Screening of *GJB2 (*35delG, W24X, W77X) and *GJB6* (D13S1830) Mutations in Patients with Non-syndromic Hereditary Hearing Impairment

By

Sana Rasul

NUST201463545MASAB92514F

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

H-12, Islamabad, Pakistan

2016

Screening of *GJB2* (35delG, W24X, W77X) and *GJB6* (D13S1830) Mutations in Patients with Non-syndromic Hereditary Hearing Impairment

A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science

In

Healthcare Biotechnology

By

Sana Rasul

NUST201463545MASAB92514F

Supervisor

Dr. M. Jawad Hassan

Atta-ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

H-12, Islamabad, Pakistan

2016

Dedicated to my Grandfather,

Col.(R) R.B Baloch

Acknowledgements

I would like to express my sincere gratitude to the efforts of my supervisor, Dr. Jawad Hassan, who has been highly supportive and helpful during the course of my research. Without his help and constant guidance, this project would have been incomplete. Special thanks to my GEC members including Dr. Peter John (Principal ASAB), Dr. Attya Bhatti (HoD Healthcare Biotechnology Department) and Dr. Aisha Mohyuddin (Professor of Biochemistry at Shifa College of Medicine, Shifa Tameer e Millat University, Islamabad) for their guidance and support.

All those who have helped me in my research, especially Hammad Rai, who has been extremely cooperative in helping me with sample collection, deserve a part in the successful comple*t*ion of this project. My lab mates and friends Simra Arshad, Gulsanga Zahid, Yousuf Kamran and Tahir Ghori have always been a guiding source and helping hand to me.

I would like to thank all participants of this study.

Lastly, I would like to thank my friends and family, who have always had my back and pushed me towards achieving greatness at every step of my career.

Sana Rasul

TABLE OF CONTENTS

Chapter 3: MATERIALS AND METHODS

Chapter 4: RESULTS

LIST OF ACRONYMS

%	Percentage
°C	Degree Celcius
Bp	Base pair
GJB2	Gap Junction B*et*a-2
GJB6	Gap Junction B*et*a-6
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
EDTA	*Et*hylenediamine*tet*raace*t*ic acid
HCL	Hydrochloric Acid
KCL	Potassium Chloride
MgCl2	Potassium Chloride
SDS	Sodium Dodecyl SµLphate
TAE	Tris Ac*et*ate EDTA
TE	Tris EDTA
Mg	Milligram
mL	Milliliter
mM	Millimolar
PCR	Polymerase Chain Reaction
Rpm	Revolutions per minute
µg	Microgram
µL	Microliter
ARNSHL	Autosomal Recessive Non-syndromic Hearing Loss
HHI	Hereditary Hearing Impairment

List of Tables

List of Figures

Abstract

Hereditary hearing impairment affects approximately 1 in 1000 children worldwide. In most populations, the main causative agent is *GJB2* gene (MIM#121011) located at chromosome 13q12.11 which encodes for a gap junction protein called connexin 26. Mutations in gap junction proteins, *GJB2* and *GJB6*, another protein residing near the former with similar function, affect the K+ concentration in the hair cells of cochlea, a major functional player within inner ear. In this study, we screened three already reported *GJB2* mutations (35delG, W24X, W77X) and a reported *GJB6* mutation (delD13S1830) in a group of Pakistani individuals suffering from congenital non-syndromic, severe to profound hearing impairment through PCR using mutation specific and normal primers. In 43 out of 77 patients tested, one of three *GJB2* mutations was present (55.83%). Out of these, W24X was found to be the most frequent (27/77), accounting for 35.06% of the studied sample, while frequency of W27X was 10/77 (12.98%) and of 35delG 6/77 (7.79%). No samples were reported carrying the *GJB6* deletion encompassing microsatellite marker D13S1830. There are mixed reports about the prevalence of *GJB2* mutations in Pakistan which may be due to the different in ethnicities of the highly diverse Pakistani population. This suggests the important role of ancestry in disease heterogeneity.

INTRODUCTION

1.1. Background

Hearing impairment (HI) is a disability causing diminished or complete inability to hear sound. It may occur due to a variety of factors such as exposure to noise, birth complications, infections, certain medications, age-related or genetic. Humans hear sound between 20-20,000 Hz frequency or 0 to 120 decibels. A tuning fork evaluation or an audiometer test helps determine the degree of hearing loss in a person.

This disease affects approximately 1 in 1000 children worldwide, half of which generally have hereditary forms. (Putcha et al., 2007; Ouyang et al., 2009). It is highly heterogeneous genetically and phenotypically. It can be graded on the basis of the degree of hearing loss, from mild to severe or profound.

1.2 Genetics

There are two main forms in which hereditary hearing impairment (HHI) occurs. Cases which are coupled with other clinical symptoms are called syndromic forms of deafness (Gorlin et al., 1995; Rabionet et al., 2000). They are less frequent in number, accounting for only 30% of the HHI patients. Non-syndromic forms have various patterns of inheritance such as autosomal recessive, autosomal dominant, X-linked or having mitochondrial inheritance. Autosomal dominant forms of inheritance accounts for fewer cases (22%), presenting on the DFNA locus and are mostly syndromic forms such as Usher and Pendred syndromes. However, up to 80% of the cases are non-syndromic autosomal recessive, typically involving the locus *DFNB1* (Putcha et al., 2007; Ouyang et al., 2009).

More than 100 loci have been reported for autosomal recessive non syndromic hearing loss (ARNSHL), out of which the main causative agent is *GJB2* gene **(MIM#121011)** on the DFNB1 locus. It is positioned at chromosome 13q12.11 and encodes for connexin 26, a gap junction protein (del Castillo et al., 2002; Elbagoury et al., 2014). *GJB6* **(MIM 604418)**, another gene found responsible for ARNSHL is present in the vicinity of *GJB2* on the same locus.

Mutations in the genes *GJB2* and *GJB6* (connexin 26 and connexin 30 respectively) are responsible for disorders other than non-syndromic hereditary hearing loss as well (Salman et al., 2015). There are 20 different types of connexins described in mammals (Harris, 2001). These connexin proteins (Cx26 and Cx30) are arranged in a hexagonal arrangement to form a connexon. Two opposite connexons on adjacent cells join to form a gap junction channel. Gap junctions cause intercellular interaction by the influx of different ions and small molecules between the cells. In the cochlea, Cx26 and Cx30 are responsible for maintaining the proper concentration of K+ ions by reprocessing it (del Castillo et al., 2003; Putcha et al., 2007). Mutations in connexin genes cause disruption in K+ homeostasis, leading to hearing loss.

1.2.1 *GJB2*

There are several types of mutations in the *GJB2* genes, with 35delG (rs80338939) being the most common in many populations. Most mutations in *GJB2* gene result in a truncated protein due to the generation of a pre-mature stop codon (Bukhari et al., 2013).

Two other mutations, W24X (rs104894396) and W77X (rs80338944), both of which are transition mutations, gain a stop codon and result in truncated protein. They are commonly found in South Asia (Salman et al., 2015).

1.2.2 *GJB6*

GJB6 gene is present approximately 35-kb upstream to the *GJB2*. Because of its close proximity to *GJB2*, it is believed that a large deletion in the *GJB6* may affect the cis regulatory elements of *GJB2*, therefore causing a change in gene expression for *GJB2* (Zaidieh et al., 2015). A 309-kb deletion in the *GJB6* gene (encompassing the microsatellite region D13S1830) is known to cause severe to profound hearing impairment (Snoeckx et al., 2005).

Autosomal Recessive Non Syndromic Hereditary Hearing Impairment (ARNSHI) affects many individuals born in Pakistan mainly due to consanguineous marriages. The four variants of the two genes mentioned above are widely associated with disease worldwide. There are contradictory reports on prevalence of *GJB2* variants in Pakistani patients with profound deafness and no data is available for *GJB6* variants (Shafique et al., 2014; Santos et al., 2005).

In this research we have screened three already reported *GJB2* mutations (35delG, W24X, W77X) and a reported *GJB6* deletion mutation (D13S1830) in a group of Pakistani individuals suffering from congenital non-syndromic, severe to profound hearing impairment, allowing us to predict more precisely about the prevalence of *GJB2* and *GJB6* variants in Pakistan. The prevalence of *GJB2* mutations in our sample population was found to be 56%. Amongst these, W24X was found to be the most prevalent in our sample population. The deletion D13S1830 was not found in any of our HI patients. Screening for prevalent mutations will allow us to start genetic testing in this highly heterogeneous disease. Also, we would be able to diagnose timely and counsel affected families for future reproductive decisions.

Aims and Objectives

The main objectives of this study are to:

a) Screen variants of *GJB2* (35delG, W24X, W77X) and *GJB6* (D13S1830) in Non-Syndromic Hereditary Hearing Impairment patients through ARMS-PCR.

b) Ascertain mutation frequency in sample population to pave the way for developing a genetic test.

LITERATURE REVIEW

2.1 Autosomal Recessive Non-Syndromic Hearing Impairment (ARNSHI)

Approximately 80% of the cases of hearing loss have autosomal recessive non-syndromic pattern of inheritance and only 18% are inherited in autosomal dominant fashion (Cohin and Gorlin, 1995). Being a highly heterogeneous disease, it involves up to 120 loci and several disease genes including *GJB2*, *PDS*, *MYO7A*, *TECTA*, *OTOF*, *GJB3*, *GJB6* (http://hereditaryhearingloss.org/). Out of these, three genes are found in the formation of gap junctions including *GJB2*, *GJB3*, and *GJB6*.

2.2 Syndromic forms of Hearing Impairment

Several disorders have been found to be involved in syndromic forms of hearing by using positional cloning techniques and murine model analysis, including those for Usher's and Pendred's (Li et al., 1998) and Waardenburg syndrome (Tassabehji et al., 1992, 1994)

2.3 Hearing Impairment in Pakistan

Due to the trend of consanguineous marriages in Pakistan, the number of genetic disorders has multiplied. The high diversity based on ethnicity found in Pakistani population is a goldmine for genetic studies. Almost 10% of congenital disorders worldwide are caused due to consanguineous marriages; however, the percentage increases up to 40% in Pakistan (Alwan et al., 1997).

2.4 Connexins

Connexins are a part of the transmembrane and form gap junction channels to ease cellular interaction such as the movement of small ions across the membrane. Each connexon

comprises of a hexamer of connexins joined together. They consist of a multigenic family of membrane proteins, broadly classified into two groups (alpha and beta), GJA and GJB (Bruzzone et al., 1996).

