GENETIC AND MOLECULAR PROFILING OF *GJB2* GENE IN DEAF MUTE POPULATION OF NORTH KARNATAKA

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DOCTOR OF PHILOSOPHY
IN
ALLIED HEALTH SCIENCES

(Human Genetics)

By

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LIST OF ABBREVIATIONS

Abbreviation	Full form
WHO	World health organisation
NSHL	Non syndromic hearing loss
HL	Hearing loss
ARNSHL	Autosomal recessive non syndromic hearing loss
DFNB	Autosomal recessive deafness
BOA	Behavioural observation audiometry
VRA	Visual reinforcement audiometry
ISO	International standard organisation
BERA	Brainstem evoked response audiometry
PTA	Pure tone audiometry
Db	Decibel
12SrRNA	12S Ribosomal ribonucleic acid
GJB2	Gap junction beta 2 gene
CX26	Connexin 26
GJB6	Gap junction beta 6
TMC	Transmembrane
OMIM	Online mendelian inheritance of man
KDa	Kilo Dalton
ICMR	Indian council of medical research
NSS	National service scheme
US	United states
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
DNA	Deoxyribose nucleic acid
SSCP	Single stranded conformational polymorphism
IEC	Institutional ethical clearance
KIDNAR	Karnataka institute for DNA Research
BLDE	Bijapur liberal development education association
EDTA	Ethylenediaminetetraacetic acid
ENT	Ear nose throat

Hz	Hertz
PTA	Pure tone audiometry
RNA	Ribose nucleic acid
µl	Micro litter
Nm	Nanomolar
OD	Optical density
ng/ul	Nano gram per microliter
NCBI	National centre for
Tm	Melting point
MB water	Molecular biology water
dNTPs	Deoxy nucleotide triphosphate
V	Volt
ddNTPs	Deoxyribose nucleotide triphosphate
PROVEAN	Protein variation effect analyser
PHD- SNP	Predictor of human deleterious single nucleotide polymorphism
DVD	Deafness variation database
3D	3 dimensions
SD	Standard deviation
dsDNA	Double standard DNA
DM- EX	Deaf mute exon
BP	Base pair
L	Ladder
G	Gradient
NSRD	Non syndromic recessive deafness
HI	Hearing impaired
TM	Transmembrane
AA change	Amino acid change
WT	Wild type
Temp	Temperature
TP3	Inositol triphosphate 3
ER	Endoplasmic reticulum
GjCh	Gap junction channel

Chapter 1

INTRODUCTION

1.1 INTRODUCTION

Hearing is a crucial component of human communication. Rarely its significance it is recognised until it is damaged or lost. The development of speech and language is impacted by hearing loss. The outer, middle, and inner ear are the three separate anatomical compartments that make up the human ear. The pinna, located on the outer ear, absorbs sound waves, and directs them into the ear canal, where they vibrate the ear drum. The three middle ear ossicles transport the vibrations as mechanical waves, which are then sent to the oval window of the inner ear where they are transformed into nerve impulses. The auditory nerve carries these impulses to the brain, where they are converted to sounds. Hearing impairment or hearing loss is a more accurate term for the inability to hear sounds[1].

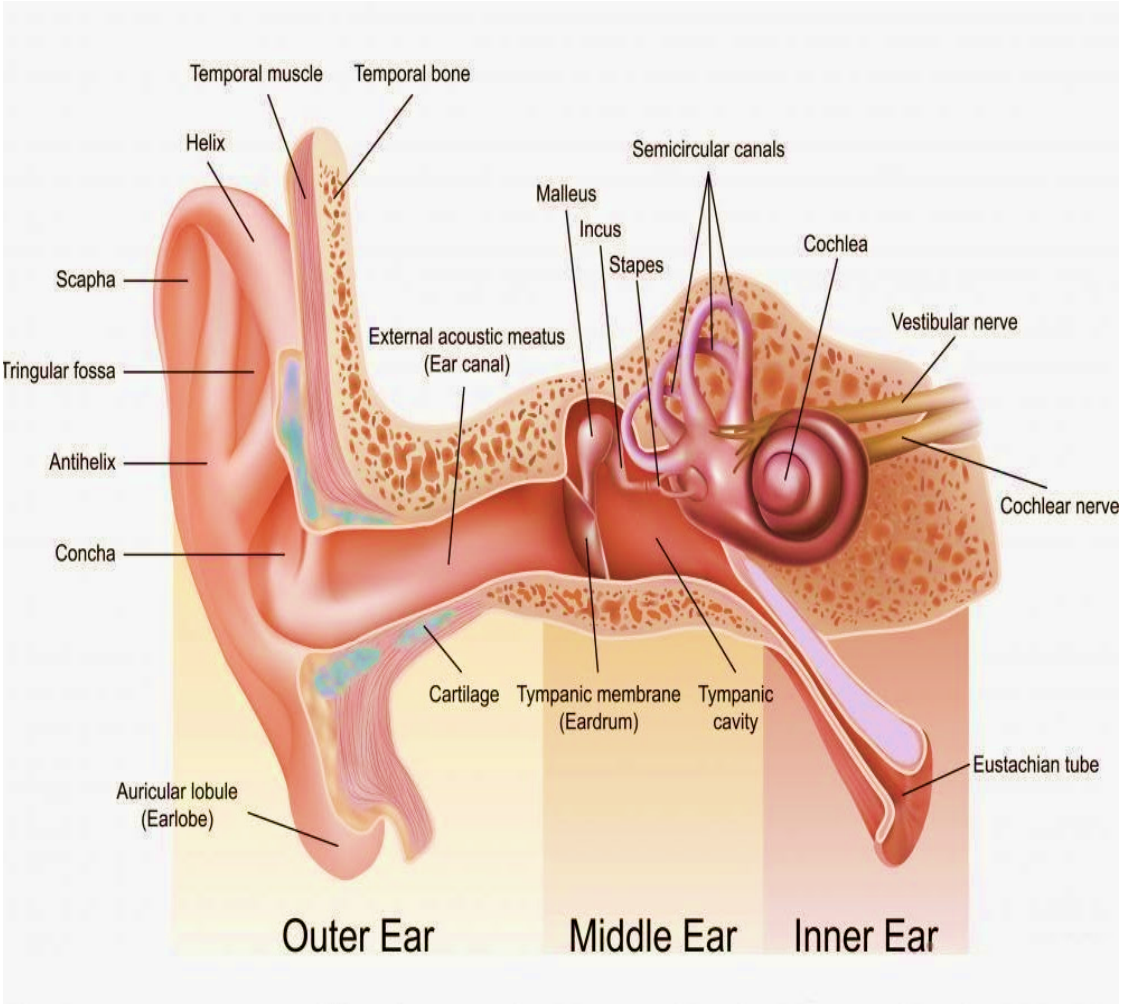

Figure 1.1: Anatomy of Human ear. (Photo credit: Simple bio)

1.2 HEARING PHYSIOLOGY

1. 2.1 Sound is transmitted by air conduction: Beyond the outer ear, the tympanic membrane picks up sound waves from the surroundings. Sound waves are partially absorbed and partially reflected when they enter the tympanic membrane. The umbo, the central region of the tympanic membrane, vibrates as a rigid cone and bends inward and outward when sound waves are absorbed by it. The malleus, whose tip is connected at the umbo, receives the motion of the membrane. Due to their near proximity to one another, the malleus head and incus body move in unison with the tympanic membrane. Low sound pressure levels cause the stapes to be vibrated, and the entire ossicular chain moves as a single mass[2].

Mechanical stapes vibrations produce pressure waves in the cochlea's scala vestibuli that travel through the cochlea as sound waves. The endolymph in the cochlear duct receives these waves when they enter the Scala tympani. The tectoral membrane and the organ of corti collide as a result of the basilar membrane's vibration, which also helps nerve messages reach the brain[2].

1.2.2 Mechanical vibration transduction: The hair cells in the organ of Corti transform mechanical sound vibrations into nerve impulses. The hair cells are held in place by the reticular lamina, which is joined to the basilar fibres. The tip of the hair cell has a hair bundle with stereocilia extending upward into the tectorial membrane. The exact mechanism by which the hair cell turns sound into an electrical stimulus is unknown, however one of the most important factors in this process is the endocochlear potential, which is located between the endolymph and perilymph. The difference in potassium level between the two fluids causes this direct current potential difference, which is around 14 +80 millivolts. It is thought that the striavascularis continuously transfers potassium ions from the perilymph into the cochlear duct, maintaining its health. The cochlear duct contains the endolymph, which has a high potassium content and a positive potential. Consequently, it cleans the hair cells' tops. The perilymph, which is kept in the scala vestibuli and scala tympani and has a low potassium content and a negative potential, soaks the lower regions of the hair cells. According to Human ear | Structure, Function, & Parts, the intracellular potential of the hair cell is negative, measuring -60 millivolts for the perilymph and -140 millivolts for the endolymph (2022, November 30). In order to

allow the lesser stereocilia to move with the taller ones against the tectorial membrane, the stereocilia are interconnected and graded in height. An alternating potential for the hair cell receptor is produced by the mechanical movement of this hair bundle. Ion channels in the membrane open, allowing potassium ions to enter the cell, when the stereocilia are twisted in a way that lengthens the stereocilia. The hair cell is excited or depolarized when potassium ions enter. When the stereocilia are deflected in the opposite direction, the ion channels close, inhibiting or hyperpolarizing the hair cell. The release of substances known as neurotransmitters from the hair cell's base is stimulated by the depolarization of the cell. An electrical signal is sent along the cochlear nerve by the neurotransmitters, who also activate the nerve fibres at the base of the hair cell. The electrical signal is then transmitted into the central nervous system through the 5th cranial nerve[2].

Hearing loss is one of the important disabilities. All kinds of communication depend on the hearing sense, which is also critical for a child's proper brain development. A concealed condition with serious, complex problems is hearing loss. Given that a large number of those affected are youngsters, who represent the future productive age group and the potential of a nation, this issue is of much more importance. Globally, there are around 5 million people who are profoundly deaf, and another 200 million people are expected to suffer hearing loss that is less severe[3]. Two thirds of all deaf individuals live in underdeveloped nations, where a lack of luxury makes their disability even worse. According to WHO (2015), 360 million people throughout the world suffer from a crippling hearing impairment, or those who have moderate to profound hearing loss in both ears[4]. Deafness is a problem that is disproportionately common in Southeast Asia, with prevalence rates ranging from 4.6% to 8.8%[5]. According to the studies[6,7], profound deafness is a frequent condition that affects 1 in 1000 new-borns and has genetic causes in both syndromic (one third) and non-syndromic (two thirds) forms. There are two types of deafness: syndromic (associated with certain defects) and nonsyndromic (without other abnormalities), which can be distinguished. Hearing loss, also known as prelingual loss, affects the kid before the age of speech development. Prelingual hearing loss has epidemiological characteristics that indicate recessive disorders are present in certain loosely nonsyndromic forms (three quarters). Genotype-phenotype relationships have attracted the interest of medical experts and scientists since these hypotheses were

acknowledged to exist. The extent to which genetic variations and health impacts have been linked has greatly improved the public's awareness of inherited illnesses [8]. Understanding these relationships, however, is challenging due to the non-syndromic hearing loss (NSHL significant)'s clinical and genetic diversity. Inherited HL is frequently monogenic. Monogenic hearing loss has a wide range of characteristics[9]. More than half of the time, the hearing loss is inherited in a straightforward Mendelian manner. The loss is classified as autosomal recessive nonsyndromic hearing loss in 75–80% of hereditary cases where both parents have normal hearing (ARNSHL). About 20% of instances of hearing loss are caused by autosomal dominant non-syndromic hearing loss (ADNSHL), with X-linked and mitochondrial inheritance making only a small contribution[10]. As of today, 154 nonsyndromic deafness loci have been identified on various chromosomes, 86 of which are inherited autosomally (DFNB), and 57 of these loci contain a cloned gene[11].

1.3 TYPES OF HEARING LOSS (HL)

There are many categories for hearing loss (HL) [12]

1.3.1 Conductive HL: Conductive HL: Typically denotes an issue with the middle or outer ear. When sound waves cannot pass through the outer and middle ear, conductive hearing loss results. It could be difficult to detect subtle noises. Louder noises could be masked.

1.3.2 Sensorineural HL: An inner ear problem is referred to as sensorineural HL. Sensorineural deafness is a kind of hearing loss. It may be brought on by harm to the inner ear, the brain, or the region where the auditory nerve, which links the ear to the brain, begins. A hearing loss that is both conductive and sensorineural is referred to as mixed hearing loss[13].

1.3.3 Based on the age of onset

1.3.3.1 Pre-lingual: When the beginning of hearing loss comes before the development of speech. a person who did not learn to talk because they were either born deaf or lost their hearing as a young kid[14].

1.3.3.2 Post-lingual: Deafness that appears after the acquisition of speech and occurs post-lingually. a hearing loss that develops after language comprehension and speech are learned. Hearing loss can develop gradually or unexpectedly[15].

1.3.4 According to clinical manifestation

1.3.4.1 Non-syndromic hearing loss: Non-syndromic hearing loss refers to a partial or total loss of hearing without any other symptoms or warning signs. 70% of cases of congenital hearing loss have no other clear signs of illness[16].

1.3.4.2 Syndromic hearing loss: Other body parts are also affected by the signs and symptoms of this type of hearing loss. Other physical anomalies are linked to the remaining 30% of hearing loss.

1.4 MODES OF INHERITANCE

There are four basic ways that deafness is inherited:

1.4.1 Autosomal dominant: Unlike autosomal dominant disorders, which frequently only affect one parent per offspring, this condition has a 50% probability of affecting every child. Various degrees of an expected characteristic's penetrance or non-penetrance can be seen in some of the situations in this category. One example is the Waardenburg syndrome, where the phenotypic shows itself as varying degrees of hearing loss and clinical symptoms. In situations of genetic hearing loss that manifests in adults or with delayed start, penetration is typically age-related. In 20% of cases of deafness, this happens.

1.4.2 Autosomal recessive: A individual with an autosomal recessive condition is homozygous for the defective gene whereas their parents are often phenotypically normal carriers. When several individuals carry a certain aberrant allele, these diseases are prevalent among genetic isolates that have undergone recurrent inbreeding. In this way, around 80% of deafness is explained.

1.4.3 X-linked: X-linked illnesses have a distinct method of inheritance since the malfunctioning gene is located on the X chromosome. Due to the fact that men are not heterozygotes and females have X-inactivation, it is typically difficult to discern between recessive and dominant variations. 1% of cases of deafness are linked to the X chromosome.

