at -20°C.

10mM Tris HCl (pH: 8)

121.1g Trizma base in app 800 ml of d. H2O. Adjust the pH to 8 with conc HCl. Adjust to 1L by adding d. H_2O. Filter through 0.4um filter paper. Store at room temperature. 0.1M and 10mM Tris are prepared from this stock.

Tris EDTA (TE Buffer) 500ml

IM Tris: 5ml

0.5 M EDTA: 1ml

Mix and raise the volume to 500ml with d. H2O. Store at room temperature.

ANNEXURE B

10x TAE buffer (5000ml)

Tris base = 242grams

Glacial Acetic acid = 57ml

0.5M EDTA= 100ml

Weigh out 242g of Tris base. Add 57ml of Glacial acetic acid. Add 100ml of 0.5 M EDTA. Mix it well by placing it on magnetic stirrer. Make up the volume to 5000ml by d. H_2O. Label and keep it at room temperature.

EtBr (50mg/ml)

Add 2 grams of Ethidium Bromide in 40ml of d. H2O.