2.4.1 Connexin Structure

Connexins are the main component of gap junctions, assembled within the lipid bilayer. Polar amino acids form the transmembrane domain, forming the lining of the channel (Buehler et al., 1995). A study showed that expressing the connexin cDNA in yeast results in formation of functional channels, highly similar to those *in vivo*.

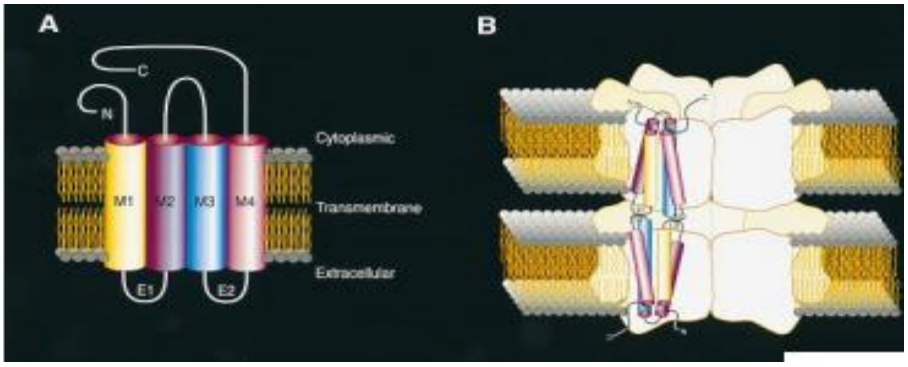

Figure 2.1. Structure of Gap Junction channels (Kumar and Gilula 1996).

Connexins are known to be categorized in the general topological model for all protein junctions. The polypeptides move towards the bi-layered lipid. During this process, both terminals, C- and N-, point in the direction of cytoplasm. Different studies conducted on Connexins have revealed that M3, transmembrane domain, has amphipathic properties. These properties of transmembrane add to the lining of the channel. The connection between the adjacent cells is initiated by E1 and E2, the two extracellular loops. Each extracellular loop has a set of three cysteine residues which are arranged in a way that portrays the properties of connexins. These residues help two adjacent connexins to connect with each other by the help of strong tertiary structures. (Kumar and Gilula, 1996).

2.5 *GJB2* (Connexin 26)

This gene, present on chromosome 13q11-q12 (DFNB1 locus), is the most important for non-syndromic autosomal recessive hearing loss (Mignon et al., 1996). It was identified in 1997 and was the first gene on the *DFNB1* locus to be identified. They were first found in two large consanguineous families from Tunisia through linkage analysis (Guilford et al., 1994). Later studies showed them to be prevalent in deaf families from New Zealand/Australia and Spanish/Italian (Maw et al., 1995; Gasparini et al., 1997). It was also found in 3 Pakistani consanguineous families with profound deafness (Kelsell et al., 1997). The gene contains two exons, and only the second exon is translated. It has a highly conserved promoter region (Kiang et al., 1997). The resultant protein is called connexin 26 which is responsible for intercellular communication via gap junctions. Mutations in this gene are common, accounting for up to 50% of the autosomal recessive, non-syndromic, hearing loss in many populations (Rabionet et al., 2000).

Connexin 26 was first cloned by Lee et al. (1992), from the cDNA library of normal mammalian epithelial cDNA using subtractive hybridization technique. This helped calculate the molecular mass of the protein which was about 26 kD. He also demonstrated the occurrence gap junction in normal mammary epithelial cells, as opposed to tumor cells which did not express them. According to this research, the late S and G2 phases of the cell cycle showed strongest expression of Cx26. The mammary tumor epithelial cell not expressing Cx26 was shown to express it after treatment with Phorbol ester (Lee et al., 1992).

Mammalian *Cx26* is 92.5% identical to that in rats. Their presence in human cochlea was found by Kelsell et al. (1997). Further studies on mouse and rat cochlea confirmed their role in the cochlea, leading scientists to believe that they had an important role in potassium homeostasis (Rabionet et al., 2000). Studies by Mese et al., (2004) depicted that other than

K+ recirculation, hearing impairment due to *Cx26* mutations were also caused by the problems in the interchange of other metabolites.

Connexins 26 and 43, which are integral gap junction subunits, were found to be expressed at specific contact places present between radial fibers and the migrating neurons. Even though neuronal migration is hindered during their downregulation, gap junctions were found to have no direct role in their mediation. Rather they interacted with the glial cell cytoskeleton to form adhesive contacts which are important in neuronal migration (Elias et al., 2007). There are various types of mutations present in this gene. It causes both autosomal dominant deafness (DFNA3) and Autosomal recessive deafness (DFNB1A), as showed by Kelsell et al (1997). Mutations in *GJB2* are also responsible for sporadic cases of non-syndromic deafness. They were first detected in patients from UK and Belgium (Lench et al., 1998).

The most common worldwide is believed to be 35delG, which is present in a vast majority of the Caucasian population. It is a deletion in a repeat of six G residues on the 35th position, which causes a frameshift and eventually leads to premature termination of the protein (Zelante et al., 1997). This gene has the highest carrier frequency in Italian and Greek populations (Antoniadi et al., 1999). A highly contrasting carrier frequency amongst European countries has been observed with an average being 2.8% in southern Europe against 1.3% in northern Europe (Gasparini et al., 2000). The original idea proposed the presence of a mutational hotspot resulting in a high frequency of occurrence of this mutation but further studies on homozygous 35delG carriers showed that a common ancestral origin (Van Laer et al., 2001).

Approximately 90 mutations in the *GJB2* have been found to lead to ARNSHI (http://www.crg.es/deafness). Other than the 35delG, various mutations in *GJB2* have been

found to cause ARNSHI in different racial groups. 167delT was found prevalent in Ashkenazi Jews (Morell et al., 1998), R143 in African population (Brobby et al., 1998), and 35delC in Japanese (Fuse et al., 1999).

Another mutation in the *GJB2* gene, resulting from a G-to-A transition, causing trp24-to-ter substitution (W77X) at the seventy seventh amino acid position, was found first in two individuals of a large consanguineous Pakistani family suffering from non-syndromic profound deafness (DFNB1A). The two affected individuals were found to be homozygous for W77X and their parents, who were unaffected, were heterozygous (Brown et al., 1996, Kelsell et al., 1997).

Kelsell et al., (1997) also found another mutation caused by a G-to-A transition in the *GJB2* gene, leading to a trp24-to-ter (W24X) substitution. This also resulted in the production of truncated protein which was unable to perform fully. In a study of Indian families by Maheshwari et al., (2003), it was found that W24X was responsible for 13.3% of the mutation amongst 45 families, establishing that it is a common allele in Indian population.

A screening study of Spanish Romani (gypsies) families suffering from ARNSHL found that half of the causes of deafness are due to *DFNB1* locus. W24X was found to be the predominant allele, occurring at a staggering 79% out of the *DFNB1* alleles. The second most common allele was the 35delG (17%) (Alvarez et al., 2005). In a study correlating the genotype and phenotype amongst deaf patients, it was shown that truncating mutations in both alleles usually have a more severe effect on an individual's phenotype than a truncating mutation combined with a missense mutation. (Snoeckx et al., 2005)

Although *GJB2* gene is primarily responsible for causing non-syndromic autosomal recessive hearing loss, however there is some debate regarding its role in autosomal dominant hearing loss also (Chaib et al., 1994). There are several lines of evidences that report a

number of heterozygous *GJB2* mutations specifically located in the first extracellular domain of the protein. These mutations have been found to segregate with various forms of autosomal dominant hearing loss in quite a number of families with varying phenotype.

GJB2 mutations are also involved in syndromic forms of hearing loss. Diseases such as autosomal-dominant palmoplantar keratoderma with deafness, ectodermal dysplasia keratitis–ichtyosis–deafness syndrome or mutilating keratoderma with sensorineural deafness, also called Vohwinkel syndrome. (Richard et al., 1998; Kelsell et al., 2001). Therefore, we can say that *GJB2* is like *GJB3* and *GJB6* which are involved in both major forms of hearing impairment.

2.6 *GJB6* (Connexin 30)

GJB6 encodes the connexin 30 protein and is located adjacent to *GJB2*. Being a part of the connexin family, its function is the formation of gap junction channels for the distribution of ions between the cells. Connexin 30 has been found profusely in the brain and cochlea (Lautermann et al., 1998). It was identified when a cDNA of a novel mouse connexin protein was cloned by Dahl et al. (1996). He found that the *GJB6* gene encoding the connexin 30, forms a protein with 261 amino acids, and was 77% similar to connexin-26 of mice. Later studies found that human connexin-30 shared 93% similarity with mouse connexin-30. (Grifa et al., 1999). *GJB6* was mapped to chromosome 14 in mouse cells, which is homologous to a region on the human chromosome 13q12 (Dahl et al., 1996, Grifa et al.,1999).

GJB6 causes both autosomal dominant and autosomal recessive forms of deafness. The T5M mutation (thr5-to-met) was identified by Grifa et al. (1999), which is responsible for autosomal dominant bilateral hearing loss. However, no other significant mutations were

found in *GJB6* gene in American and Japanese families in a study conducted by Kelley et al. (1999).

Autosomal recessive cases of *GJB6* were found mostly in conjunction with *GJB2* mutations that had a single mutant allele. The second allele was often unidentified, and later identified by Del Castillo et al. (2002). At first, the deletion was found to be of 342-kb in size, extending up to the *GJB2*. Sequencing of the breakpoint junctions revealed most patients with the deletion were heterozygous for it. It was found to be the second most common cause of pre-lingual hearing loss in Spanish population. Due to the presence of *GJB2* and *GJB6* genes in close proximity, the pattern of inheritance for both may be monogenic or digenic. Later, more accurate studies have shown that the actual size of the deletion is 309-kb (del Castillo et al., 2005).

A 309- kb deletion, involving the microsatellite marker D13S1830 was found by Del Castillo et al (2005). The mutation was present in a compound heterozygous form, with one allele containing a *GJB2* mutation and the other carrying a large deletion in the *GJB6* (del Castillo et al., 2002). It was found to be present in high frequencies in Spain, Israel, the UK and France, as found by a multicenter study (del Castillo et al., 2003). In Ashkenazi Jews and those living in Western Europe, a clear founder effect was shown by haplotype analysis. However, it has not been detected in some countries such as Italy, Turkey and Austria (Uyguner et al., 2003). Structurally, this deletion extends to the *GJB2* gene, which makes the control regions of *GJB2* more likely to be a part of this deletion. Patients carrying this mutation are more likely to be heterozygous, having one *GJB2* mutation and the carrying the other deletion. Because both the *GJB2* and *GJB6* genes are expressed in the inner ear, digenic inheritance may also occur. Teubner et al. reported the deficiency of endo-cochlear potential in *GJB6*-deficient mice due to cell death in the sensory epithelium of the cochlea. (Teubner et

al., 2003) More recently, another 232-kb deletion (D13S1854) has been reported, arising due to homologous recombination and usually occurring alongside of *GJB2* mutations. (del Castillo et al., 2005).