1.4.4 Mitochondrial: There has been a rise in the significance of mitochondrial inheritance in a variety of genetic diseases, including both syndromic and non-syndromic hearing loss. For instance, it has been shown that the point mutation A1555G in the protein coding gene 12SrRNA predisposes to amino glycoside cochlear damage[17,18].

When a child has a hearing loss that has been detected, both genetic and nongenetic aetiologies need to be investigated. Due to hypoxia, hyperbilirubinemia, very low birth weight, and ototoxic drugs, graduates of the neonatal intensive care unit are more likely to experience hearing loss. Despite a decline in frequency, meningitis remains a risk factor for hearing loss. The most frequent congenital infection and a frequently occurring cause of progressive hearing loss is cytomegalovirus. Hearing loss can be prevented by avoiding head injuries, loud noises, and ototoxic drugs. The creation of a therapy and care plan can be made easier by determining the cause of hearing loss (table 1.1).

Table 1.1: The etiological factor of acquired hearing loss

Prenatal infectious disease	Rubella, Measles, Mumps, Poliomyelitis, pneumonia caused by a virus, Herpes zoster, Toxoplasmosis, Hepatitis, other severe virus infections.
Non-infectious disease	Alcohol, Drugs, Aminoglycosides, Diabetes mellitus, X-rays, Thalidomide, Hypoxic events nephropathy
Perinatal	Asphyxia, Rhesus-incompatibility, Congenital heart failure, Herpes, Premature birth, Birth trauma with intracerebral haemorrhage hyperbilirubinemia.
Postnatal	Meningitis, Sepsis, Measles, Herpes zoster, Ototoxic drugs, Sound exposure.

1.5 MAJOR EAR DEFECTS IN HEREDITARY HEARING LOSS

The principal ear defects linked to inherited hearing loss fall into three groups[19,20]

i. Morphogenic flaws
ii. Cochleo-sacular abnormalities
iii. Neuroepithelial abnormalities.

Defects in morphology takes place as a result of the labyrinth's early developmental processes being disrupted, which leads to an inner ear deformity[19,20]. It has been established that the inner ear develops inductively from the neural tube. The area that sustains injury most commonly is the lateral semi-circular channel of the vestibule. Multiple gene mutations have been linked to morphogenic disorders in animals, and at least four of these genes are transcription factor-coding[21,22,23]. A significant stria vascularis defect, a breach of the Reissner's membrane, the degeneration of hair cells, and spiral ganglion cells are all indicators of cochlea problems[19,20]. These characteristics are typically seen in patients who have non-syndromic hearing loss, according to observations. A neuroepithelial cell deficiency is the main cause of corti defect. Despite the addition of stria vascularis, endochlear potential, and Reissner's membrane deterioration[19,20].

1.6 DIAGNOSIS OF HEARING IMPAIRMENT

Audiometry is a subjective method for assessing hearing or the ability to comprehend auditory information. This includes behavioural assessments and pure tone audiometry. Two forms of behavioural testing are behavioural observation audiometry (BOA) and visual reinforcement audiometry (VRA). The use of BOA on new-borns from birth to six months of age is possible, although it is heavily reliant on the examiner's expertise. A credible full audiogram may be obtained with VRA on children between the ages of six months and two and a half, although this depends on the tester's expertise and the child's developmental stage. Both approaches are error-prone and have substantial drawbacks. In accordance with International Standards Organization (ISO) standards, congenital hearing loss is frequently discovered utilising Pure Tone Audiometry (PTA) or Brain stem Evoked Response Audiometry (BERA) using a diagnostic audiometer in a soundproof environment. Table 1.2 displays a scale for diagnosing hearing impairment.

1.6.1 Pure Tone Audiometry (PTA): It evaluates a person's frequency-dependent ability to recognise pure tones (pitch). 131 frequencies between 250 and 8000 Hz are measured using headphones, and the results are recorded together with a measurement of the intensity or loudness in decibels (dB). Bone conduction and air conduction, which indicate the condition of the inner ear, respectively reflect the condition of the middle ear, ossicles, and external ear canal[24].

1.6.2 Brainstem Evoked Response Audiometry (BERA): In reaction to click noises provided via the ear, it is an objective test to ascertain how the VIIIth cranial nerve transfers electrical impulses to the brainstem. This test is frequently performed while the patient is sleeping. An earphone or headset is required to provide stimulation in the form of a click sound or tone pip. The stimulus's loudness ranges from 1000 to 4000 Hz and its frequency changes. The brainstem produces impulse waves in response to auditory information. Electrodes are applied to the scalp and used to record these waves. Several electrical approaches are used to amplify the produced waves. It is simple to record waveforms[25,26]

Table1.2: scale for identification of hearing impairment (according to WHO)

Grade of impairment	Corresponding audiometric ISO value	performance	Recommendation
0-No impairment	25dB or better (better ear)	No or very slight hearing problems. Able to hear whispers	
1-slight impairment	26-40dB	Able to hear and repeat words spoken in normal voice at 1 meter distance.	Counselling. Hearing aids may be needed.
2-Moderate impairment	41-60dB	Able to hear and repeat words spoken in raised voice at 1 meter distance	Hearing aids usually recommended
3-Severe impairment	61-80dB	Able to hear some words when shouted when shouted in better ear.	Hearing aid needed. If no hearing aid available, lipreading and signing should be taught.
4-Profound impairment	81 dB or greater (better ear)	Unable to hear and understand even a shouted voice.	Hearing aid may help understanding words. Additional rehabilitation needed. Lip reading and signing needed.

1.7 GLOBAL PREVALENCE OF HEARING LOSS

WHO updated their estimates of the severity of hearing loss in 2012. The following are the estimates: 360 million people worldwide (5.3% of the world's population) suffer from a hearing loss that is incapacitating. 32 (9%) million of them are children, whereas 328 million (91%) of these are adults (183 million men, 145 million women). South Asia, the Asia-Pacific region, and Sub-Saharan Africa are the regions with the highest prevalence of debilitating hearing loss in children

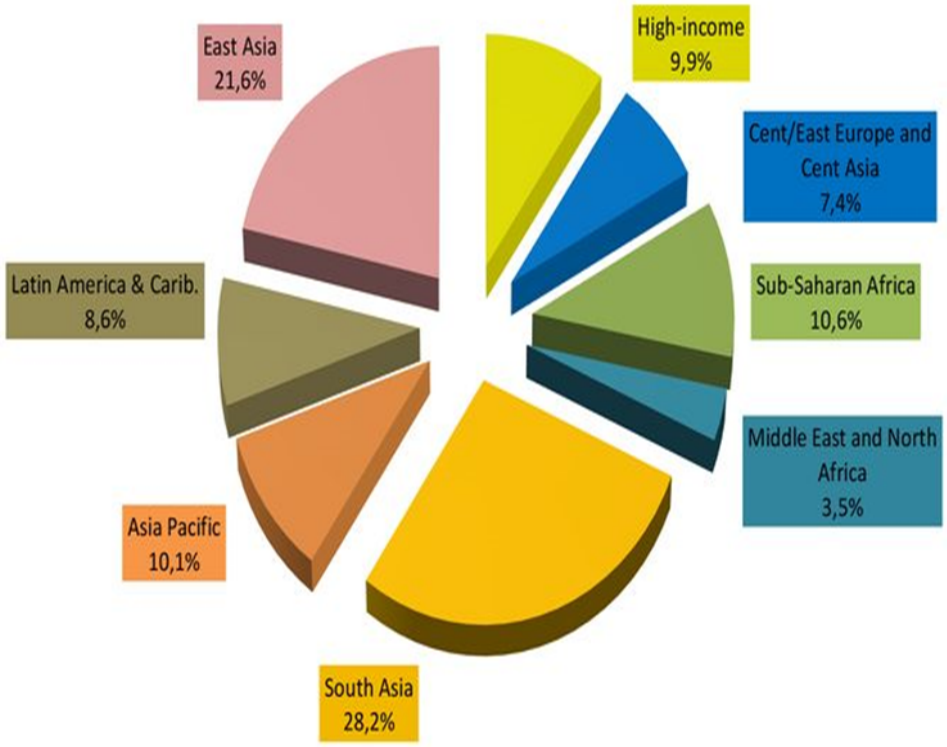

Figure 1.2: Pie chart showing the Prevalence of disabling hearing loss around the Globe (WHO, 2018)[27]

1.8 PREVALENCE OF HEARING LOSS IN INDIA

Congenital hearing loss is projected to affect 10 and 20 people out of every thousand people in India's rural and urban areas, respectively, according to a community-based disability survey that was funded by the Indian Council of Medical Research (ICMR). In India, 6.3% of people have hearing impairment, according to WHO estimates from 2005[28]. India has implemented a variety of initiatives to prevent deafness, including the National Program for Prevention and Control of Deafness, which is now in its trial phase. Hearing impairment was shown to be the second most prevalent birth defect in the 58th round of the National Sample Survey (NSS), which

examined disability in both urban and rural households. Figure 4 depicts the global increase in hearing loss prevalence.

1.9 GENETICS OF DEAFNESS

In India, there are presently known to be 11 autosomal recessive loci (DFNB1, DFNB3, DFNB4, DFNB5, DFNB6, DFNB7, DFNB11, DFNB15, DFNB17, DFNB18, and DFNB95) and 1 autosomal dominant locus (DFNA59) linked to hearing loss[11]

Despite genetic variation, up to 50% of NSHL is accounted for by a single locus, DFNB1, on chromosome 13q11–12[23,29]. The GJB2 gene is thought to be the main cause of non-syndromic recessive hearing loss at this location. Globally, it has been discovered that mutations in the gap junction beta 2 gene (GJB2), which produces the protein Connexin 26 (Cx26), are the main cause of hereditary hearing loss. This transmembrane protein helps the cochlea's hair cells recycle potassium by forming connexons. Varied populations have different frequencies of the various GJB2 mutations. Due to founder effects, it has been demonstrated that the main mutations in GJB2 are population-specific. These variants include c.35delG, which affects Caucasians[30], c.167delT, which affects Ashkenazi Jews[31], and c.235delC, which affects East Asians[32]. Studies on GJB2, however, have not revealed that it significantly contributes to deafness in Africans or African Americans and that the prevalent variants are only occasionally found[33,34]. However, only one mutant allele exists at that locus in a significant portion (10–42%) of patients with GJB2 mutations, and other family instances exhibit evidence of linkage to the DFNB1 location but no mutation in GJB2 gene. It was thus proposed that a different gene located adjacent to GJB2 may be to blame for these cases and that mutations in the GJB6 gene, which is also located close to GJB2, may be a factor in these cases of hearing loss. GJB6 mutations have been linked to both autosomal dominant and autosomal recessive hearing loss, according to studies [35-37]

It has long been understood that environmental and genetic factors play a role in "deafness," one of the most prevalent and maybe the most upsetting conditions to impact people. The genetic factors have been disputed despite the identification and

control of the environmental causes (intra-uterine viral infections, medications, trauma, and noise). Due to assortative mating and significant genetic variability, genetic study of deafness evaded scientists and medical geneticists for a long time. However, there has been an increase of publications on various chromosomal regions and genes associated to deafness in recent years due to improved knowledge and linkage study methodology. Globally, the GJB2 gene has come to be the most common cause of deafness. The study of the functional role of the genes and the identification of mutations in families, have made a substantial contribution to our understanding of the cellular and molecular processes driving deafness. The incidence of severe hearing loss is thought to be 1 in 1000 live births globally, with genetics accounting for 50% of the segregation as monogenic characteristics. The scope of the issue in India is still completely unknown, despite recent tremendous progress in our understanding of the molecular causes of hereditary deafness.

The prevalence of deafness in our nation, given the extrapolation of the global incidence to our 1 billion people and the high prevalence of consanguineous marriages, is expected to be a major public health problem. Approximately 50% of hearing loss may be genetic or inherited; this condition may be syndromic, meaning it is linked to problems in other organ systems, or nonsyndromic, meaning it occurs on its own. The non-random relationship with problems in other organ systems may often be used to identify syndromic hearing loss, which accounts for 30% of all hereditary causes. More than 500 disorders are connected to deafness. On the other hand, non-syndromic deafness is a mystery that can only be addressed through genetic tests. Only 20–22% of nonsyndromic hearing loss appears in dominant forms, while X-linked and mitochondrial inheritance are only seen in 2% of instances. In contrast, 70–80% of nonsyndromic hearing loss segregates into autosomal recessive inheritance. Conventionally, the recessive deafness loci are marked with a suffix 'B', i.e., DFNB, and the dominant loci are designated with a suffix 'A', i.e., DFNA, signifying the order of their discovery and DFN for X-linked inheritance.

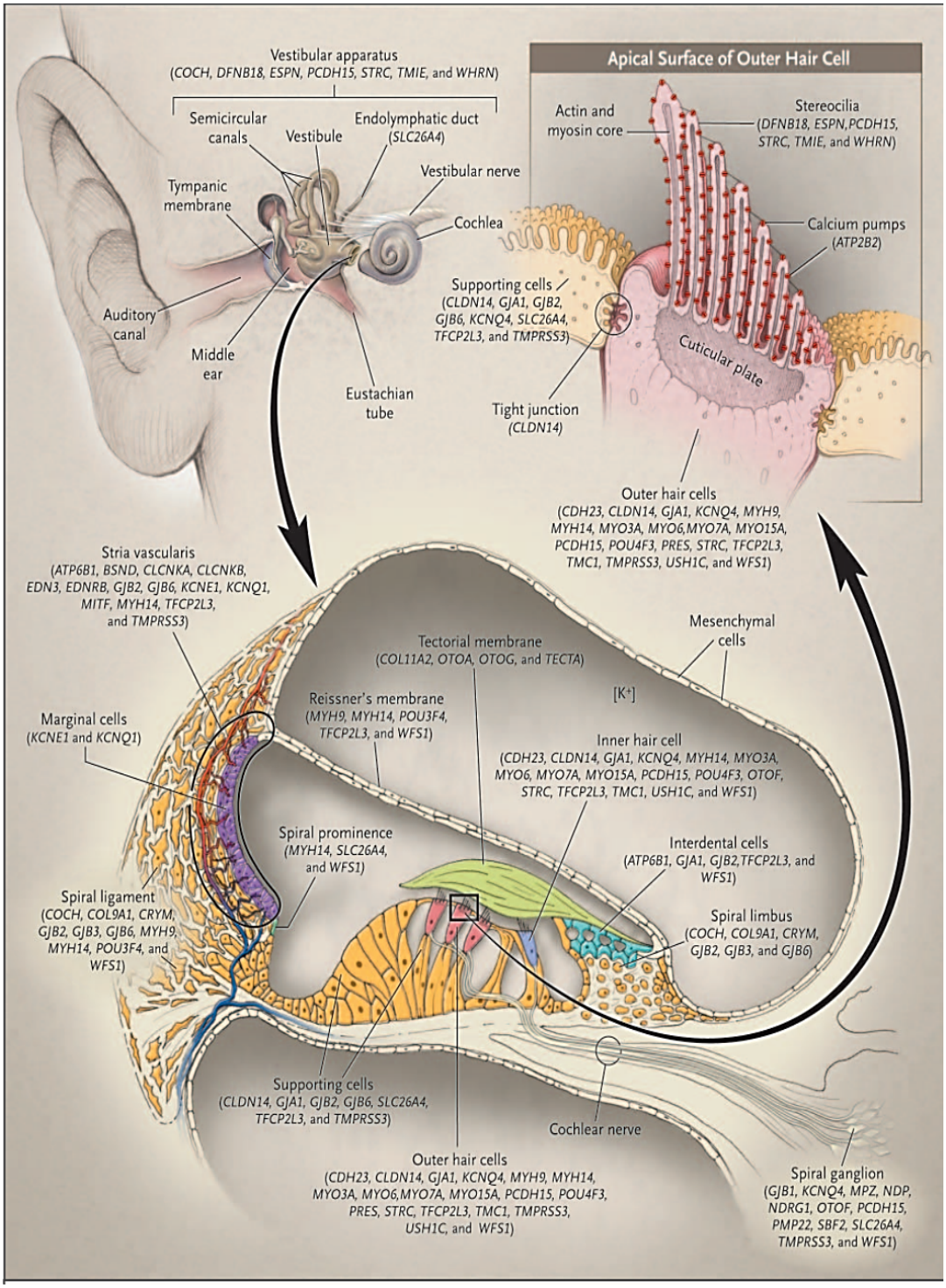

Figure 1.3: View of outer, Middle, and Inner Ear with a cross-sectional view of the cochlear duct[38].