Dominant forms of *GJB6* mutations in non-syndromic patients have rarely been seen. Hidrotic ectodermal dysplasia (Clouston syndrome), an inherited autosomal dominant skin disorder is often caused by several missense mutations in the *GJB6* gene which is sometimes involved with deafness. (del Castillo et al., 2005)

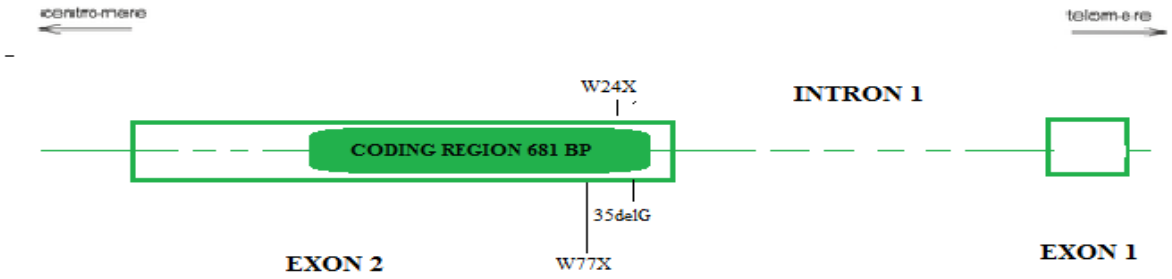

Figure 2.2. Positions of the mutations on the GJB2 gene. 35delG is a point mutation resµLting from a single G residue deletion on amino acid 12. It is followed by W24X (transition mutation, G→A resµLting in premature stop codon) on the 24th amino acid. W77X is a G→A transition resµLting in premature stop codon at 77th amino acid.

Figure 2.3. Total area of fragment deleted in GJB6 mutation D13S1830 (shown by dotted lines)

MATERIALS AND METHODS

3.1. Sample Size and IRB Approval

Our study was conducted on 77 patients suffering from congenital, pre-lingual; sever to profound hearing impairment, diagnosed by a trained clinician. This research has been duly approved by the Internal Review Board (IRB) of Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology. Approved IRB form has been attached for reference (**Appendix I**)

3.2. Study Population

Blood samples were taken from hearing impairment patients of different parts of Punjab province (Multan and Gujranwala regions). The inclusion criteria of the patients were as follows:

- Unrelated patients showing symptoms of Pre-lingual, Non-Syndromic, Sever to Profound Hereditary Hearing Impairment, diagnosed by a trained ENT specialist.
- In families with multiple affected individuals, only one patient was selected.

All those patients with any environmental cause (viral, bacterial infection, drugs etc.) or with any chronic illness (HCV etc.) were not considered for this study. To ensure this, proper disease history of each patient was taken and genetic background of the disease in their families was noted.

3.3. Blood Sample Collection

The selected patients were required to give blood sample, taken using 10 ml syringes by a practiced phlebotomist in standard potassium EDTA vacutainer tubes (BD, USA).

Written consent was taken before drawing blood (Attached, **Appendix II**). For minors, the consent was obtained from their parents. These blood samples were then processed for genomic DNA extraction at Immuno-genetics Lab in Atta-ur-Rahman School of Applied Sciences, National University of Sciences and Technology, Islamabad.

3.4. Genomic DNA Extraction

Genomic DNA was extracted using the standard phenol-chloroform method, using Proteinase-K digestion and SDS treatment. This method can take one or two days, depending on the time the samples are incubated for during the process. The first step was to take 750µL of blood in a micro centrifuge tube and mix with equal volume of Solution A (composition given in Table 3.1). The tubes were kept at room temperature for 15 minutes and then centrifuged for 1 minute at 13000 rpm. The resulting supernatant was discarded, leaving a pellet behind. The DNA pellet was re-suspended in 400µL of Solution A, vortexed to mix properly and centrifuged again as before. After discarding the supernatant for the second time, 400µL of Solution B (composition given in Table 3.1), 12µL of 20% SDS and 5µL of Proteinase-K was added to the pellet. The tubes were incubated at 65ºC for 3 hours or at 37ºC overnight. After incubation, a fresh mixture of Solution C and Solution D was prepared and 500µL of this mixture was added to the tubes. The tubes were thoroughly inverted to mix the solutions and were then centrifuged for 10 minutes at 13000 rpm. The resulting aqueous phase (upper layer) was collected in a newly labelled tube and 500µL of Solution D was added to it. After inverting the tubes a few times, they were centrifuged again as before. The aqueous layer was separated in a new tube again and 55µL of 3M (pH 6) Sodium Acetate along with 500µL of chilled isopropanol or 1 ml of 100% Ethanol was added to precipitate the DNA. At this stage, DNA strands are visible after inverting the tubes a few times. The tubes were then centrifuged for 10 minutes at 13000 rpm and the supernatant was discarded.

The tiny DNA pellet was seen as a white speck on the bottom or wall of the micro centrifuge tube. To the pellet, 200µL of 70% Ethanol was added and it was centrifuged at 13000 rpm for 7 minutes. The ethanol was discarded after and tubes were dried in an incubator at 37ºC for 30 minutes. DNA was dissolved in 150µL of TE (Tris-EDTA) buffer for storage and kept at 4ºC.

3.5. Agarose Gel Electrophoresis for DNA Quantification

The presence and quality of DNA was checked by running it on 1% agarose gel. To make the gel, 1 gram of agarose powder was dissolved in 100ml of 1% TAE (Tris-acetate and EDTA, pH 8) buffer. The mixture was heated until the agarose dissolved, upon which 5µL of Ethidium Bromide was added. Ethidium Bromide binds to the DNA and makes it visible under UV light. The mixture as poured into a casting tray and left to set. After the gel had set, the DNA was run at 80V for 25 minutes in 1% Tris-Acetate EDTA buffer (running buffer). The bands were observed under UV light. A single bright intact band shows that the DNA is pure and in good quantity, whereas disintegrated bands or smears show protein contamination.

3.6. Amplification Refractory Mutations System (ARMS) PCR

ARMS PCR is an efficient and sensitive method to detect the presence of single nucleotide polymorphisms (SNPs) and point mutations. In our case, three of the mutations selected (35delG, W77X and W24X) are point mutations, which means that change in one nucleotide base results in the changed phenotype. Therefore, to detect them accurately, we used ARMS PCR technique.

3.6.1. Primers for ARMS PCR

The primer sequence for the mutations 35delG and W77X were taken from Scott et al., (1998)**,** their sequence **s**hown in Table 1. For W24X, the normal and mutated primer were taken from the same paper, but instead of the common primer described there, we used reverse primer of W77X, resulting in a product size of 334 base pairs.

3.6.2. ARMS PCR Process

The PCR was carried out in 0.2 ml PCR tubes (Axygen, California, USA) containing 25µl of reaction mixture. The reaction mixture consisted of 2.5µl of $MgCl_2$ (25 mM, Thermo Fisher Scientific, USA), 2.0µl of dNTPs' (2mM, Thermo Fisher Scientific, USA), 1µl of forward (normal) primer (0.5µM, Macrogen), 1µl of reverse primer (0.5µM, Macrogen), 2.5µl of 10X buffer (100mM Tris-HCl, pH 8.3, 500mM KCl) (Thermo Fisher Scientific, USA) and 1µl of extracted genomic DNA (40ng) in 14.5µL nuclease free water (PCR water), making up a total of 25µL of reaction mixture in each tube. The PCR tubes were spun and 0.5µL of 1 unit Taq polymerase was added to each tube.

The PCR was performed using normal primer, and then repeated with mutated primer to confirm the presence of mutation. All the reactions were replicated to avoid chances of error.

The PCR was carried out in an Applied Biosystems Thermocycler and the optimized protocol is as follows: 95ºC for 5 minutes, followed by 35 cycles of 94ºC for 40 seconds, 60ºC for 30 seconds (annealing temperature of 35delG and W77X primers) and 55.6ºC for 30 seconds (for W24X); 72ºC for 30 seconds. The final extension was given at 72ºC for 10 minutes. The PCR products were then stored at 4ºC until analysis by gel electrophoresis.

3.7. PCR to detect Deletion *GJB6*- D13S1830

Simple PCR was used to detect the presence of the large *GJB6* deletion involving microsatellite marker D13S1830. Due to the large size of the deleted fragment, the primers were designed such that they contained parts of flanking regions of the mutation. Therefore, in samples containing the deletion, a small fragment (460 bp) would be amplified. In patients not having the deletion, no amplification would occur as a 309 kb fragment cannot be amplified by PCR. To ensure that error was avoided, negative and positive controls were run with every PCR reaction. Negative control lacked the template, whereas the positive control contained a DNA sample having the deletion (given by a foreign lab).

The optimized PCR program used was: Denaturation at 95ºC for 5 minutes, 30 cycles of denaturation at 94ºC for 40 seconds, annealing at 55.6ºC for 40 seconds, and extension at 72ºC for 30 seconds. The final extension was given at 72ºC for 7 minutes.

3.8. Analysis of PCR products on Agarose Gel

Agarose gel electrophoresis was used to determine the presence/absence of the mutations in the PCR products, using 1.5% agarose gel. Gel was prepared as mentioned above for DNA quantification, except that 1.5g of agarose powder was added to 100ml of TAE buffer. The PCR products were run at 70V for 20 minutes. 100bp DNA ladder was also run to confirm the size of band.

3.9. *In silico* Analysis: Homology Modelling

The 3D protein model of GJB2 protein was constructed using the PyMOL Molecular Graphics System (Version 1.7.4.4 Schrödinger, LLC) and the chain A of 2ZW3 pdb file (resolution 3.50 Å) for the crystal structure of GJB2 protein.

Table 3.1. Composition of solutions used for DNA extraction

Solution A	Solution B	Solution C	Solution D
0.32M Sucrose 10mM Tris (pH 7.5) 5mM $MgCl_2$ Autoclave, add 1% Triton X-100 (v/v)	10mM Tris (pH 7.5) 400mM NaCl 2mM EDTA (pH 8)	Phenol	Chloroform and Isoamyl alcohol (24:1)

Table 3.2. Sequence of primers used in ARMS PCR for 35delG, W77X and W24X and simple PCR for D13S1830

Mutation	PRIMER SEQUENCE	Annealing temp.	Product size
35delG	Normal:5'- TTGGGGCACGCTGCAGACGATCCTGGGGGG-3' Mutated:5'- TTGGGGCACGCTGCAGACGATCCTGGGGGT-3' Common: 5'- GAAGTAGTGATCGTAGCACACGTTCTTGCA-3'	60ºC	202 bp
W77X	Normal: 5'- TACTTCCCCATCTCCCACATCCGGCTATGG-3' Mutated:5'- TACTTCCCCATCTCCCACATCCGGCTATGA-3' Common:5'- GATGACCGGGAAGATGCTGCTTGTGAT-3'	60ºC	234 bp
W24X	Normal: 5'-CTCCACCAGCATTGGAAAGATCTG-3' Mutated: 5'-CTCCACCAGCATTGGAAAGATCTA-3' Common:5'- GATGACCGGGAAGATGCTGCTTGTGAT-3'	55.4ºC	388 bp
D13S18-30	F: 5'- TTTAGGGCATGATTGGGGTGATTT-3' R: 5'- CACCATGCGTAGCCTTAACCATTTT-3'	54.1ºC	Amplifies 460 bp in samples having deletion

RESULTS

4.1. Clinical Findings:

All the patients were found to have pre-lingual, congenital, sever to profound, hearing impairment as diagnosed by trained clinical specialist. All affected individuals were healthy and had no other abnormalities.

4.2. Mutation Screening:

ARMS PCR was done on all 77 samples separately for all *GJB2* mutations, as all three of these are resulted by change in a single base pair, resulting in stop codon which produces truncated protein. PCR reaction was first run using normal primer and then repeated with the mutated primer for all samples to confirm the mutation. For *GJB6* deletion D13S1830, simple PCR was performed using forward and reverse primers. All PCR products were checked for results by gel electrophoresis.

From a total of 77 HI subjects, 43 showed at least one of the three *GJB2* mutations. This signifies that *GJB2* mutations are widely prevalent in our sample population, accounting for approximately 56% of the cases. The rest of the samples did not test positive for these three mutations so we can assume that other mutations, either in the *GJB2* gene or in other genes are responsible for causing those.

4.2.1 Prevalence of W24X

The mutation W24X was found to be the most prevalent amongst this particular group of Pakistani individuals, with a frequency of 27/77 (35%). Previous studies found this

mutation to be more prevalent among the *GJB2* mutations in Pakistani and Indian populations.

4.2.2 Prevalence of W77X

This mutation was less widespread, and was detected in 10 out of the 77 individuals (12.9%).

4.2.3 Prevalence of 35delG

Being the least common mutation in our sample size, this mutation had a frequency of 6/77, making up 7.7% of the total.

4.2.4 Prevalence of *GJB6*-delD13S1830

None of the samples tested positive for this deletion. This is the first study checking the prevalence of del-D13S1830 in a Pakistani population.

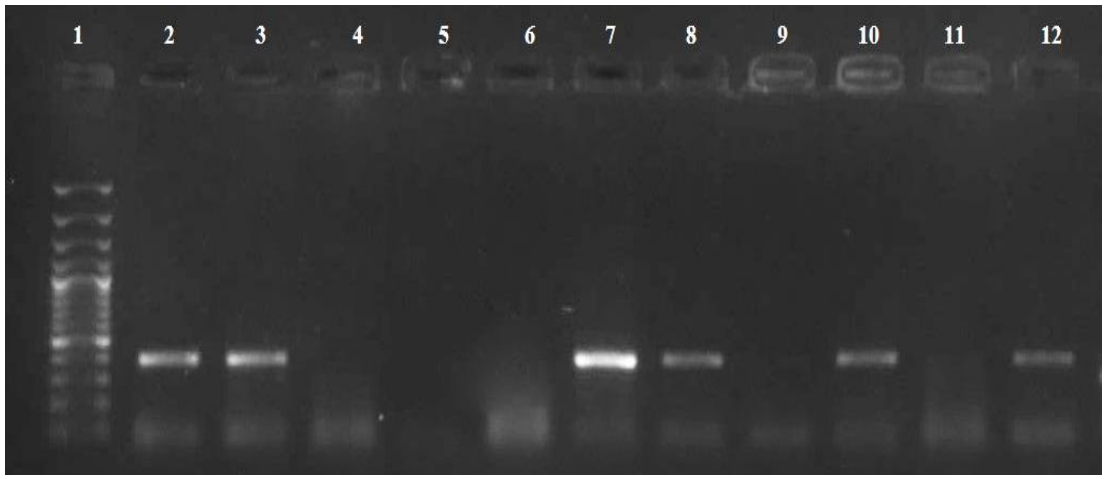

Figure 4.1 Electropherogram of PCR products of W24X with normal primer. The 1st well contains the 100 bp ladder. Presence of band suggests mutation not present in that sample. Absence of band in well no. 4, 5, 6, 9 and 11 suggests presence of mutation which was confirmed by running a PCR with mutated primer (shown below).

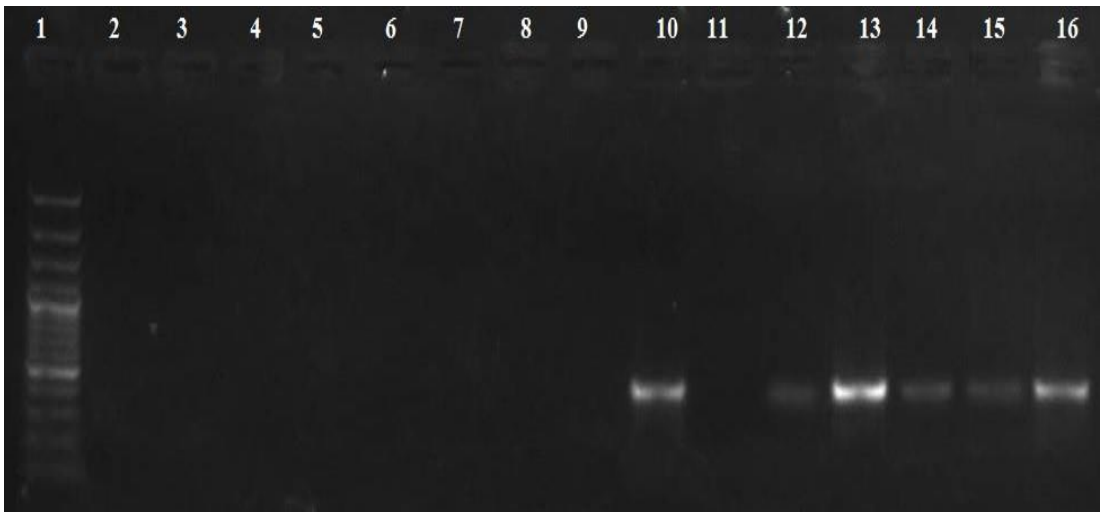

Figure 4.1 (b) Electropherogram of PCR products of W24X with mutated primer. The 1st well contains the 100 bp ladder. Presence of band confirms mutation in the sample and vice versa.

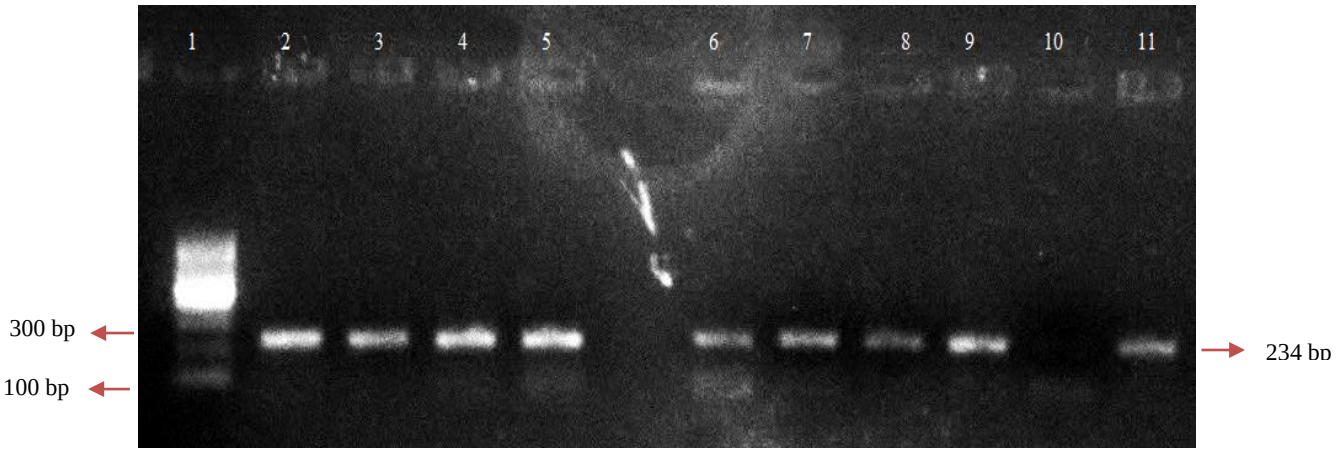

Figure 4.2 (a) Electropherogram of PCR of W77X with normal primer. The 1st well contains the 100 bp ladder. Presence of band indicates absence of mutation in that sample. Each well is numbered at the top. The 1st well contains a 100 bp ladder. The rest of the wells have been loaded with PCR product of each sample. In samples where no band is present (Well no. 10), it suggests that the mutation might be present.