It is becoming more and more obvious that many genes play a role in coordinating the intricate process of sound transduction in the auditory system as the genes responsible for deafness are identified and their gene products are investigated. Around 80 chromosomal sites containing genes associated in non-syndromic hearing loss have already been identified[11], and around half of the genes in these loci have been mapped (Fig 2). Transcriptional factors (POU3F4, EYA4, TFCP2L3), motor molecules (myosin 2, 6, and 7), ion channels, transporters (pendrin, KCNQ4), integral membrane proteins (TMC1, TMIE), adhesion molecules (cadherin and protocadherin), gap junction proteins (connexins 26, 30, 31 and 43), extracellular protein, and numerous other novel molecules are among the protein products of these genes. Understanding how these molecules work has helped researchers better understand the intricate mechanics of sound transmission in the inner ear. Gene discovery and mutation studies in families with recessive and dominant inheritance patterns have identified various mutant alleles with distinctive behaviour. A gene that expressed as recessive in some families was shown to be responsible for other families' autosomal dominant segregation. One such gene that has been shown to be recessive in a number of Indian and Pakistani families as well as in sizable American kindred that segregates as dominant is the TMC1 gene, which is encoded by the DFNB7/11 interval on chromosome 9. In addition, it was shown that other genes linked to non-syndromic deafness in some families were also linked to syndromic deafness in other families. Numerous genes that cause Usher Syndrome, an incredibly heterogeneous condition, have been shown to present themselves both as non-syndromic deafness and as progressive vision loss from retinitis pigmentosa. It has been hypothesised that this phenomenon, in which mutations in the same gene have been shown to cause a range of clinical symptoms with various routes of inheritance, is caused by the variable behaviour/effect of the mutant alleles. It has been shown that deletion and nonsense mutations cause more severe symptoms (syndromic) due to a dominantly negative impact of the mutant allele, whereas missense and splicing mutations cause non-syndromic deafness.

1.10 MISCELLANEOUS GENES

This category consists of a collection of genes whose functions have not yet been fully clarified or which do not fall into any of the aforementioned classes. These include GPSM2, TPRN, PDZD7, GRXCR1, and others. Various factors affect how

genes linked to hearing loss are expressed[38]. The protein that a gene produces and its role in the hearing mechanism are tied to the expression of that gene[39]. Different genes are preferentially expressed, up-regulated, and down-regulated in the ear at every stage of development. Gene deficiencies may cause early apoptosis or function loss[39]. More than half of cases of autosomal recessive non-syndromic hearing loss are caused by the gene GJB2, followed by SLC26A4, GJB6, MYO15A, OTOF, CDH23, and TMC1. However, the majority of cases are not caused by any of the genes connected to autosomal dominant nonsyndromic hearing loss. WFS1, KCNQ4, GJB2, and COCH mutations are reported considerably more often. One of these most often occurring causes of autosomal dominant nonsyndromic hearing loss is KCNQ4[9]. In several industrialised nations during the past ten years, systems for the universal neonatal hearing screening have been devised and put into place. The rationale behind this programme is the notion that early detection and intervention for children with hearing loss improves their odds of language and speech development, which subsequently makes it simpler for them to develop typical social, cognitive, and physical capacities. The auditory cortex has to be activated before the age of six months in order for the auditory tracts to develop appropriately.

1.11 RESEARCH ON GENETIC DEAFNESS IN INDIA

It is unknown what the full spectrum and frequency of GJB2, SLC26A4, and KCNQ4 gene variations among people with non-syndromic hearing loss in India exist, despite the fact that there has been a substantial amount of study on the genetics of hereditary deafness in various parts of the world. Only a few studies have particularly looked at how the GJB2 gene's genetic signature has altered in various regions of India, but never in the eastern region. Maheswari et al. for the first time identified GJB2 mutations associated with ARNSHL in a study cohort from south India[40]. Ram Shankar et al. examined deaf individuals for GJB2 mutations in a subsequent investigation and noted that p.W24X is the founder mutation in the Indian population[41]. A large research cohort from south India was later subjected to genetic screening for GJB2, and four unique mutations—-23G>T, p.I33T, 377–385 dup TCCGCAT, and p.W172R—were discovered. He also demonstrated the functional impact of new connexin 26 mutations linked to ARNSHL in this work[42]. When Joseph and Rasool in Kerala investigated the genetics of deafness, they found that the p.W24X mutation was highly prevalent in their research population (32.5%)[43]. On the

other hand, it was discovered that GJB2 mutations were uncommon in NSHL patients from Western India[44]. It is evident from the findings of the earlier investigations that the incidence and extent of GJB2 mutations differ significantly depending on the population's ethnicity or place of origin. Two dominant GJB2 variants, p.R75Q and p.R184Q, one from a south Indian family and the other from a north Indian family, have also been described from India in addition to recessive mutations[45,46]. Despite the fact that these two genes have been identified as the main causes of non-syndromic deafness around the world, no research has been done to yet to ascertain the mutation spectrum of the SLC26A4 and KCNQ4 genes among NSHL patients in India.

1.12 *GJB2* GENE

The basic genomic structure of GJB2 (Gap Junction protein, Beta-2 OMIM 121011), which encodes the connexin 26 (Cx26) protein, consists of 2 exons[47]. Its location on chromosome 13 is known (Figure 1.5). The GJB2 functional protein exon is 681 bp long and codes for a polypeptide of 226 amino acids. In vertebrates, connexins are essential transmembrane proteins that help to construct intercellular channels. A minimum diameter of 1.2 nm aqueous pore is defined by a hexamerical assembly of six connexins, also known as a connexon or hemichannel. When two hemichannels from adjacent cells dock and combine, leaving a gap of around 2 to 3 nm, an intercellular gap junction channel may be created. The exchange of cytoplasmic molecules up to around 1 kDa is allowed through this channel, which connects the two plasma membranes[48]. Gap junction channels have a significant role in electrical and chemical signalling, which is generally acknowledged[49]. It is hypothesised that the activity of this connexion 26 protein is essential for K+ ions recycling back to the endolymph of the cochlear duct after activating sensory hair cells in the corti organ. When K+ enters through mechanically regulated ion channels, hair cells become depolarized, and neurotransmitters are produced when Ca+2 enters through the basilar membrane. K+ ion recycling is crucial for the cochlea's proper operation[50]. As a result, both autosomal recessive and autosomal dominant hearing loss are caused by mutations in this gene, which also affect the ionic composition of endolymphatic fluid. The GJB2 loci are categorised as either DFNA (autosomal dominant), DFNB (autosomal recessive), or DFN depending on the mode of inheritance of deafness (X – linked). Autosomal recessive nonsyndromic deafness is connected with the Connexin 26 gene mutation (DFNB1). In autosomal recessive

deafness, DFNB1 was the first locus implicated; in 1997, GJB2, which is fully heterogeneous, was discovered to be the pathogenic locus. At least 19 DFNB and 18 DFNA loci have been identified on the human genome so far[51], and 30-100 genes are thought to be involved. GJB2 is a short gene with a length of around 5.5 kilobases that is found on chromosome 13q.11. There are two exons, but only one (exon 2) has the 2.2 kilobases-long coding sequence that results in a protein with 226 amino acids, while the other exon (exon 1) is non-coding. Connexin26 is encoded by the Gap Junction Beta2 gene, also known as GJB2. Deaf Mutism is the most prevalent type of the congenital condition caused by a mutation or deficiency in this gene. Historically, the term "deaf-mute" was used to describe a person who was either deaf (hearing loss)

Cytogenetic Location: 13q12.11, which is the long (q) arm of chromosome 13 at position 12.1

Molecular Location: base pairs 20,187,463 to 20,192,975 on chromosome 13

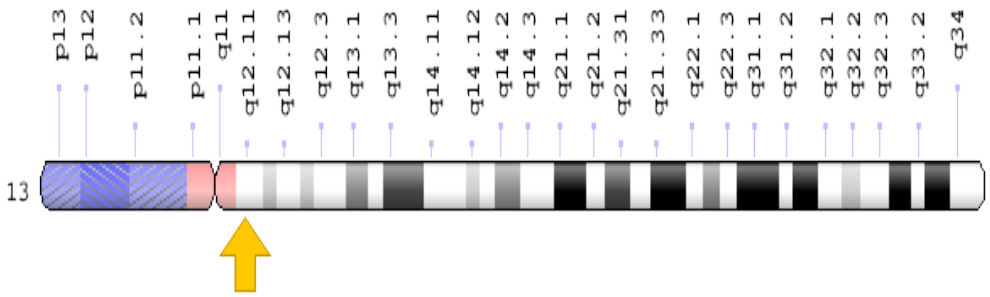

Figure 1.4: Molecular location of the *GJB2* gene

This gene, which comprises a single short coding exon, has been extensively researched in practically all ethnic communities. It has been estimated that up to 50–80% of the deaf population, in both sporadic and familial instances, is present in several ethnic groups in the Mediterranean area and among Askenazi Jews. The main mutant allele detected was a founder mutation, which was 35delG in Caucasians and 187delT in Ashkenazi Jews[32].

1.13 HEARING LOSS AND THE GJB2 GENE

As of today, non-syndromic hearing loss has been related to more than 60 genes. One of the most important genes for autosomal recessive non-syndromic hearing loss (ARNSHL) has been identified as GJB2[9]. In many parts of the world,

including Europe [52], Taiwan [53], Slovakia [54], Argentina[55], the United States[56], China[57], Studies on the GJB2 gene have been carried out in China[57] and Japan[58]. GJB2 mutations have been related to up to 50% of cases of ARNSHL in Caucasians and North Americans, however their prevalence is lower in Japanese (20%-30%) and Korean (5%) deaf persons [59-62]. As genetic testing for the deaf has advanced, it has become clear that GJB2 mutations also contribute to dominant deafness. 92 mutations in the GJB2 gene have so far been associated with ARNSHL[11]. There have been several common and essentially population-specific GJB2 mutations found (Human Gene Mutation Database professional edition, accessed in June 2010). The c.35delG mutation is the most prevalent pathogenic variant among them, constituting up to 70% of all GJB2 mutations in the populations of Europe and America[63]. The c.167delT mutation in Ashkenazi Jews[32], the c.235delC mutation in Japanese[58], the p.R143W mutation throughout Ghana [64,65], and the non-coding variation IVS1+1G>A mutation[61] in Mongolians are other recurring population-specific mutations[63]. Numerous investigations in the Middle East, such as those in Iran, have revealed that only 11% of ARNSHL is caused by hearing loss caused by the GJB2 gene[66], while GJB2 mutation causes 21.4%–30% hearing loss in 26 surrounding populations in Turkey[67,68]. The GJB2 gene was also examined in African deaf patients, and it was shown that Sudanese and Kenyan deaf children had a low rate of GJB2-related hearing loss [33]. In addition, no GJB2 mutation was discovered in Limpopo-region deaf South Africans [34]. Nine GJB2 mutations, in contrast to ARNSHL, have been linked to autosomal dominant deafness. Six of them have non-syndromic penetration (p.W44C, p.W44S, p.R143Q, p.D179N, p.R184Q, and p.C202F), while the other three (p.G59A, p.R75W, and p.R75Q) are linked to a syndromic type of deafness [69]. The first and second extracellular domains of GJB2, which are remarkably conserved and play a crucial role in voltage gating and connexin-connexin docking, have been revealed to be the sites of the majority of dominant mutations[70]. Two French families were the first to find the p.W44C, f the p.W44C was originally discovered by two French families, then by a family from the United States[71,72]. At the same site, p.W44S, a second GJB2 mutation was shown to be related to ADNSHL. followed by a family from the United States [71,72]. Another GJB2 mutation was discovered to be connected to ADNSHL at the same location, p.W44S[73]. Iranian Azeri Turkish people as well as Austrian patients were found to have the dominant GJB2 mutation, p.R143Q[74,75]. First-ever reports of the p.D179N dominant mutation came from a family in southern

Italy who had post lingual deafness[76]. In Ghana, ADNSHL patients were found to have two more dominant mutations, p.R184Q and p.C202F[65]. Recently, ADNSHL in a Cuban family was linked to the new GJB2 mutation p.G21R[30]. The severity of hearing loss appears to vary with the kind of GJB2 mutations, indicating that the phenotype of these mutations is varied. Although dominant GJB2 mutations can cause mild to profound, progressive, or non-progressive hearing loss, recessive GJB2 mutations often cause severe to profound hearing loss [71,77-81].