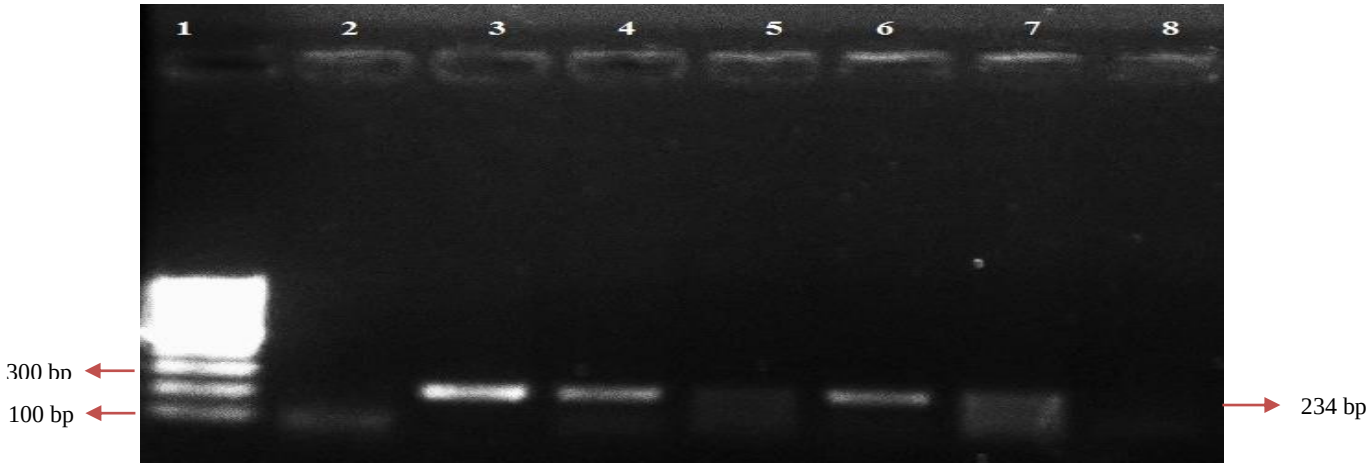

Figure 4.2 (b) Electropherogram of PCR of W77X with mutated primer. The 1st well contains 100 bp ladder. Presence of band (Well no. 3, 4 and 6) indicates presence of mutation in that sample.

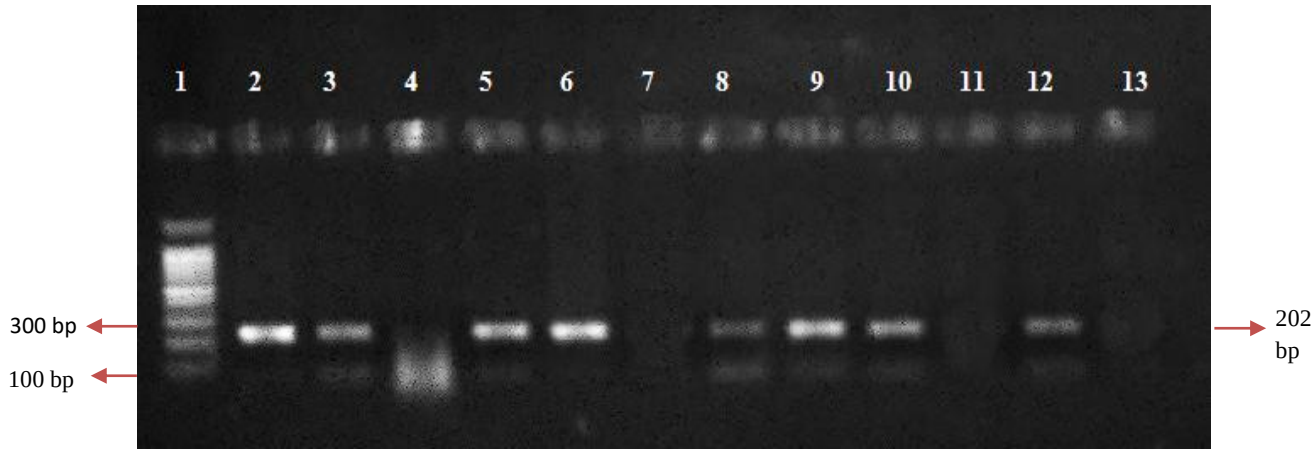

Figure 4. 3 (a). Electropherogram for checking 35delG mutation with normal primer. The 1st well contains 100 bp ladder. Presence of band indicates that mutation is not present in that sample. Absence of band (well 4,7, 11 and 13) indicates that mutation may be present in that sample.

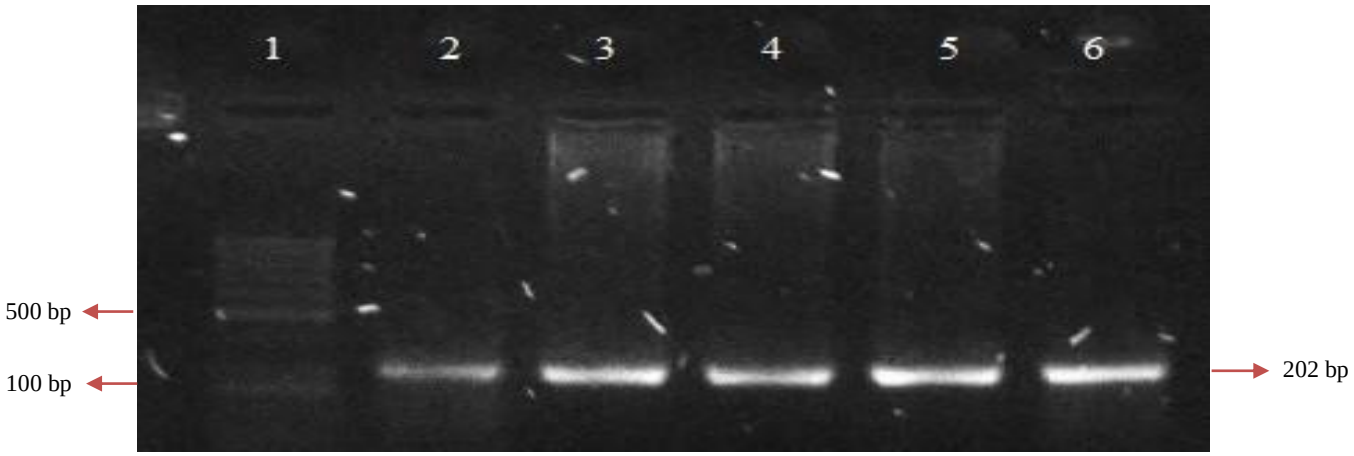

Figure 4.3 (b) Electropherogram for confirming the presence of mutation 35delG with mutated primer. The 1st well contains 100 bp ladder. Presence of band confirms presence of mutation in sample.

Table 4.1. *GJB2* Mutation Frequency in sample population

Mutations	Frequency
35delG	6 (7.7%)
W24X	27 (35.06%
W77X	10 (12.9%)
Total	43/77 (55.84%)

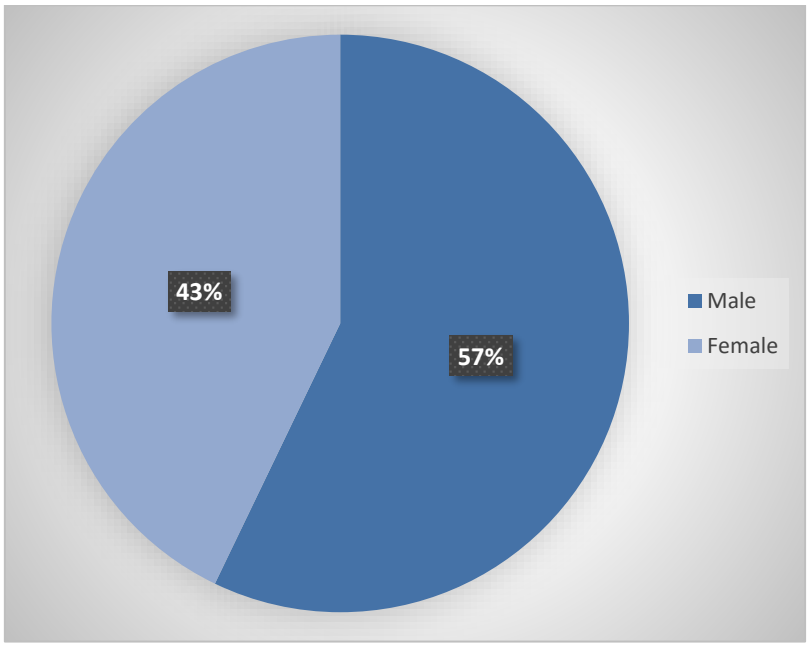

Figure 4.5. Pie chart showing gender distribution in our sample population. 57% of our samples were male and 43% were female.

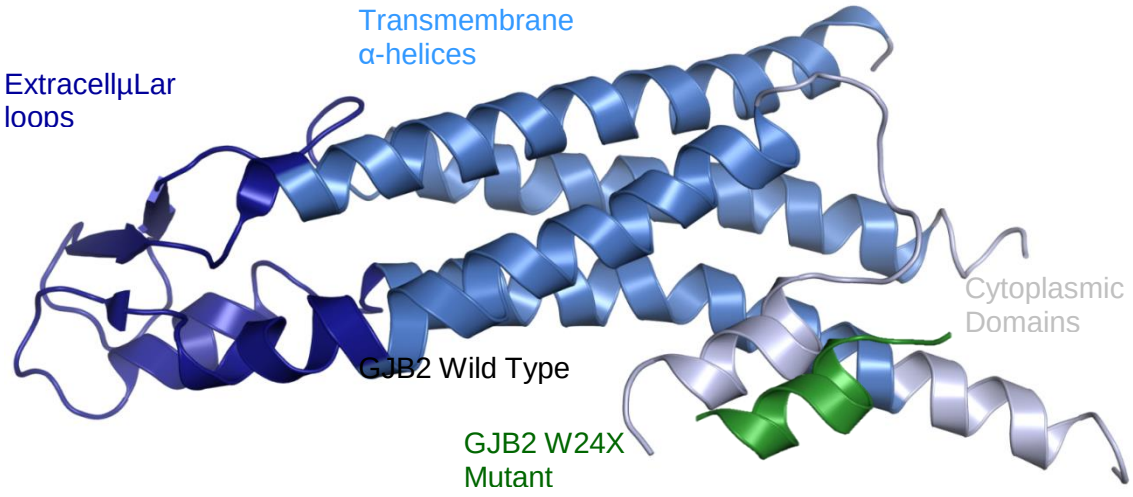

Figure 4.6. Comparison of wild type and p.W24X mutant GJB2 proteins: The topology of wild type GJB2 protein is displayed, consisting of three cytoplasmic domains (grey), four transmembrane α-helices (royal blue) and two extracellular loops (dark blue). The p. W24X mutation results in a very short truncated GJB2 protein (green) which lacks two cytoplasmic domains in addition to the entire transmembrane and extracellular portion.

Discussion

Pakistani society offers a large genetic diversity due to its long history and the intermingling of different races such as the Arabs, Persians. Due to the predominance of consanguineous marriages in our culture, recessive disorders such as ARNSHL are rampant. Therefore, our population is a rich source of data for research on genetic diseases such as ARNSHL. Although there is no long term solution of hearing impairment, there is a chance for correcting the disorder by cochlear transplant but only in the case of connexin mutations. Furthermore, it can help in managing the disease by proper genetic counselling. But without prior knowledge about the mutations currently prevalent in our country, this is impossible.

ARNSHL is difficult to diagnose through molecular testing because of the complex involvement of various genes and their heterogeneous nature. It is not feasible to perform a genetic test for all genes involved in HI as the causative mutations are caused by hundreds of genes (many of them unknown) and is very costly. Therefore, more epidemiological data is needed for cheaper and accurate molecular diagnosis.

W77X and W24X have already been reported to be more prevalent in Pakistan (Santos et al., 2005) Spanish Romani families were found to have a very high prevalence of *GJB2* mutations, especially W24X (Alvarez et al., 2005). In North Indians, the occurrence of *GJB2* mutations was found to be low, with 35delG being the most common amongst those (Bhalla et al., 2009).

In our study, we chose to screen three *GJB2* mutations (which had been previously reported in Pakistan and other parts of the world) and one *GJB6* deletion (not studied in Pakistan before this). This research aims at identifying the most common causative agent for ARNSHL in a group of Pakistani individuals from Punjab province. According to our study,

the most common mutation was W24X, which coincides with previous studies held on an Indian population (Padma et al., 2009).

ARMS PCR was used because these mutations have a single base pair change which can be efficiently d*et*ected through ARMS PCR. 35delG, which has been found to be very common in most Caucasian populations, was not as common in Pakistan. Previous studies on Pakistani population shows conflicting results as some have reported *GJB2* mutations to have a low prevalence in Pakistan (Santos et al., 2005; Bukhari et al., 2013; Salman et al., 2015), whereas another study by Shafique et al. (2014) on deaf subjects from various areas of Punjab reports a high frequency of *GJB2* mutations, similar to our results. This difference may have arisen due to the variance in ethnicities between the sample population because each race has a different ancestry, arising from various parts of the world.

In our sample population, the occurrence of W24X mutation was higher than that of W77X, whereas a previous study found W77X to be more widespread (Santos et al., 2005).

It has been shown that *GJB6* mutation D13S1830 is often found in compound heterozygosity with *GJB2* mutations. But it did not appear in any of our samples. This mutation has not been found in Pakistani and Indian populations. However, it is relatively frequent in Israel, Spain and France (Seeman et al., 2005). These results propose that *GJB6* mutations are limited to specific populations only.

Table 5. 1. Comparison between the mutation frequency for GJB2 in different studies on Pakistani population.

Bukhari et al., 2013	Low prevalence of *GJB2* mutations in Hazara region
Shafique et al., 2014	*GJB2* mutations accounted for 53% of the thirty families studied (different regions of Punjab)
Salman, Bashir et al., 2015	*GJB2* mutations contribute to 9.52 % of HI among familial participants and 4.65 % among sporadically affected individuals.
Santos et al., 2005	Low prevalence of *GJB2* in 192 Pakistani families (mainly Hindko, Seraiki and Sindhi speaking)
Our study	• GJB2 mutations account for upto 56% of cases studied • W24X found to be most prevalent *GJB2* deletion (35%) • D13S1830 inexistent in Pakistani population

Conclusion/ Future Prospects

In the light of our findings, we conclude that *GJB2* mutations are found in a high frequency in our sample population and out of those, W24X is the most prevalent. The D13S1830 deletion (*GJB6*) was not found in our studied samples. These results coincide with studies conducted on an Indian population, which suggest common ancestral lineage amongst Indian and Pakistani population groups. However, to find out more about the prevalence of these mutations accurately, we need large scale studies in future. Screening of these variants of *GJB2* and *GJB6* genes will provide a basis of genetic testing for affected individuals. This will help decide if cochlear implants may be the best treatment form available, as these gap junctions are expressed in the cochlea.

References

Alwan, A. D., Modell, B., Bittles, A., Czeilel, A. and Hamamy, H. (1997). *Community control of genetic and congenital disorders*, World Health Organisation. Office for the Eastern Mediterranean.

Antoniadi, T., Rabion*et*, R., Kroupis, C., Aperis, G. A., Economides, J., P*et*mezakis, J., Economou-P*et*ersen, E., Estivill, X. and P*et*ersen, M. B. (1999). High prevalence in the Greek popµLation of the 35delG mutation in the connexin 26 gene causing prelingual deafness. *Clin Genet,* 55 (5)**:** 381-2.

Bhalla, S., Sharma, R., Khandelwal, G., Panda, N. K. and KhµLlar, M. (2009). Low incidence of GJB2, GJB6 and mitochondrial DNA mutations in North Indian patients with non-syndromic hearing impairment. *Biochem Biophys Res Commun,* 385 (3)**:** 445-8.

Brobby, G. W., MµLler-Myhsok, B. and Horstmann, R. D. (1998). Connexin 26 R143W mutation associated with recessive nonsyndromic sensorineural deafness in Africa. *N Engl J Med,* 338 (8)**:** 548-50.

Chaib, H., Lina-Granade, G., Guilford, P., Plauchu, H., Levilliers, J., Morgon, A. and P*et*it, C. (1994). A gene responsible for a dominant form of neurosensory non-syndromic deafness maps to the NSRD1 recessive deafness gene interval. *Hum Mol Genet,* 3 (12)**:** 2219-22.

Cryns, K., Orzan, E., Murgia, A., Huygen, P. L., Moreno, F., Del Castillo, I., Chamberlin, G. P., Azaiez, H., Prasad, S., Cucci, R. A., Leonardi, E., Snoeckx, R. L., Govaerts, P. J., Van De Heyning, P. H., Van De Heyning, C. M., Smith, R. J. and Van Camp, G. (2004). A genotype-phenotype correlation for GJB2 (connexin 26) deafness. *J Med Genet,* 41 (3)**:** 147-54.

Dahl, E., Manthey, D., Chen, Y., Schwarz, H. J., Chang, Y. S., Lalley, P. A., Nicholson, B. J. and Willecke, K. (1996). MolecµLar cloning and functional expression of mouse connexin-30,a gap junction gene highly expressed in adµLt brain and skin. *J Biol Chem*, 271 (30)**:** 17903-10.

Del Castillo, F. J., Rodriguez-Ballesteros, M., Alvarez, A., Hutchin, T., Leonardi, E., De Oliveira, C. A., Azaiez, H., Brownstein, Z., Avenarius, M. R., Marlin, S., Pandya, A., Shahin, H., Siemering, K. R., Weil, D., Wuyts, W., Aguirre, L. A., Martin, Y., Moreno-Pelayo, M. A., Villamar, M., Avraham, K. B., Dahl, H. H., Kanaan, M., Nance, W. E., P*et*it, C., Smith, R. J., Van Camp, G., Sartorato, E. L., Murgia, A., Moreno, F. and Del Castillo, I. (2005). A novel del*et*ion involving the connexin-30 gene, del(GJB6-d13s1854), found in trans with mutations in the GJB2 gene (connexin-26) in subjects with *DFNB*1 non-syndromic hearing impairment. *J Med Genet*, 42 (7)**:** 588-94.

Del Castillo, I., Moreno-Pelayo, M. A., Del Castillo, F. J., Brownstein, Z., Marlin, S., Adina, Q., Cockburn, D. J., Pandya, A., Siemering, K. R., Chamberlin, G. P., Ballana, E., Wuyts, W., Maciel-Guerra, A. T., Alvarez, A., Villamar, M., Shohat, M., Abeliovich, D., Dahl, H. H., Estivill, X., Gasparini, P., Hutchin, T., Nance, W. E., Sartorato, E. L., Smith, R. J., Van Camp, G., Avraham, K. B., P*et*it, C. and Moreno, F. (2003). Prevalence and evolutionary origins of the del(GJB6-D13S1830) mutation in the *DFNB*1 locus in hearing-impaired subjects: a mµLticenter study. *Am J Hum Genet*, 73 (6)**:** 1452-8.

Del Castillo, I., Villamar, M., Moreno-Pelayo, M. A., Del Castillo, F. J., Alvarez, A., Telleria, D., Menendez, I. and Moreno, F. (2002). A del*et*ion involving the connexin 30 gene in nonsyndromic hearing impairment. *N Engl J Med*, 346 (4)**:** 243-9.

Fuse, Y., Doi, K., Hasegawa, T., Sugii, A., Hibino, H. and Kubo, T. (1999). Three novel connexin26 gene mutations in autosomal recessive non-syndromic deafness. *Neuroreport,* 10 (9)**:** 1853-7.

Gasparini, P., Estivill, X., Volpini, V., Totaro, A., Castellvi-Bel, S., Govea, N., Mila, M., Della Monica, M., Ventruto, V., De Bened*et*to, M., Stanziale, P., Zelante, L., Mansfield, E. S., Sandkuijl, L., Surrey, S. and Fortina, P. (1997). Linkage of *DFNB*1 to non-syndromic neurosensory autosomal-recessive deafness in Mediterranean families. *Eur J Hum Genet,* 5 (2)**:** 83-8.

Gasparini, P., Rabion*et*, R., Barbujani, G., Melchionda, S., P*et*ersen, M., Brondum-Nielsen, K., M*et*spalu, A., Oitmaa, E., Pisano, M., Fortina, P., Zelante, L. and Estivill, X. (2000). High carrier frequency of the 35delG deafness mutation in European popµLations. Gen*et*ic Analysis Consortium of GJB2 35delG. *Eur J Hum Genet,* 8 (1)**:** 19-23.

Grifa, A., Wagner, C. A., D'ambrosio, L., Melchionda, S., Bernardi, F., Lopez-Bigas, N., Rabion*et*, R., Arbones, M., Monica, M. D., Estivill, X., Zelante, L., Lang, F. and Gasparini, P. (1999). Mutations in GJB6 cause nonsyndromic autosomal dominant deafness at DFNA3 locus. *Nat Genet,* 23 (1)**:** 16-8.

Guilford, P., Ben Arab, S., Blanchard, S., Levilliers, J., Weissenbach, J., Belkahia, A. and P*et*it, C. (1994). A non-syndrome form of neurosensory, recessive deafness maps to the pericentromeric region of chromosome 13q. *Nat Genet,* 6 (1)**:** 24-8.

Harris, A. L. (2001). Emerging issues of connexin channels: biophysics fills the gap. *Q Rev Biophys,* 34 (3)**:** 325-472.

Kelsell, D. P., Dunlop, J., Stevens, H. P., Lench, N. J., Liang, J. N., Parry, G., Mueller, R. F. and Leigh, I. M. (1997). Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature,* 387 (6628)**:** 80-3.