1.14 OTHER DEAFNESS GENES IN INDIAN FAMILIES

Other deafness genes segregating non-syndromic/ syndromic autosomal recessive inheritance, contributed to 15% of genetic cause of deafness in India. In order of precedence, the genes associated with deafness in Indian families that were identified in the study were (1) Myo7A, segregating with UshlB, in the DFNB2 interval on chromosome 11q13.5, (2) Myo 15 (DFNB3) on chromosome 17p11.2. 21 (3) SLC26A4 (DFNB4) on 7q31, also cause of Pendred syndrome, 16 (4) TMC1 gene (DFNB7/11) on chromosome 9q13, 6~~ (5) OTOF located in DFNB9 interval on chromosome 2p22-23, 6) Cadherin 23(DFNB12) on chromosome I0q21-q22 segregating with UshlD, 7) Harmonin (DFNB18) on 11p14-15.1, also causing UshlC, 17, ~9 and 8) Protocadherin 15 (DFNB23) underlying UshlF syndrome? It may be noted that many genes segregating nonsyndromic deafness overlap Usher syndrome, type1, which are of 3 types clinically but very heterogenous at the molecular level. Only five causal genes have been discovered despite the discovery of seven Usher type 1 loci. After the GJB2 gene, Usher syndrome is the second most frequent cause of deafness. A number of additional genes implicated in the aetiology of deafness need to be uncovered, as the GJB2 gene and the other genes found in Indian families account for around 40% of the molecular causes of deafness in India. Given the size of our population and the widespread practise of consanguineous marriage in India, the scope of the issue has to be clarified. High-risk families must have proper genetic counselling and education, which calls for genetic testing of "at risk" couples for the frequent deafness mutations seen in ethnic cultures. In some nations with a high frequency of GJB2 gene mutations, new-born screening has been suggested since an early diagnosis would allow for better medical treatment and therapeutic alternatives.

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Chapter 2

AIMS & OBJECTIVES

2.1 AIM

Identification of the genetic biomarker for deaf mute populations of North Karnataka

2.2 OBJECTIVES

1. To Screen the *GJB2* gene in Deaf and a dumb population of north Karnataka.
2. To analyse the *GJB2* Genetic variants in Deaf and Dumb population.
3. To identify the novel mutations in *GJB2* gene in Deaf and Dumb population.
4. To correlate the molecular alteration of *GJB2* gene with clinical features of Deaf and Dumb population.
5. To propose the management strategies for the individuals suffering from *GJB2* deficiency.

2.3 HYPOTHESIS

Many studies from various parts of the world have documented the incidence of *GJB2* mutation in the deaf population. Many of those studies involved individuals from the Indian population. Though they included the Indian individuals, none of those reports provided a systematic study of the prevalence of connexin 26 mutation in the Indian population.

Mutation in *GJB2* is the single most frequent cause of inherited deafness, in a population in which the genetic epidemiology of deafness has been evaluated. Identification of genes and mutation analysis in children's segregating recessive and dominant inheritance may reveal the unique behaviour of several mutant alleles. Besides, the overall high involvement of CX26 mutation in autosomal recessive non syndromic forms of deafness, and even in sporadic causes, makes mutation analysis distinctly worthwhile, and CX26 mutation analysis provides a good starting point in the molecular diagnosis of patients with non-syndromic congenital deafness. “There is a relationship between the Hearing and *GJB2* gene, Defect in *the GJB2* gene which thereby may affect the hearing mechanism in Sensorineural Deafmute patients. Therefore, the present study has been undertaken to evaluate the role of *the GJB2* gene in the North Karnataka population of Karnataka, India.”

Chapter 3

REVIEW OF LITERATURE

3.1 NATIONAL

128 (24%) of 530 southern, northern, and western Indians with nonsyndromic hearing loss contained GJB2 mutations, according to 2009 study [1]. Biallelic mutations made up around 21% of the mutations (112 individuals). W24X, the most prevalent mutation, with a 16.4% allelic frequency. Study discovered that several GJB2 mutations generated a variety of detrimental impacts on gap junction activity in HeLa cells using in vitro functional expression experiments. In contrast to the R75W mutation, which produced membrane localization but did not result in the formation of a functional gap junction channel, the R184P mutation blocked protein trafficking to the plasma membrane. A further dominant-negative effect was seen with the R75W mutation. The truncating mutation W24X was shown to allow the production of a full-length protein, however it was predominantly detected in the cytoplasm, possibly because of a stop codon read-through mechanism [1].

In 2016, study [2] conducted on mutation analysis of the connexon 26 genes to find monoallelic differences in 36 prelingually deaf children from Raipur, India. The positions in their analysis where changes occur most frequently are 60 and 30-35. The most prevalent mutation was discovered to occur between nucleotides 30 and 35 of exon 2 of the GJB2 gene, which is one of the two locations where it occurs most frequently. Furthermore, they were unable to locate a single mutation indicating a G deletion at positions 30-35; instead, they found a G insertion at those locations in the coding area of the GJB2 gene. The most prevalent dangerous mutation, 235delC, which affects the Asian population, was also not present [2].

In a study of 45 Indian families from three distinct Indian States, namely Karnataka, Tamil Nadu, and Delhi, discovered that the W24X mutation caused 13.3% of autosomal recessive nonsyndromic hearing loss. The W24X mutation was also found in all six families, whether homozygous or heterozygous, proving that it is a typical GJB2 variant in India [3].

The recycling of potassium ions is one of the critical stages in the mechano-transduction of hearing, and Connexin 26 (Cx-26), a gap junction protein encoded by the GJB2 gene, is important in this process. Mutations in the GJB2 gene have been associated to both autosomal recessive hearing loss and dominant non-syndromic hearing loss. Because this gene is involved to skin homeostasis, mutations in the Cx-

26 gene are infrequently linked to syndromic kinds of hearing loss that display skin abnormalities. One of the hearing-impaired partners, their hearing-impaired sibling, and their hearing-impaired offspring all displayed compound heterozygosity in the GJB2 gene, involving a dominant mutation, p.R184Q, and two recessive mutations, p.Q124X and c.IVS 1+1G>A. This hearing-impaired non-consanguineous assortative mating family was reported in 2017 study [4].To their knowledge, this is the first report from India on the uncommon compound heterozygosity displaying nonsyndromic presentation associated with the p.R184Q mutation in the GJB2 gene.

Connexin26 mutations were widely distributed in the Indian population, according to a 2003 study [5]. Up until 2003, a small number of Indian people were engaged in studies to explore for genes other than connexin26 that cause deafness. The researchers discovered the mutations in the coding region (W24X, W77X) in a limited number of individuals from the Indian subcontinent, involving cases from India, Pakistan, Bangladesh, and Sri Lanka. However, ivs1(+1)GA is the first reported from India. There was no discovery of the 35delG and 167delT mutations, which are carriers at rates as high as 2-4% in European and Ashkenazi Jewish populations. Compared to 35 to 50% of congenital cases of deafness in southern Europe and the US, only 17.7% of congenitally deaf probands in India have biallelic GJB2 mutations. They thus assumed that there could be more genes that are common in India in addition to the deafness-causing genes already identified. There are 57 potential genes linked to deafness, according to study done on nuclear, consanguineous families from Tamil Nadu, India. They identified many amino acid changes, which we categorised as polymorphisms as they were observed in both the hearing and deaf groups. They used Conseq, an algorithmic tool that calculates the evolutionary rate at each amino acid site, to analyse all the polymorphisms. It is predicted that I111T, E114G, R127H, and R165W won't have any functional repercussions because all of the wild type amino acids at these sites are mutable. Although V27I and V153I are kept, it is anticipated that they will also be polymorphic since these are conservative modifications [5]. The R127H mutant was transfected into HeLa cells in two recent experiments to examine gap junction channel function. Immunolocalization in the second case indicates that R127H does produce gap junctions, but the gap junction's activity is reduced. R127H was found in a high frequency in the hearing population of

Indians, clearly indicating that this is not a causal mutation for deafness. In the first instance, R127H behaves like wild type connexin26 and 43.

In 2018, research [6], 19.4% of NSHL in the Indian population is linked to mutations in connexin 26 (GJB2). The majority of GJB2 mutations, which accounted for 72.2% (234 of 324 total mutant alleles from 7 studies) and 15.4% (50 of 324 total mutated alleles from 7 studies), respectively, were found to be c.71G > A(W24X) and c.35delG. They concluded that employing PCR and RFLP to test for these two common mutations in the GJB2 gene would significantly help in providing rapid genetic diagnoses and help with genetic counselling for families with NSHL.

3.2 INTERNATIONAL

Tested 53 unrelated Italians were tested with nonsyndromic sensorineural hearing loss for CX26 mutations in 1999 [7]. A mutation was present in 53% of instances, 35% of cases with autosomal recessive inheritance, and 60% of cases with sporadic inheritance. Three new mutations were discovered. Even within the same family, there were variations in the degree of hearing loss. Gene mutations were detected in the DNA of 20% of patients with severe hearing loss, 35.5% of patients with profound hearing loss, and 33.3% of patients with moderate hearing loss.

Out of more than 50 reports, in 2003 study [8] identified three GJB2 mutations: 35delG, 167delT, and 235delC, which collectively account for 70% of the deleterious alleles in whites, Ashkenazi Jews, and Asians, accordingly. The aqueous gap between the cytoplasm of two neighboring cells is created by gap junctions, which are large-diameter channels made up of two hemichannels on opposing membranes, each comprised of six connexin subunits. These channels merge through hydrophobic contacts. The developing brain expresses the gap junction component (GJB2) (2007) [9]. 1997 study discovered that the GJB2 gene has two exons [10], one of which is untranslated. In the promoter regions of the mouse and human genes, there are six GC boxes, two GT boxes, a TTAAAA box, a YY1-like binding site, and a consensus mammary gland factor binding site.

Work done in 1995 looked at a family of Bedouins that was extremely inbred and had autosomal recessive deafness [11]. The family belonged to a Bedouin-Arab clan that was founded some 200 years ago by a man who travelled from Egypt to what is

now southern Palestine. Five of his seven children who were born after he wed a local lady grew up. Consanguineous marriage has been customary since the third generation of the tribe. Around 3,000 people made up the tribe, which was then in its seventh generation. They resided in an area of Israel that was apart from other Bedouin tribes. The tribe's high birth rates and widespread polygamy. In the preceding generation, two of the founder's five adult sons were the ancestors of all 80 congenitally deaf individuals. Prelingual neurosensory hearing loss, which was a severe form of deafness, resulted in much higher audiometric thresholds all around. All of the deaf individuals had phenotypic characteristics that were otherwise normal, including normal intelligence levels, no retinopathy, renal issues, or abnormalities of the external ear. Discovery done in 1997 CX26 mutations creating premature stop codons at locus 13q11-q12 in three autosomal recessive nonsyndromic sensorineural deafness pedigrees (DFNB1A)[12].

Using immunohistochemistry in1997 discovered that human cochlear cells express GJB2 at high levels [12]. Connexins 26 and 30 (604418) are expressed in the cochlea's supporting cells in mice and rats, suggesting that they have a role in the recycling of endolymph potassium [13]. Connexin mutations in skin disease and hearing loss were thoroughly studied in 2001 [14]. They evaluated the dominant connexin diseases of keratoderma and/or hearing loss as well as the autosomal recessive nonsyndromic hearing loss brought on by connexin mutations.

Prelingual sensorineural deafness was diagnosed in 26 unrelated Greek individuals (2000) [15] in order to rule out environmental and syndromic causes of deafness. 28 chromosomes (53.8%) had the 35delG mutation, and an additional three sequence variations accounted for 7.6% of alleles. With the use of dye transfer, according to study (1992) [16], tumor cells lack functional gap junctions in contrast with normal mammary epithelial cells that express CX26 and CX43. Cell cycle control of GJB2 expression led to modest expression throughout the G1 and S phases and a significant increase during the late S and G2 S phases in synchronised cells. Throughout the cell cycle, CX43 was expressed at a constant low level. In response to phorbol ester, the two CX26 transcripts, but not CX43, were re-expressed in breast tumor epithelial cells.

In Greece, 210 instances of nonsyndromic prelingual sensorineural deafness were examined by in 2002 [17]. In 70 of the instances, biallelic GJB2 mutations were discovered (33.3 percent). In addition to the 63 persons who were homozygous for the 35delG mutation, seven patients were compound heterozygous for the 35delG gene and another mutation. In addition to 35delG, there were seven alleles with four additional mutations. This means that 95% of the GJB2 deafness alleles were caused by the 35delG mutation. The coding area of the GJB2 gene was sequenced for six people heterozygous for the 35delG mutation, but no further mutation was discovered. This ratio was the same as the carrier frequency of 3.5% seen in the Greek population in good health.

In a study of 65 Caucasian families with prelingual deafness from three geographical populations—New Zealand/Australian, French, and British (1997)[18], discovered that the 30delG mutation accounted for more than 70% of the CX26 mutant alleles. For families with a single deaf kid, genetic counselling may be suggested due to the high occurrence of this mutation. Importantly, study noted that several individuals homozygous for the 30delG mutation only experienced little hearing loss.

In 534 Mongolian probands with nonsyndromic sensorineural deafness, the GJB2 gene was evaluated in 2010 [19], and 23 (4.5%) of them had biallelic GJB2 mutations. The most common mutation, IVS1+1G-A (121011.0029), appears to have a number of origins based on multiple related haplotypes. Research hypothesises that the lower occurrence of GJB2 deafness in Mongolia results from the deaf in Mongolia having a lower frequency of assortative mating (37.5%) and inferior genetic fitness (62%) than in Western populations.

In 2002 research conducted on the GJB2 variants 167delT (121011.0010), 35delG (121011.0005), and 235delC that have previously been researched globally (121011.0014). The results showed that these alleles had 100% penetrance but varied expressivity for nonsyndromic prelingual sensorineural hearing loss. The researchers also investigated the relationship between GJB2 variation and variations in the connexin-26 allele type [20].

Sequenced the whole coding region and neighbouring regions of the GJB2 gene in 324 juvenile deafness patients using a PCR-based DNA sequencing technique

in 2002[21]. 127 children (or 39.2%) out of the 324 cases had at least one mutant connexin 26. 70 percent of familial cases and 36.1% of sporadic cases. On average, 57 (44.8%) of the 127 children were homozygotes or compound heterozygotes. Wu et al. discovered 34 mutations in 2002 [21], including 10 unique variants, six of which could be harmful.

In 2002, study [22] examined the functional importance of six prevalent CX26 variants, including 35delG and M34T, that cause hearing loss. The changed protein expression, subcellular location, and/or functional activity seems to classify the related impairments into three categories. In 2002 [23]another study demonstrated that mutations in the GJB2 coding area, which were identified in deaf persons and effectively transfected in human HeLa cells, have functional importance. According to the research, changes in the Connexin-26 gene can impair gap junctional intercellular communication by affecting protein translation, trafficking, and hemichannel construction.