Kumar, N. M. and GilµLa, N. B. (1996). The gap junction communication channel. *Cell,* 84 (3)**:** 381-8.

Lautermann, J., Ten Cate, W. J., Altenhoff, P., Grummer, R., Traub, O., Frank, H., Jahnke, K. and Winterhager, E. (1998). Expression of the gap-junction connexins 26 and 30 in the rat cochlea. *Cell Tissue Res,* 294 (3)**:** 415-20.

Lee, S. W., Tomas*et*to, C., PaµL, D., Keyomarsi, K. and Sager, R. (1992). Transcriptional downregµLation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. *J Cell Biol,* 118 (5)**:** 1213-21.

Li, X. C., Evere*tt*, L. A., Lalwani, A. K., Desmukh, D., Friedman, T. B., Green, E. D. and Wilcox, E. R. (1998). A mutation in PDS causes non-syndromic recessive deafness. *Nat Genet,* 18 (3)**:** 215-7.

Maestrini, E., Korge, B. P., Ocana-Sierra, J., Calzolari, E., Cambiaghi, S., Scudder, P. M., Hovnanian, A., Monaco, A. P. and Munro, C. S. (1999). A missense mutation in connexin26, D66H, causes mutilating keratoderma with sensorineural deafness (Vohwinkel's syndrome) in three unrelated families. *Hum Mol Genet,* 8 (7)**:** 1237-43.

Maheshwari, M., Vijaya, R., Ghosh, M., Shastri, S., Kabra, M. and Menon, P. S. (2003). Screening of families with autosomal recessive non-syndromic hearing impairment (ARNSHI) for mutations in GJB2 gene: Indian scenario. *Am J Med Genet A,* 120a (2)**:** 180-4.

Maw, M. A., Allen-Powell, D. R., Goodey, R. J., Stewart, I. A., Nancarrow, D. J., Hayward, N. K. and Gardner, R. J. (1995). The contribution of the *DFNB*1 locus to neurosensory deafness in a Caucasian popµLation. *Am J Hum Genet,* 57 (3)**:** 629-35.

Mignon, C., Fromag*et*, C., Mattei, M. G., Gros, D., Yamasaki, H. and Mesnil, M. (1996). Assignment of connexin 26 (GJB2) and 46 (GJA3) genes to human chromosome

13q11-->q12 and mouse chromosome 14D1-E1 by in situ hybridization. *Cytogenet Cell Genet,* 72 (2-3)**:** 185-6.

Morell, R. J., Kim, H. J., Hood, L. J., Goforth, L., Friderici, K., Fisher, R., Van Camp, G., Berlin, C. I., Oddoux, C., Ostrer, H., Keats, B. and Friedman, T. B. (1998). Mutations in the connexin 26 gene (GJB2) among Ashkenazi Jews with nonsyndromic recessive deafness. *N Engl J Med,* 339 (21)**:** 1500-5.

Padma, G., Ramchander, P. V., Nandur, U. V. and Padma, T. (2009). GJB2 and GJB6 gene mutations found in Indian probands with congenital hearing impairment. *J Genet,* 88 (3)**:** 267-72.

Putcha, G. V., Bejjani, B. A., Bleoo, S., Booker, J. K., Carey, J. C., Carson, N., Das, S., Dempsey, M. A., Gastier-Foster, J. M., Greinwald, J. H., Jr., Hoffmann, M. L., Jeng, L. J., Kenna, M. A., Khababa, I., Lilley, M., Mao, R., Muralidharan, K., Otani, I. M., Rehm, H. L., Schaefer, F., Seltzer, W. K., Spector, E. B., Springer, M. A., Weck, K. E., Wenstrup, R. J., Withrow, S., Wu, B. L., Zariwala, M. A. and Schrijver, I. (2007). A mμLticenter study of the frequency and distribution of GJB2 and GJB6 mutations in a large North American cohort. *Genet Med,* 9 (7)**:** 413-26.

Ramchander, P., Nandur, V., Dwarakanath, K., Vishnupriya, S. and Padma, T. (2004). Study of families of nonsyndromic hearing impairment segregating with mutations in Cx26 gene. *Indian Journal of Human Genetics,* 10 (2)**:** 58.

Ramshankar, M., Girirajan, S., Dagan, O., Ravi Shankar, H. M., Jalvi, R., Rangasayee, R., Avraham, K. B. and Anand, A. (2003). Contribution of connexin26 (GJB2) mutations and founder effect to non-syndromic hearing loss in India. *J Med Genet,* 40 (5)**:** e68.

Salman, M., Bashir, R., Imtiaz, A., Maqsood, A., Mujtaba, G., Iqbal, M. and Naz, S. (2015). Mutations of GJB2 encoding connexin 26 contribute to non-syndromic moderate and severe hearing loss in Pakistan. *Eur Arch Otorhinolaryngol,* 272 (8)**:** 2071-5.

Santos, R. L., Wajid, M., Pham, T. L., Hussan, J., Ali, G., Ahmad, W. and Leal, S. M. (2005). Low prevalence of Connexin 26 (GJB2) variants in Pakistani families with autosomal recessive non-syndromic hearing impairment. *Clin Genet,* 67 (1)**:** 61-8.

Seeman, P., Bendova, O., Raskova, D., Malikova, M., Groh, D. and Kabelka, Z. (2005). Double h*et*erozygosity with mutations involving both the GJB2 and GJB6 genes is a possible, but very rare, cause of congenital deafness in the Czech popµLation. *Ann Hum Genet,* 69 (Pt 1)**:** 9-14.

Shafique, S., Siddiqi, S., Schraders, M., Oostrik, J., Ayub, H., Bilal, A., Ajmal, M., Seco, C. Z., Strom, T. M., Mansoor, A., Mazhar, K., Shah, S. T., Hussain, A., Azam, M., Kremer, H. and Qamar, R. (2014). Gen*et*ic spectrum of autosomal recessive non-syndromic hearing loss in Pakistani families. *PLoS One,* 9 (6)**:** e100146.

Snoeckx, R. L., Huygen, P. L., Feldmann, D., Marlin, S., Denoyelle, F., Waligora, J., Mueller-Malesinska, M., Pollak, A., Ploski, R., Murgia, A., Orzan, E., Castorina, P., Ambros*et*ti, U., Nowakowska-Szyrwinska, E., Bal, J., Wiszniewski, W., Janecke, A. R., Nekahm-Heis, D., Seeman, P., Bendova, O., Kenna, M. A., FrangµLov, A., Rehm, H. L., Tekin, M., IncesµLu, A., Dahl, H. H., Du Sart, D., Jenkins, L., Lucas, D., Bitner-Glindzicz, M., Avraham, K. B., Brownstein, Z., Del Castillo, I., Moreno, F., Blin, N., Pfister, M., Sziklai, I., Toth, T., Kelley, P. M., Cohn, E. S., Van Maldergem, L., Hilbert, P., Roux, A. F., Mondain, M., Hoefsloot, L. H., Cremers, C. W., Lopponen, T., Lopponen, H., Parving, A., Gronskov, K., Schrijver, I., Roberson, J., Gualandi, F., Martini, A., Lina-Granade, G., Pallares-Ruiz, N., Correia, C., Fialho, G., Cryns, K., Hilgert, N., Van De Heyning, P., Nishimura, C. J., Smith, R. J. and Van Camp, G. (2005). GJB2 mutations and degree of hearing loss: a mµLticenter study. *Am J Hum Genet,* 77 (6)**:** 945-57.

Tassabehji, M., Newton, V. E. and Read, A. P. (1994). Waardenburg syndrome type 2 caused by mutations in the human microphthalmia (MITF) gene. *Nat Genet,* 8 (3)**:** 251-5.

Tassabehji, M., Read, A. P., Newton, V. E., Harris, R., Balling, R., Gruss, P. and Strachan, T. (1992). Waardenburg's syndrome patients have mutations in the human homologue of the Pax-3 paired box gene. *Nature,* 355 (6361)**:** 635-6.

Teubner, B., Michel, V., Pesch, J., Lautermann, J., Cohen-Salmon, M., Sohl, G., Jahnke, K., Winterhager, E., Herberhold, C., Hardelin, J. P., P*et*it, C. and Willecke, K. (2003). Connexin30 (Gjb6)-deficiency causes severe hearing impairment and lack of endocochlear potential. *Hum Mol Genet,* 12 (1)**:** 13-21.

Uyguner, O., Emiroglu, M., Uzumcu, A., Hafiz, G., Ghanbari, A., Baserer, N., Yuksel-Apak, M. and Wollnik, B. (2003). Frequencies of gap- and tight-junction mutations in Turkish families with autosomal-recessive non-syndromic hearing loss. *Clin Genet,* 64 (1)**:** 65-9.

Van Laer, L., Coucke, P., Mueller, R. F., Ca*et*hoven, G., Flothmann, K., Prasad, S. D., Chamberlin, G. P., Houseman, M., Taylor, G. R., Van De Heyning, C. M., Fransen, E., Rowland, J., Cucci, R. A., Smith, R. J. and Van Camp, G. (2001). A common founder for the 35delG GJB2 gene mutation in connexin 26 hearing impairment. *J Med Genet,* 38 (8)**:** 515-8.

Zelante, L., Gasparini, P., Estivill, X., Melchionda, S., D'agruma, L., Govea, N., Mila, M., Monica, M. D., Lutfi, J., Shohat, M., Mansfield, E., Delgrosso, K., Rappaport, E., Surrey, S. and Fortina, P. (1997). Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (*DFNB*1) in Mediterraneans. *Hum Mol Genet,* 6 (9)**:** 1

Appendix 1

ASAB

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES
NATIONAL UNIVERSITY OF SCIENCES & TECHNOLOGY

Ref.No:26IRB- Date:20April, 2016

IRB APPROVAL LETTER

Project Title: Screening of GJB2 (35delG, W24X, W77X) and GJB6 (D1s1830) Mutation in patients with Non-syndromic Hereditary Hearing Impairment

Name of Principal Investigator I: Dr. M. Jawad Hassan

Name of Principal Investigator II: _

Field and Subfield of Project: Biosciences

Duration: 12 Month

Name of the Department: Health Care Biotechnology, ASAB, NUST

The aforesaid project has been reviewed by Institutional Review Board (IRB) Committee, ASAB, keeping in view the following selection criteria:

- Qualification, Expertise and Scientific Caliber of the Principal Investigators
- Proposed Goals of the Study
- Subject Selection
- Selection Criteria of Subjects
- Informed Consent Process
- Potential Problems
- Research Design and Methods
- Potential Benefits of the Study
- Risks of the Study
- Management of Risks
- Assessment of Risk
- Confidentiality
- Conflict of Interest

The committee thus **APPROVES** the project on "**Screening of GJB2 (35delG, W24X, W77X) and GJB6 (D1s1830) Mutation in patients with Non-syndromic Hereditary Hearing Impairment"** on the scales and criterion set by IRB.