In an Italian study [24], (2002) looked at GJB2 mutations in 179 unrelated patients who had sporadic or familial hearing loss. Hearing loss was handed down vertically in 18 of the 57 families that were examined, impacting two or three generations in each case. 24 of the 179 people showed extra-auditory clinical symptoms, whereas 155 were non-syndromic. In 19 people with GJB2 mutations, the anamnestic history of prenatal risk factors for acquired hearing loss was also examined. The incidence of the 35delG mutation was 41 percent in autosomal recessive pedigrees and 44.4 percent in pseudodominant pedigrees; it was discovered in 22.1 percent of sporadic cases and 39.4 percent of familial cases. Two distinct GJB2 mutations were discovered in compound heterozygosity with the 35delG allele: an asp159 to val (D159V; 121011.0024) and a 5-bp duplication at codon 96. (121011.0025). Two 35delG homozygous individuals were discovered among hearing loss cases that were determined to be of environmental origin. Four patients who were compound heterozygotes for 35delG and another GJB2 mutation, as well as two patients who were homozygotes, all experienced extra-auditory clinical symptoms affecting numerous organs (skin, vascular system, hemopoietic lineages, and thyroid). A sizable portion of those with 35delG heterozygous hearing loss (52%) had no second GJB2 mutation.

In 777 children with hearing loss who were unrelated to them, 12 percent had GJB2 or GJB6 mutations, and 20 percent of those who had a sibling with the condition did as well. According to the researchers, GJB2/GJB6 mutations were found in 4% of individuals whose medical records identified an environmental cause for their deafness and 11% of those whose etiology was unclear. Otoacoustic emissions testing for the detection of active outer hair cells in 76 children (10%) revealed positive emissions, which suggested auditory neuropathy. Five individuals with auditory neuropathy had GJB2 gene mutations that were homozygous or compound heterozygous. According to study (2005) [25], the loss of all functioning gap junctions is not always the result of GJB2 mutations.

In 610 hearing-impaired Asians and 294 healthy individuals, the GJB2 gene was examined in 2006 [26]. Due to the discovery of unique, unidentified, or controversial coding sequence changes or just one recessive mutation in GJB2, they found causal mutations in 10.3% of the cases and inconclusive findings in 1.8% of the instances. The controls had thirteen sequence variations, and 47% of the Asian controls had two to four sequence variations in the GJB2 coding area. The controls also had complicated genotypes.

1,294 deaf patients with hearing loss who had been referred for a DFNB1 diagnosis were tested. in 2004 [27]. (220290). The coding area of GJB2 exon 2 was examined for allelic variants. If two deafness-causing mutations in GJB2 were discovered, more testing was not conducted. Researchers searched for mutations in the noncoding area of GJB2 and a large GJB6 deletion identified as GJB6-D13S1830 if only one mutation causing deafness was discovered. 205 individuals with two GJB2 exon 2 mutations were identified to have DFNB1, whereas 100 individuals had just one deafness-causing exon 2 allelic variation. 37 of these individuals in total carried the 35delG mutation (121011.0005). Average hearing impairments were 88 percent and 37 percent, respectively, in individuals with two truncating/nonsense mutations in DFNB1 compared to those with two missense variants (p less than 0.05). When compared to controls with normal hearing, the number of 35delG carriers who are deaf was higher than expected, indicating the possibility of at least one additional mutation outside the GJB2 coding region that does not complement.

GJB2 deafness causing allelic variations are Compared to 35delG/non-35delG compound heterozygotes, which in turn had a considerable higher hearing loss than those with two non-35delG mutations, 35delG (121011.0005) homozygotes had a considerably higher hearing loss, according to study done in 2004 [28]. Homozygosity for V37I (121011.0023) or the 35delG with L90P (121011.0016), V37I, or IVS1+1G-A was linked to hearing loss (121011.0029). The GJB2 gene was sequenced in 2000 [29] using 63 persons with normal hearing, 39 Japanese patients with prelingual deafness, 39 patients with post lingual progressive sensorineural hearing loss, and 39 patients with prelingual deafness. 5 of the 39 people with prelingual deafness (about 12%) had GJB2 mutations. The most frequent mutation, present in 7 out of 10 mutant alleles, was 235delC (121011.0014). The 30delG allele was absent in none of the cases (121011.0005). No GJB2 mutation was found in the group of patients with post lingual hearing loss. The frequency of hereditary prelingual hearing impairment is highest in the world among different Palestinian tribes. In 2002 [30] examined CX26 mutations in 48 Palestinian probands with nonsyndromic hearing loss who were all identified separately. For mutations in the GJB2 gene, they found homozygosity or compound heterozygosity in 11 cases (23 percent). The 167delT mutation (121011.0010), which seems to be exclusive to Israeli Ashkenazi and Palestinian groups, and the 35delG mutation (121011.0005), which is distributed globally, were demonstrated to have a shared origin in Palestinian and Israeli populations, according to linkage disequilibrium study. The other nine deaf probands were homozygous, whereas only two of them were compound heterozygous.

In 2008 study [31] included 95 babies from California with hearing loss in a study comparing children with and without connexin-related hearing loss. From these newborns, both exons of Cx26 were sequenced, and the Cx30 deletion was evaluated. Three of the 82 babies who underwent hearing tests passed; twelve of them had hearing loss associated to connexin. There were no differences in newborn hearing screening pass rate, neonatal morbidity, or hearing loss severity between neonates with and without connexin-related hearing loss. According to study not all babies with connexin-related hearing loss will fail newborn hearing screening (2008). Hearing loss caused by connexin is strongly correlated with family history [31]. 2002 study [32] reported that individuals with autosomal recessive nonsyndromic deafness (10-42%) who had a mutation in the GJB2 gene were mistakenly identified with the second

mutation. These individuals were from Spain, Israel, and the United States. Double heterozygosity for both the GJB2 gene mutation (35delG; 121011.0005) and the GJB6 gene deletion was discovered in 22 of 33 unrelated individuals with GJB2 and GJB6 gene mutations (604418.0004). The GJB6 mutation was present in two individuals who were homozygous for it. The GJB6 deletion was the second most common mutation causing prelingual deafness in the Spanish population. The researchers found that mutations in the GJB2 and GJB6 genes can cause prelingual deafness to be inherited either monogenetically or di-genetically. The deletion was reported to be 342 kb by 2002 study [32], however study done on 2005 [33]claimed that more current sequencing data showed the loss to be 309 kb. Lowa City, United States, in 2000 [34], studied 209 congenitally deaf people using allele specific PCR, SSCP, and direct sequencing. In 74 people, the GJB2 allele variant was discovered. Additionally, they identified two novel mutations that cause deafness (R32C, 645-648delTAGA). They discussed how the study's conclusions may improve genetic counselling for patients and healthcare professionals.

In 2001 [35] looked at 23 Japanese families that had the 1555A-G mutation in the mitochondrial 12S rRNA gene (561000.0001), which results in late-onset progressive hearing loss in the affected people. Mutations in the gene for GJB2 were found in eight of these families (4 frameshift, 2 nonsense, and 2 missense). GJB2 mutations were statistically far more common than in the general population, according to statistics. The authors postulate that the phenotypic manifestation of nonsyndromic hearing loss linked to the 1555A-G mitochondrial mutation may be made worse by exposure to aminoglycosides and GJB2 mutations.

In research on 2011 [36], vestibular impairment was present in 127 (54%) of 235 persons with DFNB1 due to mutations in the GJB2 and/or GJB6 genes, compared to 25 (41%) of 61 deaf controls who did not have DFNB1 deafness (p less than 0.03). 48% of DFNB1 patients reported that their daily activities were impacted by their vertigo, and the majority of them had to lie down for it to go away. Vertigo was noticed in considerably more cases with truncating mutations than non-truncating mutations, and it was also connected to a family history of the condition. Vestibular dysfunction causes DFNB1 deafness more commonly than previously believed, according to study [36] .

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Chapter 4

MATERIALS AND METHODS

4.1 STUDY DESIGN: Cross-Sectional observational study.

4.2 INSTITUTIONAL ETHICAL CLEARANCE: Approval was taken from Institutional Ethical Committee (IEC) of BLDE (Deemed to be University), Vijayapura and KIDNAR, Dharwad respectively. The entire research protocol was according to ICMR (2006) guidelines.

4.3 SAMPLE SIZE CALCULATION: With 95% confidence level, expected prevalence of deafness in India as 6.3% [1] and margin of error of ±2%, a sample size of 613 subjects for screening was considered in the study to determine the Genetic and Molecular Profiling of *GJB2* Gene in Deaf Mute Population of North Karnataka.

By using the formula:

$$n = Z^2 p(1-p) / d^2$$

where,

Z= z statistic at 5% level of significance

d is margin of error

p is anticipated prevalence rate

4.4 INCLUSION CRITERIA: Individuals with congenital, unilateral, bilateral, severe to profound sensorineural deafness without any other dysmorphic features or systemic affection and without any known aetiology for deafness were included in the study.

4.5 EXCLUSION CRITERIA: syndromic cases and deafness due to environmental factors and infections were excluded from the study.

4.6 SOURCE OF DATA: The special schools/hospitals in North Karnataka region from which 613 deaf-mute children were screened for the study. Along with this, medical colleges and practicing, clinicians were requested to extend their support for the said study. After taking the informed consent, the samples were collected from each patient. The clinical data was collected as described in the enclosed data collection form.

4.7 SAMPLE COLLECTION

The study was performed by screening 613 deaf-mute children. Initially, the heads of all the schools/training centres were contacted and explained the objectives of the study. They were then requested to provide names of the students and their parents with residential addresses. Parents of the students were then contacted and their consent was obtained to participate in the study. Audiological information was made available from the school records (fig4.1).

Blood samples from nonsyndromic deaf population were collected after taking the consent form of the subjects. Samples were collected from different geographical regions of north Karnataka. Collection of the sample was done by using the EDTA (Ethylene Diamine Tetra Acetic acid) coated tubes, which is stored at 4^0C during the fieldwork, and kept in 4^0C for further analysis. Cluster random sampling was carried out to collect the samples followed by the approval of the institutional ethical committee. Total 7 districts were chosen from north Karnataka region (Table 4.1), in that, 16 special schools/hospitals were included for screening, to get minimum 368 subjects (chosen by simple random sampling) from each schools/hospital. 2 ml of the peripheral blood sample was collected in EDTA coated vacutainers and stored at -20^0C until further use.

Figure 4.1: Figure showing the geographic areas along with the different districts in North Karnataka region.

4.8 SCREENING PROCEDURES

The present survey utilised a three-stage approach. In the first step, one of the team's doctors and secondary medical staff members interviewed and examined each deaf-mute children of a referral site using a standardised thorough questionnaire and examination method. All illnesses investigated in this survey were covered by the medical and family history questions utilised in this step. Regarding nonsyndromic hearing loss, particular attention was paid to 1) severity of hearing loss and 2) determining the age at which the patient or the patient's parents first became aware of the hearing deficiency and the age at which the issue was identified. Whispered voice testing (>6 years of age) and behavioural tests (<6 years of age) were among the techniques used to check for hearing loss at this period. In the second stage, patients with suspected hearing loss were referred to otologists and other specialists (such as paediatricians, obstetricians, ophthalmologists, dermatologists, neurologists, surgeons, and internists) in order to assess the causes of hearing loss and discover associated organ abnormalities. This required thorough history-taking, clinical examinations, and laboratory studies to look for external causes or pinpoint genetic aetiology. A thorough history was acquired, including information on the aetiology (infection, medications, etc.) and the age of onset. Triiodothyronine and thyroxine levels, urine tests for proteinuria and haematuria, electrocardiography, skull radiography, and chromosomal analysis were among the non-audiological exams done for each patient. The final step was looking into patients in whom no environmental variables that could have contributed to hearing loss had been found. A pedigree spanning at least three generations and a thorough medical history were developed, with a focus on a family history of hearing loss and other related congenital defects. All accessible family members had routine physical examinations and otologic tests, including oto-immittance and pure tone audiometry.

4.8.1 Audiometric evaluation: With the assistance of an ENT specialist and an audio metrician, clinical characterisation, and audiological profile for children with hearing impairment were reported. Using a Graphic Digi 1-S Audiometer, pure-tone audiometry was performed in an acoustically isolated environment (Graphic Electronics, India). Decibel (dB) hearing level thresholds for pure tone frequencies of 250, 500, 1000, 2000, 4000, and 8000 Hz were established (based on normal hearing subjects). The sum of the audiometric thresholds at 500, 1000, and 2000 Hz served as

the basis for calculating the pure tone averages (PTA). The following definition of hearing loss was based on the mean hearing level: normal90 dB. Additionally, it was divided into three categories: conductive, sensorineural, or mixed, unilateral, or bilateral, and dependent on kind.

Table 4.1: List of special schools visited for the blood sample and data collection

Sl No	District	Name of the School
1	Dharwad	1. Honnamma education society's residential school for deaf children, Dharwad
2	Belgaum	1. Deaf and dumb gov.school, Vidyagiri Belagavi 2. Birds deaf and dumb school, Tukanatti 3. Jemur deaf and dumb school, Munavalli 4. Nitin memorial, Akkul road, Nippani 5. Integrated rural developmental society, Gokak
3	Bagalkot	1. Shri Muragendra Shivacharya Mahaswani Vidya Samsthe, Hunagund.
4	Haveri	1. Shri Renuka Yellamma deaf and dumb school, Ranebennur 2. Residential school for deaf
5	Bijapur	1. S.S high school, Vijayapura 2. Swapna deaf and dumb residential school **3.** BLDE hospital, Vijayapura
6	Kalburgi	1. Government deaf school, Kalburgi 2. Vinayak educational trust, Sharan nagar, Kalburgi
7	Gadag	1. Annadaneshwara deaf and dumb school, Naregal 2. Pandit Panchakshari Andhara Vasatiyuta Vishesha Sangeeta Patashale, Gadag 3. Sri B.T Tatti(annavaru) memorial charitable trust, Laxmeshwara

4.9 MUTATION ANALYSIS OF *GJB2* GENE

4.9.1 Isolation of Genomic DNA

The process of removing chromosomal DNA from the cellular matrix in which it was housed is known as genomic DNA extraction. The opening of the nuclei and cell walls (if relevant) was necessary for genomic DNA extraction, which often entails the addition of a suitable detergent and mechanical shearing.

Total genomic DNA was isolated from above-collected blood samples by DNeasy Blood & Tissue (QIAgen, Germany) kit method. The isolated DNA samples were further analysed for quality as well as quantity and store at -20^{0}C for further use. The main procedures for isolating DNA were as follows: 1) disrupting the structure of the cell to produce a lysate; 2) protecting the DNA from degradation during processing; 3) separating the soluble DNA from cell debris and other insoluble substances; and 4) eluting the pure DNA (Fig 4.2).

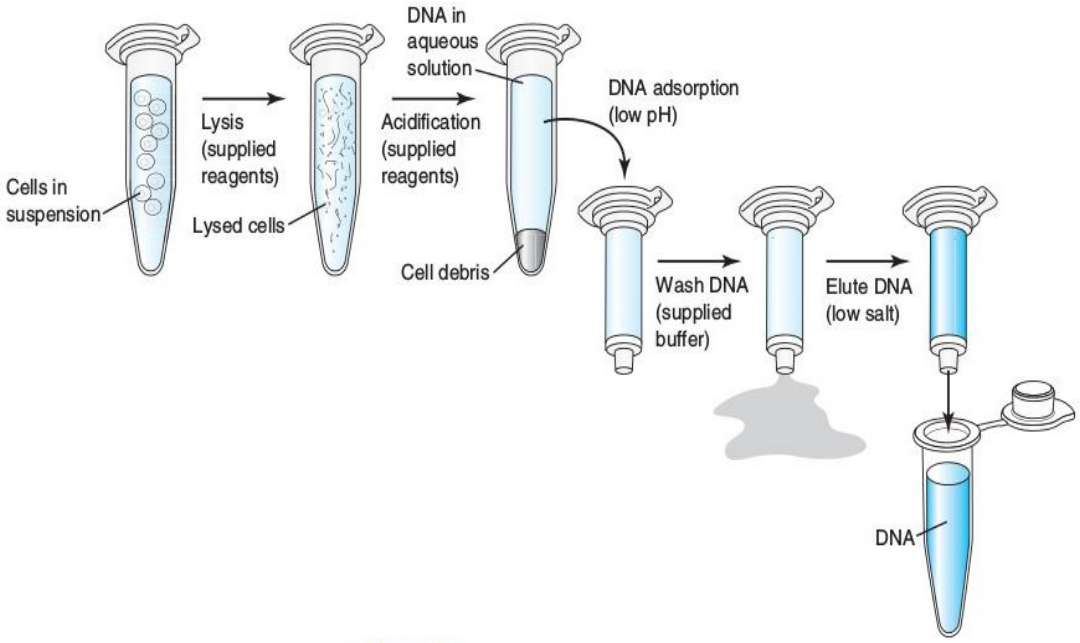


Figure 4.2: Schematic representation of DNA isolation from Blood sample [2].

Protocol for DNA isolation

1. 20ul proteinase K into was pipetted out a 1.5ml micro centrifuge tube. Added 100ul anticoagulant treated blood. Adjusted the volume to 220ul with PBS.
2. 200ul of buffer AL was added. Mixed thoroughly by vortexing. Incubated the blood samples at 56^0C for 10 min.
3. After 200ul ethanol (96-100%) addition, mixed thoroughly by vortexing.
4. Pipetted the mixture into DNeasy mini spin column placed in a 2ml collection tube. Centrifuged at ≥ 6000 * g (8000 rpm) for 1 min. discarded the flow-through and collection tube.
5. Placed the spin column in a new 2ml collection tube and added 500ul buffer AW1. Centrifuged for 1 min at ≥6000*g (8000rpm). Discarded the flow through and collection tube.
6. Placed the spin column in a new 2ml collection tube, added 500ul buffer AW2, centrifuged the mixture for 3min at 20,000*g (14,000rpm). Discarded the flow-through and collection tube.
7. Transferred the spin column to a new 1.5ml or 2 ml micro centrifuge tube.
8. Eluted the DNA by adding 200ul buffer AE to the centre of the spin column membrane. Incubated for 1 min at room temperature (15-25^0C). Centrifuged for 1 min at ≥6000*g.
9. DNA sample was stored at -20^0C until further use (figure 4.2).

4.9.2 Quality analysis of isolated genomic DNA

Concentrations of the extracted DNA samples were measured using Nanodrop spectrophotometer. The amount of DNA, RNA, and protein in a 2-µL drop on a pedestal was measured using a Nanodrop spectrophotometer, a typical lab tool. Compared to using a conventional 1-cm cuvette, a tiny sample volume means less preparation and clean-up time and allows you to measure multiple samples in under a minute. It measures DNA, RNA and Protein concentration and sample purity also. The ratio 260/280 measures the purity of the samples.

Note: Pure DNA sample gives 260/280 ratio ~1.8, for pure RNA 260/280 ratio was ~2 and for Protein 260/280 ratio was~1.6. Protein contamination of nucleic acids was indicated by a low A260/280 ratio or a significant peak at A280; high A230: contaminated with phenol, EDTA, or carbohydrates.

Procedure

1. 1-2ul of deionized water was pipetted out to cover the surface, clean the lever arm and optical surface with tissue paper [m between each measurement, it is important to wipe to prevent the sample carry over residue].
2. Required nucleic acid molecule was selected from nanodrop software 3000.
3. Blank measurement was carried out by loading l ul of de-ionized water.
4. Once the blank reading was noted down, optical surface was carefully cleaned with a tissue.
5. Since the buffer does not contribute to absorbance at 260nm, it was always made sure that the instrument is set to zero.
6. Similarly, 1-2ul of the DNA sample to be analysed was loaded. The quantity of DNA was displayed on the screen and the quality is measured using absorbance at 260/280nm.

4.9.3 Quantification of isolated genomic DNA

The quality of isolated genomic DNA was determined by using agarose gel electrophoresis (at proper agarose concentration). The intact double-stranded DNA forming a thick single band of high molecular weight confirms the presence of good quality of genomic DNA. This good quality genomic DNA was used for downstream processes.

Gel electrophoresis is a method used in molecular biology to separate a mixture of DNA and RNA fragments by length, to estimate the size of DNA and RNA fragments or to separate proteins by charge. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving.

Nucleic acids absorb light at a wavelength of 260nm. If a 260nm light source shines on a sample, the amount of light that passes through the sample can be measured, and the amount of light absorbed by the sample can be inferred. For double-stranded DNA, an Optical Density (OD) of 1 at 260 nm correlates to a DNA

concentration of 50 ng/ul, so that DNA concentration can be easily calculated from OD measurements.

Here we used agarose gel electrophoresis to check isolated genomic DNA from whole blood. Presence of bright DNA bands stained with Ethidium Bromide confirmed the presence of DNA in all the Deaf-Mute samples.

4. 10 PRIMER DESIGNING

Primers are short DNA sequences, usually composed of 18 to 24 base pair, which act as the starting points for DNA amplification by DNA polymerase enzyme during the PCR process. Because DNA polymerase enzymes are only able to add nucleotides to the end of a DNA strand under construction, primers were essential components of the DNA replication process. Therefore, designing suitable primers are essential for performing a PCR reaction correctly and replicating the desired part with high efficiency. To design a primer, the sequence of the studied gene was obtained in FASTA format from nucleic acid sequence databanks (like NCBI). In the next step, different software's such as Oligo, gene runner, primer3, beacon designer, etc. can be used to design primers. We used primer 3 input version (0.4.0) for our study.

4.10.1 Basic principles of primer designing: Primer length

Typically, a length of 18 to 22 nucleotides was usually suitable for a primer. If the length of the primer was shorter, the probability of non-specific bindings of the primers to the template DNA strand increases, and if the length of the primer was longer than this range, the binding of the primer to the template becomes more difficult at the annealing temperature.

4.10.2 The Flow Chart of Primer Designing

The sequences of the primers were generated using "Bioinformatics Primer Designing Tool" (Primer3) for the *GJB2* gene as given below. These designed primers were confirmed through Insilico PCR (Genome Build 36) method.

Go to http://www.ncbi.nlm.nih.gov.

↓

Search nucleotide sequence of HBB gene.

↓

Select FASTA and convert to FASTA format.

↓

Mark all the exonic regions in nucleotide sequence using Genbank format.

↓

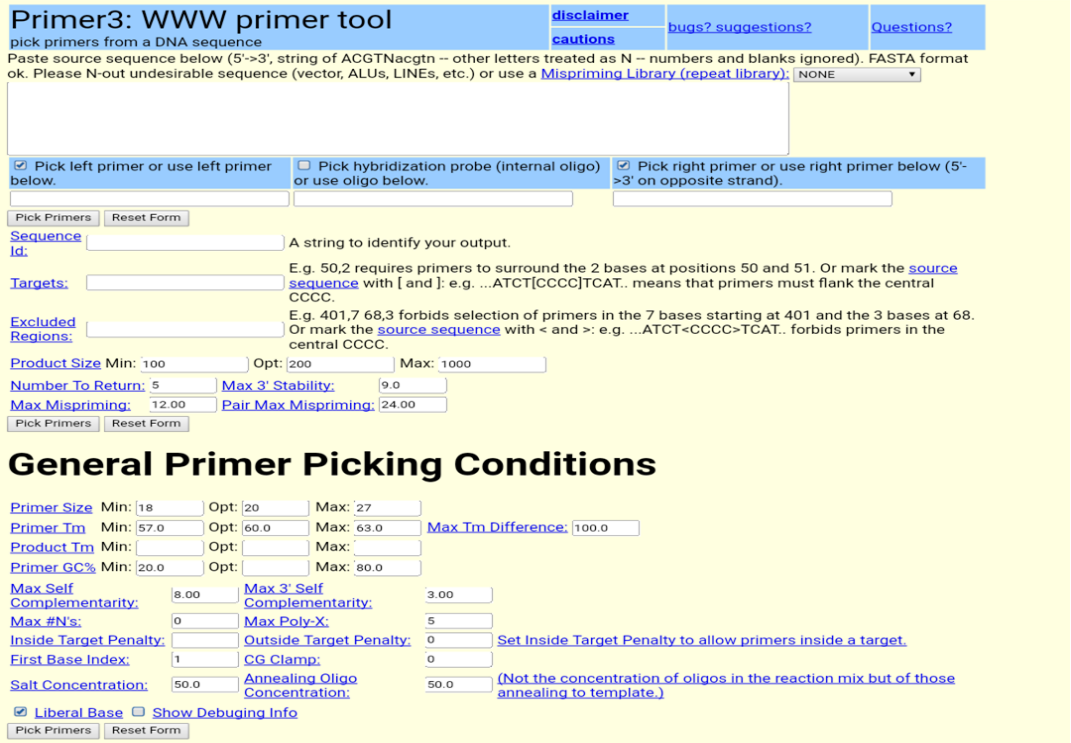
Google search for primer 3 input version (0.4.0).

↓

Enter the exon regions and enter the product size depending upon the length of the Sequence and give GC% content as 50%.

↓

Then select pick primers and the Mark the forward and the reverse primers in the nucleotide sequence from primer 3



Go to Insilico PCR 36 (Genome Build 36).

↓

Open In Silico PCR (Genome Build 36)

↓

Enter left and right primer sequence

↓

Compare the product size with that of primer 3 output.

↓

PCR output is obtained.

Primer3 Output

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len       tm      gc%   any   3'  seq
LEFT PRIMER         23   20    59.04    50.00  3.00  3.00 CCCCAAAGACCAACTCTGTT
RIGHT PRIMER       473   21    60.31    57.14  8.00  2.00 TCTCACTGGGCAGTGGTACTC
SEQUENCE SIZE: 490
INCLUDED REGION SIZE: 490

PRODUCT SIZE: 451, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

    1 AATAGCCCAAGCCCAGCAGGGCCCCCAAAGACCAACTCTGTTGCCCTCCCAGACCATCTT
                           >>>>>>>>>>>>>>>>>>>>

   61 GGATGACGCATACTTCCCTCTTTCCACAGGCCTGTCTGGCCCTGAGGGAGTCCCCTTTCT

  121 GAAGCTGTGGTGCTTGGACGACCTGCTCTCTACATTGCTGGGCACCTGTAGGTGTCCCTC

  181 GAGAGCTCAGTTTTGAGGTTCAAGTCAGTGTGGCCATGAAGGGGCTGCCTATTGGGCTGA

  241 TGCTGTGACCCTGGAGTCTGCCTCTCCTGCCAGTCCCCCTGCCCGGAACATGTGGCTGCG

  301 GCTTGGCCCGCCCTCGCTGTCCCTGAGCCCCAAGCCCACGGTTGGCAGGAGCCTGTGCCT

  361 CACCCTGTGGTTCCTCAGTTTGGCGCTGAGGGCCAGTACCCAGGCCCCAGCACCCACAGT

  421 CAACACTCACTTTGGGAAGCTAAGGGGTGCCCGAGTACCACTGCCCAGTGAGATCCTGGG
                                        <<<<<<<<<<<<<<<<<<<<