Dr. Najam us Sahar Zaidi
Member, IRB
ASAB, NUST

Dr. Muhammad Tahir
Member, IRB
ASAB, NUST

Dr. Hajra Sadia
HoD Research, Head of IRB
ASAB, NUST

Atta-Ur-Rahman School of Applied Biosciences, National University of Sciences & Technology, H-12, Islamabad, Pakistan.
Ph: +92 5190856101, Fax: +92 5190856102.

Appendix II

INFORMED CONSENT FORM

"Screening of *GJB2 (*35delG, W24X, W77X) and *GJB6* (D13S1830) Mutations in Patients with Non-Syndromic Hereditary Hearing Impairment"

AIMS AND OBJECTIVES

To screen and analyse the above mentioned mutations in the genes *GJB2* and *GJB6* in patients suffering from Non-Syndromic Hereditary Hearing Impairment.

SAMPLE COLLECTION

Five ml blood sample will be collected from patients with profound non- syndromic hereditary hearing impairment.

INFORMED CONSENT

You are being asked to participate in a research study to find out the gen*et*ic cause of the disease. You will be asked to donate 5ml of blood. This will not cause any physical injury. Your samples will be preserved in the laboratory and will be tested for gen*et*ic mutations of specific genes involved in the disease running in your family. Your identity in this study will be protected. You can terminate your participation at any time in the course of this study. This research project will be carried out solely on a non-commercial basis. Your participation is voluntarily. Further, if it becomes necessary, in your interest, counselling will be provided to your family. The scientific information will only be shared among the collaborating scientists. The resµLts of the study if novel or of medical interest will be published in scientific journals without disclosing your identity.

اجازت نامہ

مجوزہ تحقیقي منصوبہ آپ میں پائی جانے والي بیماري کي وجوہات معلوم کرنے کے لیئے خالصتا غیر تجارتي بنیادوں پر ترتیب دیا گیا ہے. آپ سے٥ ملي لیٹر خون کے عطیے کي درخواست کي جاتي ہے. خون دینے سے آپ کو کسي قسم کا درد یا زخم نہیں ہو گا. آپ کي شناخت کو مکمل طور پر صیغۂ راز میں رکھا جائے گا. آپ کا دیا ہوا عطیہ تجربہ گاہ میں محفوظ رکھا جائے گا اور ہم ان خاص جینیاتي تبدیلیوں کا مشاہدہ کرتے ہوئے آپ کي بیماري کو سمجھنے کي کوشش کریں گے. جس سے آپ اور آپ جیسے دوسرے مریضوں کا بہتر علاج ممکن ہو سکے گا. آپ اس تحقیق کے دوران کسي بھي وقت اپني شمولیت سے دستبردار ھو سکتے ہیں. آپ کي شمولیت رضاکارانہ ہے. دوران تحقیق اگر ضروري ہوا تو آپ کو بیماري سے متعلق مشاورت بھي کي جائے گي. حاصل شدہ سائنسي معلومات کا تبادلہ صرف تحقیق میں شامل سائنسدانوں کے مابین کیا جائے گا.

I hereby confirm that I fµLly understand what has been stated above. I voluntarily donate blood sample from myself for research purposes only.

میں تصدیق کرتا/ کرتي ھوں کہ جو کچھ بھي مجھ سے بیان کیا گیا ھے، میں اسے مکمل طور
پر سمجھ گیا/گي ھوں. نیز میں اپنے خون کا نمونہ رضاکارانہ طور پر صرف تحقیق کے لئے بطور عطیہ دیتا/دیتي ھوں
مجھے میرے تمام سوالات کے جواب مل گئے ہیں اور في الوقت میرے ذہن میں کوئی اور سوالات نہیں.

Signature/Thumb impression of the patient: ----------

Name: ---------- **Participant ID----**

Appendix III

#	Case #	Gender	Age	Location	35delG	W77x	W24x
1	SaG2CSE-002	M	16	Gujranwala Punjab	-	-	-
2	SaG2CSE-003	M	8	Gujranwala Punjab	√	-	√
3	SaG2CSE-004	F	14	Gujranwala Punjab	-	-	-
4	SaG2CSE-005	F	16	Gujranwala Punjab	-	-	√
5	SaG2CSE-006	F	13	Gujranwala Punjab	-	-	√
6	SaG2CSE-008	F	15	Gujranwala Punjab	-	-	√
7	SaG2CSE-013	F	7	Gujranwala Punjab	-	-	√
8	SaG2CSE-014	M	18	Gujranwala Punjab	-	-	√
9	SaG2CSE-015	M	16	Gujranwala Punjab	-	-	-
10	SaG2CSE-016	M	06	Gujranwala Punjab	-	-	-
11	SaG2CSE-017	M	14	Gujranwala Punjab	-	-	-
12	SaG2CSE-019	F	---	Gujranwala Punjab	√	-	√
13	SaG2CSE-020	M	14	Gujranwala Punjab	-	-	√
14	SaG2CSE-021	M	14	Gujranwala Punjab	-	√	√
15	SaG2CSE-022	F	14	Gujranwala Punjab	√	-	√
16	SaG2CSE-023	F	14	Gujranwala Punjab	-	-	√
17	SaG2CSE-024	F	16	Gujranwala Punjab	-	-	√
18	SaG2CSE-025	F	14	Gujranwala Punjab	-	-	-
19	SaG2CSE-026	F	14	Gujranwala Punjab	-	-	√
20	SaG2CSE-027	M	10	Gujranwala Punjab	-	-	√
21	SaG2CSE-033	F	6	Gujranwala Punjab	-	-	√
22	SaG2CSE-034	F	16	Gujranwala Punjab	-	-	-
23	SaG2CSE-	F	12	Gujranwala	-	-	√

	035			Punjab			
24	SaG2CSE-036	F	14	Gujranwala Punjab	-	-	-
25	SaG2CSE-037	M	10	Gujranwala Punjab	-	-	-
26	SaG2CSE-038	M	10	Gujranwala Punjab	-	-	√
27	SaG2CSE-039	M	6	Gujranwala Punjab	-	√	-
28	SaG2CSE-040	F	17	Gujranwala Punjab	-	-	-
29	SaG2CSE-041	M	14	Gujranwala Punjab	-	√	√
30	SaG2CSE-042	F	16	Gujranwala Punjab	√	-	√
31	SaG2CSE-043	M	13	Gujranwala Punjab	-	√	-
32	SaG2CSE-045	M	13	Gujranwala Punjab	-	-	√
33	SaG2CSE-046	M	15	Gujranwala Punjab	-	-	-
34	SaG2CSE-047	M	15	Gujranwala Punjab	-	-	-
35	SaG2CSE-048	M	16	Gujranwala Punjab	-	-	-
36	SaG2CSE-049	M	15	Gujranwala Punjab	-	-	-
37	SaG2CSE-050	M	17	Gujranwala Punjab	-	-	-
38	SaG2CSE-051	M	13	Gujranwala Punjab	-	-	-
39	SaG2CSE-052	M	15	Gujranwala Punjab	-	-	√
40	SaG2CSE-053	M	14	Gujranwala Punjab	-	-	-
41	SaG2CSE-054	M	12	Gujranwala Punjab	-	-	√
42	SaG2CSE-055	M	12	Gujranwala Punjab	-	-	-
43	SaG2CSE-056	M	12	Gujranwala Punjab	-	-	-
44	SaG2CSE-057	M	13	Gujranwala Punjab	√	-	-
45	SaG2CSE-060	M	-	Gujranwala Punjab	-	-	-
46	SaG2CSE-061	F	-	Gujranwala Punjab	-	-	-
47	SaG2CSE-062	F	12	Gujranwala Punjab	√	-	-
48	SaG2CSE-	F	12	Gujranwala	-	-	-

	063			Punjab			
49	SaG2CSE-065	F	10	Gujranwala Punjab	-	-	-
52	SaG2CSE-089	M	13	Gujranwala Punjab	-	-	-
51	SaG2CSE-090	M	15	Gujranwala Punjab	-	-	-
52	SaG2CSE-092	M	16	Gujranwala Punjab	-	-	-
53	SaG2CSE-093	M	16	Gujranwala Punjab	-	-	√
54	SaG2CSE-094	M	15	Gujranwala Punjab	-	-	-
55	SaG2CSE-095	F	8	Gujranwala Punjab	-	-	-
56	SaG2CSE-098	F	14	Gujranwala Punjab	-	-	-
57	SaG2CSE-101	F	17	Gujranwala Punjab	-	-	√
58	SaG2CSE-103	F	18	Gujranwala Punjab	-	√	√
59	SaG2CSE-115	M	13	Gujranwala Punjab	-	-	-
60	SaG2CSE-116	M	13	Gujranwala Punjab	-	-	-
61	SaG2CSE-117	M	13	Gujranwala Punjab	-	-	-
62	SaG2CSE-119	F	14	Gujranwala Punjab	-	√	-
63	SaG2CSE-121	F	13	Gujranwala Punjab	-	-	-
64	SaG2CSE-122	M	12	Gujranwala Punjab	-	√	-
65	SaG2CSE-125	F	8	Gujranwala Punjab	-	-	-
66	SaG2CSE-126	F	8	Gujranwala Punjab	-	-	√
67	SaG2CSE-127	F	7	Gujranwala Punjab	-	-	-
67	SaG2CSE-1b	M	10	MµLtan, Punjab	-	√	√
68	SaG2CSE-2b	M	11	MµLtan, Punjab	-	-	-
69	SaG2CSE-3b	M	14	MµLtan, Punjab	-	-	-
70	SaG2CSE-4b	M	13	MµLtan, Punjab	-	-	-
71	SaG2CSE-5b	M	13	MµLtan, Punjab	-	-	-
72	SaG2CSE-6b	M	13	MµLtan,	-	-	-

				Punjab			
73	SaG2CSE-7b	M	14	MµLtan, Punjab	-	-	-
74	SaG2CSE-8b	F	10	MµLtan, Punjab	-	-	-
75	SaG2CSE-9b	F	12	MµLtan, Punjab	-	-	-
76	SaG2CSE-10b	F	11	MµLtan, Punjab	-	-	-
77	SaG2CSE-11b	F	12	MµLtan, Punjab	-	-	-