  481 GCCTGTGGAC
```

4.11 POLYMERASE CHAIN REACTION (PCR)

A DNA polymerase enzyme like Taq polymerase and two particular DNA sequences known as primers were used in the Polymerase Chain Reaction, a procedure in which a specific region of the genome or cDNA (created from a cell's RNA content) was amplified during an interactive temperature programme.

Kary Mullis first developed the PCR reaction in 1983 [3], and it is scientifically highly comparable to the DNA replication mechanism. The DNA polymerase enzyme initiates the complementary strand in this process by reading single-stranded pattern

DNA from 3' to 5'. Genomic DNA samples were used to optimize the developed primers for our gene of interest and gradient PCR was employed to standardise the primers' ideal Tm (Melting temperature). Later, the DNA samples were amplified using the polymerase chain reaction in a thermocycler at the standard temperature for primer (Figure 4.3).

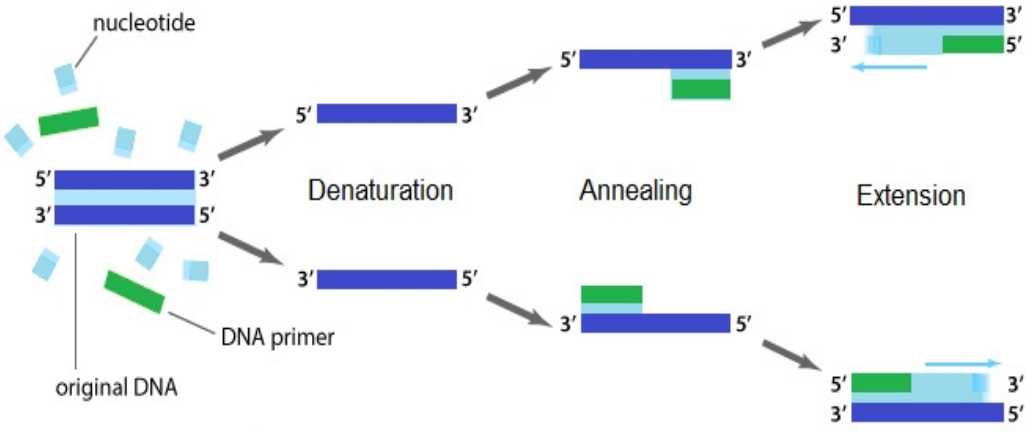


Figure 4.3: Image showing the schematic representation of PCR [4]

4.11.1 STEPS OF PCR

- **Initial denaturation:** This stage involved heating the reaction to 94–96 °C (98 °C if particularly thermostable polymerases are employed) and holding it there for 1–9 minutes.
- **Final denaturation:** The reaction was heated to 94–98 °C for 20–30 seconds at this stage, which was the first regular cycling event. By destroying the hydrogen bonds between the complementary bases, it allowed the DNA template to separate, resulting in a single strand of DNA.
- **Annealing:** To allow the primers to anneal to the single-stranded DNA template, the reaction temperature was decreased to 50–65°C for 20–40 seconds. The annealing temperature was typically 3-5 °C lower than the Tm of the employed primers. The calculation for this was done using the formula T 4(G+C) + 2(A+T) °C. The primer-template hybrid is bound by the polymerase, which then starts the DNA synthesis.
- **extension:** The DNA polymerase being used will determine the temperature at this stage. Taq polymerase functions best between 72 and 80 degrees Celsius, hence 72 degrees Celsius was the usual temperature for this enzyme. By

incorporating dNTPs that are complementary to the template in a 5-3 orientation, DNA polymerase now creates a new DNA strand that is complementary to the DNA template. The length of the DNA fragment that has to be amplified and the DNA polymerase being employed both affect how long the extension takes.

- **Final extension:** To guarantee that any leftover single stranded DNA was fully extended, this single step was occasionally carried out at a temperature of 70-74°C for 5–15 minutes following the final PCR cycle.

The reaction may be stored for a short period of time at this phase at 4°C for an infinite amount of time. PCR reaction mixture for 10µl was prepared as per the Table 4.2. The reagents were added to a vial and a short spin was given for about 30 seconds. Then PCR reactions were run on Thermal cycler (Eppendorf) using different conditions as shown in Table 4.3.

Table 4.2: PCR master mixture composition [For 10µl]

Reagents	Volume
MB water	7.35µl
Taq buffer	1.0µl
dNTP's	0.2µl
Forward primer	0.2µl
Reverse primer	0.2µl
Template	1.0µl
Taq polymerase	0.05µl

Table 4.3: Standard Cycle condition

Sl No	Steps	Temperature (^{0}C)	Time
1	Initial denaturation	94^{0}C	5 min
2	Final denaturation	94^{0}C	30 sec
3	Annealing	Primer Specific (Tm)	30 sec
4	extension	68^{0}C	1 min
5	Final extension	68^{0}C	5 min
6	Hold	4^{0}C	∞

4.11.2 Quality analysis of PCR amplified DNA

Using agarose gel electrophoresis, the quality and quality of the amplified PCR product for our gene of interest were assessed (at proper agarose concentration). The subsequent steps utilised high quality PCR products.

Based on the size and molecular weight of the DNA fragments, they were separated using this method. This was done to validate the PCR product and extracted DNA. 1X TAE Buffer was used to create the agarose gel (1%). In order to solidify, the produced gel was put into a casting tray. The isolated DNA samples were loaded for analysis. For around 30 minutes, the samples were electrophoresed at 110V. Under a gel documentation system, the bands were examined, and the results were noted.

4.12 SEQUENCING

Using forward primer and the large dye terminator cycle sequencing kit V3.1 (Applied Biosystem, USA), the PCR products for the GJB2 gene was sequenced on an ABI 3500 sanger sequencer. Sanger sequencing, sometimes referred to as the "chain termination method," was a method for determining the nucleotide sequence of DNA. Frederick Sanger, a two-time Nobel winner, and his colleagues developed the Sanger Sequencer apparatus in 1977.

Sanger sequencing can be done manually or, more often, automatically with the use of a sequencing equipment. (Figure 4.4). Each technique has three fundamental stages that are listed below.

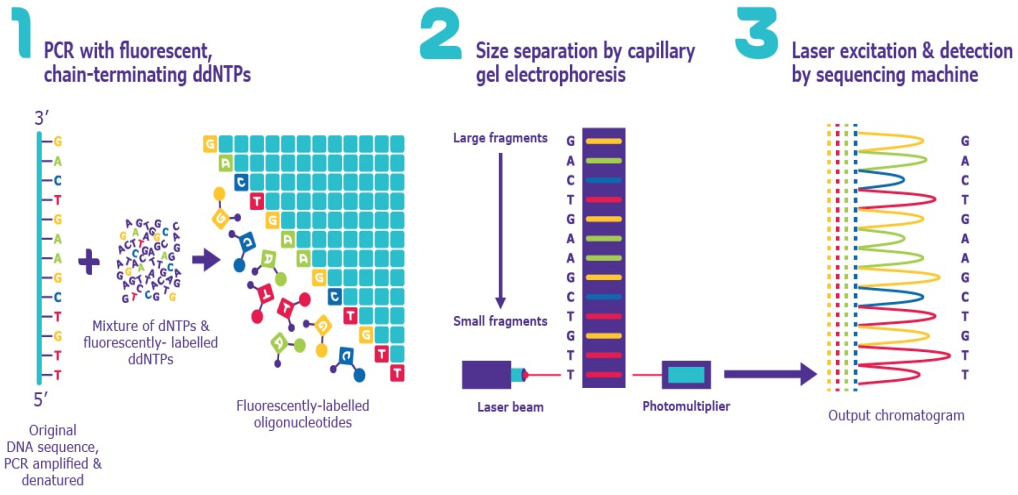


Figure 4.4: Three Basic Steps of Automated Sanger Sequencing.

The DNA sequence of interest was used as a template for a special type of PCR called chain-termination PCR. The insertion of modified nucleotides (dNTPs) known as dideoxy ribonucleotides during chain-termination PCR makes a significant difference from regular PCR (ddNTPs). In the extension stage of conventional PCR, DNA polymerase adds dNTPs to an expanding DNA strand by catalysing the creation of a phosphodiester bond between the free 3'-OH group of the last nucleotide and the 5'-phosphate of the next.

In chain-termination PCR, the regular dNTPs were combined in the PCR reaction with a small amount of chain-terminating ddNTPs. Since ddNTPs were deficient in the 3'-OH group necessary for the production of phosphodiester bonds, extension was halted when DNA polymerase randomly incorporates a ddNTPs. Millions to billions of copies of the target DNA sequence, each terminated at a random length (n) by 5'-ddNTPs, were produced as a result of chain-termination PCR.

Four PCR reactions were set up for manual Sanger sequencing, each containing just one type of ddNTP (ddATP, ddTTP, ddGTP, and ddCTP) (Table 4.4 and table 4.5). All the ddNTPs were combined in a single reaction for automated Sanger sequencing, and each of the four dNTPs has a distinct fluorescent label.

4.12.1 Size separation by gel electrophoresis

The second stage involves separating the chain-terminated oligonucleotides by size using gel electrophoresis. The oligonucleotides were drawn toward the positive electrode on the other side of the gel in gel electrophoresis because DNA was negatively charged. DNA samples were placed into one end of a gel matrix and an electric current was applied. Because each DNA fragment has the same charge per unit of mass, only size was affected how quickly oligonucleotides move. Smaller fragments travelled through the gel more quickly since there was less friction between them. The oligonucleotides were then organised in size order, reading the gel from bottom to top, as a result.

The oligonucleotides from each of the four PCR reactions were run in four different lanes of a gel during manual Sanger sequencing. This enables the user to determine which oligonucleotides match each ddNTP. All oligonucleotides used in automated Sanger sequencing run through a single capillary gel electrophoresis inside the sequencing equipment.

4.12.2 Gel Analysis & Determination of DNA Sequence

The final step consisted of simply reading the gel to ascertain the input DNA sequence. Each terminal ddNTP corresponded to a specific nucleotide in the original sequence because DNA polymerase only creates DNA in the 5' to 3' direction starting at a provided primer (for example, the shortest fragment must terminate at the first nucleotide from the 5' end, the second-shortest fragment must terminate at the second nucleotide from the 5' end, etc.). Therefore, we may ascertain the 5' to 3' sequencing of the original DNA strand by analysing the gel bands from smallest to largest.

When performing manual Sanger sequencing, the user examines the gel's four lanes sequentially from bottom to top in order to identify the terminal ddNTP for each band. The shortest PCR fragment, for instance, concludes with ddGTP if the lowest band in the column corresponding to it was discovered, and the first nucleotide from the original sequence's 5' end was the base guanine (G).

Fluorescence was used in automated Sanger sequencing to identify each terminal ddNTP by reading each band of the capillary gel sequentially. In essence, a laser stimulates each band's fluorescent tags, and a computer detects the light that was produced as a result. The fluorescent label on each of the four ddNTPs allows for easy identification of the terminal ddNTP from the light emitted. The output, known as a chromatogram, displays each nucleotide's fluorescent peak along the length of the template DNA.

Table 4.4: A. Sequencing master mix composition.

Components	Volume (in µl)
M.B. Water	6.5
Big dye Buffer	1.3
Bigdye	1.0
Forward primer	0.2
Template	1.0

Table 4.5: B. Sequencing Thermocycle condition.

	Temperature	Time
Initial denaturation	96.0 °C	60 sec
Denaturation	96.0 °C	10 sec
Annealing	…**… °C	10 sec
Extension	60.0 °C	4.0 min
Final extension	60.0 °C	5.0 min
Hold	4.0 °C	∞

4.13 FAMILIAL STUDY

Audiometric and clinical analysis in this research, the GJB2 gene was molecularly evaluated in 35 afflicted people from 20 families who had NSHL. In addition to being checked for HL, every proband was also checked for any other medical ailments. All the subject's information, including patient history, family medical history of HL or any other diseases running in the families, and information on consanguinity, was gathered prior to the audiometric test through a personal interview. After obtaining each patient's informed consent, this information was gathered. Each person underwent pure tone audiometry, and the findings were used to assign a hearing grade. The study only included NSHL probands without any acquired (associated) aetiology. Probands with signs and symptoms other than those associated with HL, as well as those with a history of infections like meningitis or rubella, as well as those who had used ototoxic medicines while pregnant, were excluded from the research. An EDTA vacutainer (BD, United States) was used to collect 2 to 3 ml of peripheral blood, along with the patients' signed consent and family tree.

4.13.1 Family pedigree analysis

To explain how human genes are carried from one generation to the next, a pedigree chart shows a family tree and the family members who are impacted by a specific genetic trait. Pedigree analysis is especially useful when there is a shortage of progeny data from several generations in any group being investigated. One may learn a lot about long-lived generations of a family by studying the pedigree of a family. Several symbols are used to denote different aspects of a lineage. After collecting phenotypic information over several generations and creating a pedigree, we can determine if a trait is dominant or recessive.

4.14 INSILCO ANALYSIS

Using bioinformatics methods, functional and structural analyses of the GJB2 protein were anticipated. Using databases, data mining, data analysis tools, homology models, machine learning, pharmacophores, quantitative structure-activity relationships, and network analysis tools, computer models known as "in silico" to assess experimental hypotheses.

4.14.1 Pathogenicity Prediction: Using the following bioinformatics, the pathogenic implications of the non-synonymous mutations were analysed:

4.14.1.1 PROVEAN- Protein Variation Effect Analyser

In order to determine if an amino acid change or indel (insertion/deletion) will have an effect on the biological activities of a protein, PROVEAN, a software tool, was utilised [5,6,7].

Web link: http://provean.jcvi.org/seqsubmit.php

Protocol:

1. The PROVEAN Web interface was accessed at http://provean.jcvi.org/seqsubmit.php
2. The “Protein sequence in FASTA format” text box left empty.
3. The position of the mutation in the protein was entered.
4. The Submit Query button was clicked.
5. The corresponding View link to browse the PROVEAN prediction (deleterious/neutral) report for our query was clicked.

4.14.1.2 PolyPhen2

A web-based programme called PolyPhen2 uses physical and comparative factors to forecast the potential effects of an amino acid change on the structure and behaviour of proteins, particularly human proteins. An updated version of the PolyPhen programme for annotating coding nonsynonymous SNPs is called PolyPhen-2 [8,9,10,11].

Web link: http://genetics.bwh.harvard.edu/pph2/index.shtml

Protocol:

1. The PolyPhen-2 Web interface was at http://genetics.bwh.harvard.edu/pph2/.
2. The "Protein sequence in FASTA format" text box was left empty.
3. The position of the substitution in the protein sequence into the Position text box was entered.
4. The appropriate boxes for the wild-type (query sequence) amino acid residue AA1 and the substitution residue AA2 were selected.
5. An optional description into the "Query description" text box was entered.
6. Submit Query button was clicked.
7. The corresponding View link to browse the PolyPhen-2 prediction report for our query was clicked.

4.14.1.3 PHD SNP- Predictor of human Deleterious Single Nucleotide Polymorphisms

A web-based programme called PHD-SNP is used to forecast how single nucleotide polymorphisms may affect human proteins. Predictor of harmful single nucleotide polymorphisms in humans based on PHD SNP Support Vector Machines [12,13,14,15]

Web link: https://snps.biofold.org/phd-snp/phd-snp.html

Protocol:

1. The PHD SNP web interface was accessed at https://snps.biofold.org/phd-snp/phd-snp.html
1. Uploaded the Protein sequence in FASTA format or the file containing protein sequence of interest or entered swiss prot code.
2. Entered the nucleotide substitution position.
3. Entered the suitable prediction viz sequence based or sequence and profile based.
4. Selected the multi SVM option.
5. The prediction was clicked.
6. A prediction result for mutations of interest was checked.

7. The output consisted of a table listing the number of the mutated position in the protein sequence, the wild-type residue, the new residue and if the related mutation is predicted as disease-related (Disease) or as neutral polymorphism (Neutral).

   The RI value (Reliability Index) is evaluated from the output of the support vector machine O as;

   RI=20*abs(O-0.5).

4.14.1.4 SNP & GO

Protein sequence, 3D structures, protein sequence profile, and protein function data are all combined into one framework by SNPs&GO. SNP & GO combines several pieces of information, such as those generated from the Gene Ontology annotation, to evaluate whether a certain variant is disease-related or neutral [14-19].

Web link: https://snps.biofold.org/snps-and-go/snps-and-go.html

Protocol:

1. The SNP & GO Web interface was accessed at https://snps.biofold.org/snps-and-go/snps-and-go.html
2. The protein sequence in FASTA format was uploaded or the file containing protein sequence of interest or entered swiss prot code.
3. Entered the nucleotide substitution

   Optional: Enter the EMAIL address.
4. The prediction was clicked
5. The prediction results for mutations of interest were checked.

4.14.1.5 SNAP2

SNAP2 is a technique for predicting how mutations over prot would affect functionality. A sophisticated classifier called SNAP2 makes use of a "neural network," a machine learning technique. To distinguish between effect variations and neutral variants/non-synonymous SNPs, it makes use of a variety of sequence and variant features. Along with structural traits like predicted secondary structure and

solvent accessibility, the evolutionary information from a machine-generated multiple sequence alignment serves as the crucial input signal for prediction [20,21].

Web link: https://www.rostlab.org/services/snap/

Protocol:

1. The SNAP2 Web interface was accessed at https://www.rostlab.org/services/snap/
2. Entered the protein sequence in FASTA format'
3. Optional: Enter the EMAIL address.
4. Clicked on run prediction.
5. The prediction results for mutations of interest were analysed.

4.14.1.6 PANTHER

PANTHER calculates the chance that a protein would be affected functionally by a coding single nucleotide polymorphism, especially a non-synonymous variation. It establishes the length of time that each amino acid was present in the protein's ancestors. The possibility of functional effect increases with preservation time [22].

Web link: http://www.pantherdb.org/

Protocol:

1. The PANTHER Web interface was accessed at http://www.pantherdb.org/
2. Protein sequence in FASTA format was entered.
3. The nucleotide substitution was entered.
4. Selected the organism, in which protein of interest belongs to
5. The prediction Clicked.
6. The prediction results for mutations of interest were analysed.

4.14.1.7 DVD &CADD

The Deafness Variation Database (DVD) offers a thorough overview of the genetic variation in the genes that are known to be connected to deafness. The DVD's objective is to compile, document, and categorise every genetic variation connected to both syndromic and non-syndromic hearing loss. Data may be gathered from all

significant public databases and used to create a single categorization that is supported by evidence for each variant, which is then edited by hereditary hearing loss specialists.

Web link: https://deafnessvariationdatabase.org/gene/GJB2

Protocol:

1. The DVD Web interface was accessed at

   https://deafnessvariationdatabase.org/gene/GJB2
2. Variant details with genomic DNA location was entered (example: Examples - 13:20763071: T>C, 13:20763044)
3. Clicked on Run the prediction
4. The prediction results for mutations of interest were checked.

4.14.2 CONSERVATION ANALYSIS: Clustal Omega and Consurf tool was used to examine the evolutionary conservation of variable residue across various species.

4.14.2.1 CLUSTAL OMEGA

Clustal Omega is a software for multiple sequence alignment that can accurately and efficiently align three or more nucleic acid or protein sequences. It creates divergent multiple sequence alignments that are physiologically significant. Cladograms and Phylograms can be used to visualise evolutionary connections. The tool for multiple sequence alignment known as Clustal Omega produces alignments between three or more sequences using HMM profile-profile methods and seeded guide trees. Two sequences will be aligned using pairwise sequence alignment methods [23].

Note:

An * (asterisk) represent positions which have a single and fully conserved residue.

A **:** (colon) represent conservation between groups of strongly similar properties.

A **:** (period) represent conservation between groups of weakly similar properties.

Web link: https://www.ebi.ac.uk/Tools/msa/clustalo/

Protocol:

1. The Clustal omega was used by following web link

   https://www.ebi.ac.uk/Tools/msa/clustalo/
2. Selected input sequences as DNA/RNA/Protein.
3. The sequence of interest in any supported format like FASTA, Genbank, and GCG etc… selected or uploaded.
4. Selected the parameters of interest [Clustal with character counts]
5. Then clicked submit.

4.14.2.2 CONSURF

The Consurf a web-based bioinformatics tool for predicting the evolutionary conservation of amino/nucleic acid positions in a protein/DNA/RNA molecule based on the phylogenetic relations between homologous sequences. It reveals the functional regions in protein/DNA by analysing the evolutionary dynamics of amino/nucleic acids substitutions among homologous sequences [24-28].

The degree to which an amino (or nucleic) acid position is evolutionarily conserved is strongly dependent on its structural and functional importance Thus, the importance of each position for the structure or function of the protein (or nucleic acid) can commonly revealed through conservation analysis of positions among members of the same family. In Consurf, the evolutionary rate is estimated based on the evolutionary relatedness between the protein (DNA/RNA) and its homologues and considering the similarity between amino (nucleic) acids as reflected in the substitutions matrix. One of the advantages of Consurf in comparison to other methods was that, the accurate computation of the evolutionary rate by using either an empirical Bayesian method or a maximum likelihood (ML) method [29].

Web link: https://consurf.tau.ac.il/consurf_index.php

Protocol:

1. The Clustal omega was opened using following web link. https://consurf.tau.ac.il/consurf_index.php
2. Either a Nucleotides or Amino Acid sequence was inserted. (Select weather was there a known protein structure for given sequence of interest. If yes, enter PDB ID or upload own PDB file. Upload Multiple Sequence Alignment (MSA) file. If no, Consurf will automatically make multiple sequence analysis)
3. Protein sequence of our interest was pasted in FASTA format.
4. Kept default parameters to homolog search algorithm.
5. Automatic Consurf analysis was selected.
6. Clicked on submit.
7. Output result was evaluated

4.14.3 TRANSMEMBRANE HELICES PREDICTION

Deep TMHMM

Deep TMHMM is a predictor of transmembrane helices in proteins. TMHMM is a membrane protein topology prediction method based on a hidden Markov model. It predicts transmembrane helices and discriminate between soluble and membrane proteins with high degree of accuracy. Deep TMHMM is currently one of the best-performing method for the prediction of the topology of both alpha-helical and beta-barrel transmembrane proteins. The model encodes the primary amino acid sequence by a pre-trained language model and decodes the topology by a state space model to produce topology and type predictions at unprecedented accuracy [30]

Web link: https://dtu.biolib.com/DeepTMHMM

Protocol:

1. The DeepTMHMM server was opened using following web link

https://dtu.biolib.com/DeepTMHMM

2. One or more proteins sequence in FASTA format uploaded or the protein file of interest in FASTA format was selected.

3. Clicked on submit and run the prediction

4.14.4 PROTEIN STABILITY PREDICTION

4.14.4.1 I-Mutant 2.0

I-Mutant 2.0 for Predictor of effects of single point protein mutation. I-Mutant2.0 support vector machine (SVM)-based tool for the automatic prediction of protein stability changes upon single point mutations. Predictions is performed for stability change upon single site mutation starting either from the protein structure or, more importantly, from the protein sequence. When the three-dimensional structure is known then I-Mutant2.0 predicts 80% of the cases correctly whether the protein mutation stabilises or destabilises. In case of only the protein sequence was available it predicts 77% accurately. The DDG value is calculated from the unfolding Gibbs free energy value of the mutated protein minus the unfolding Gibbs free energy value of the wild type (Kcal/mol) [31-34].

Web link: https://folding.biofold.org/i-mutant/i-mutant2.0.html

Protocol:

1. The I Mutant 2.0 Web interface was accessed at https://folding.biofold.org/i-mutant/i-mutant2.0.html
2. Protein structure [if available] or protein sequence option was selected.
3. Clicked enter to proceed.
4. The protein sequence of interest was pasted in query box.
5. Entered the position of amino acid substitution.
6. Entered new/mutated amino acid in One letter residue code.
7. Temperature was kept at 25 degrees Celsius and PH;7 as default
8. Selected the prediction based on Free Energy change value (DDG)
9. Email address was entered for further proceeding
10. Clicked on submit and run the prediction
11. The prediction results for mutations of interest were checked.

4.14.5 PROTEIN STRUCTURE PREDICTION

Homology modelling of wild type and mutant protein is developed using the Swiss model (https://swissmodel.expasy.org/). SWISS model is web-based server use for automated comparative modelling of three-dimensional (3D) structure of proteins. It started in 1993 and it became pioneer in the field of automated protein modelling and it is one of the most commonly used tools today. SWISS-MODEL offers different levels of user interaction. For example, in the "initial approach mode," simply the amino acid sequence of a protein is provided in order to create a 3D model. The server handles template selection, alignment, and model construction entirely automatically. In "alignment mode" the modelling is based on a user-defined target-template alignment. in "project mode" the integrated sequence-to-structure workbench DeepView (Swiss-PdbViewer) can handle complex modelling jobs. Each model is return with a thorough modelling report through email [35-40].

With the help of the downloadable version of the UCSF ChimeraX application, results are viewed and analysed. For the interactive viewing and study of molecular structures and associated data, such as density maps, trajectories, and sequence alignments, UCSF Chimera is an extendable molecular modelling application. (https://www.cgl.ucsf.edu/chimera/)

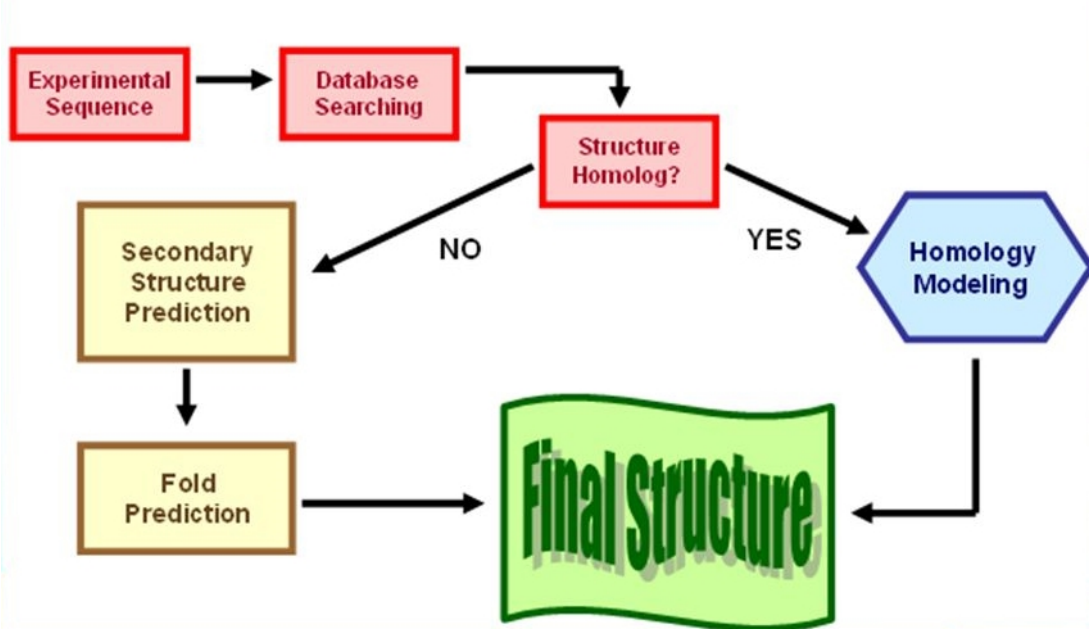


Figure 4.5: Schematic representation of protein structure prediction.

Protocol

1. Swiss model homepage was accessed using https://swissmodel.expasy.org/ web link
2. Clicked on start modelling
3. Pasted targeted protein sequence in FASTA format or entered Uniport accession number.
4. Project title and email address was entered.
5. Targeted-template alignment for proper template selection was selected
6. Structure model building of protein of interest was started.
7. Protein model was evaluated.

4.14.6 STATASTICAL ANALYSIS

The sequencing results was compared with ethnically matched samples and anthropometry. The novel changes in the DNA of deaf-mute children were analysed further using the DNA variant analysis software. All characteristics were summarized descriptively. For continuous variables, the summary statistics of mean, standard deviation (SD) was used. For categorical data, the number and percentage were used in the data summaries and data was analysed by Chi square test for association, comparison of means using t test, ANOVA and diagrammatic presentation. The connection between two category variables was tested using the Chi-square (2) test. An unpaired t-test was used to compare the means of study variables between two separate groups. ANOVA and the F test of assessing equality of variance were used to see if there was a difference in the means of analysis variables between more than two independent groups. If the p-value was less than 0.05, the results were statistically significant; otherwise, they were deemed non-significant. SPSS software version 23.0 was used to analyse the data.

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Chapter 5

RESULTS

The goal of the current study was to identify the contribution of Cx26 gene variations to childhood hearing impairment, with a focus on the North Karnataka population. The study involved 368 unrelated patients with prelingual hearing impairment. Students from special schools for the deaf in North Karnataka comprised the probands. Student records revealed that, all the affected pupils had progressive, non-progressive bilateral severe or profound non-syndromic hearing loss. The results of the present investigation have been presented in tables 5.11 to 5.21 and figures 5.1 to 5.44.

5.1 SOCIO-DEMOGRAPHIC PATTERNS IN CHILDHOOD HEARING IMPAIRMENT

The topics of religious and community backgrounds related to consanguineous marriage, socio-demographic aspects of marriages between close biological kin, fertility in consanguineous unions, and the effects of the consanguinity on rates and patterns of morbidity and mortality have been presented in the pages that followed as an introduction to the socio-cultural aspects of the study subjects.

5.2 SAMPLE COLLECTION

17 special schools (deaf and dumb) from 7 districts of North Karnataka were covered and peripheral blood samples were collected from the subjects (Table 5.1).

Table 5.1: list of special schools visited for Samples collection

Sl. No	District	Name of the School	Total Children participated in the study
1	Dharwad	1. Honnamma education society's residential school for deaf children, Dharwad	50
2	Belgaum	2. Deaf and dumb government, school, Vidyagiri Belagavi 3. Birds deaf and dumb school, Tukanatti 4. Jemur deaf and dumb school, Munavalli 5. Nitin memorial, akkul road, Nippani 6. Integrated rural developmental society, Gokak	100

3	Bagalkot	7. Shri muragendra shivacharya mahaswani vidya samsthe, Hunagund.	9
4	Haveri	8. Shri Renuka Yellamma deaf and dumb school, Ranebennur 9. Residential school for deaf	67
5	Vijayapura	10. S.S high school, Vijayapura 11. Swapna deaf and dumb residential school 12. BLDE hospital, Vijayapura	27
6	Kalburgi	13. Government deaf school, Kalburgi 14. Vinayak educational trust, Sharan Nagar, Kalburgi	35
7	Gadag	15. Annadaneshwara deaf and dumb school, Naregal 16. Pandit panchakshari andhara vasatiyuta vishesha sangeeta patashale, Gadag 17. Sri B.T Tatti(annavaru) memorial charitable trust, Laxmeshwar	80

5.3 EPIDEMOLOGICAL ANALYSIS OF DEAFNESS

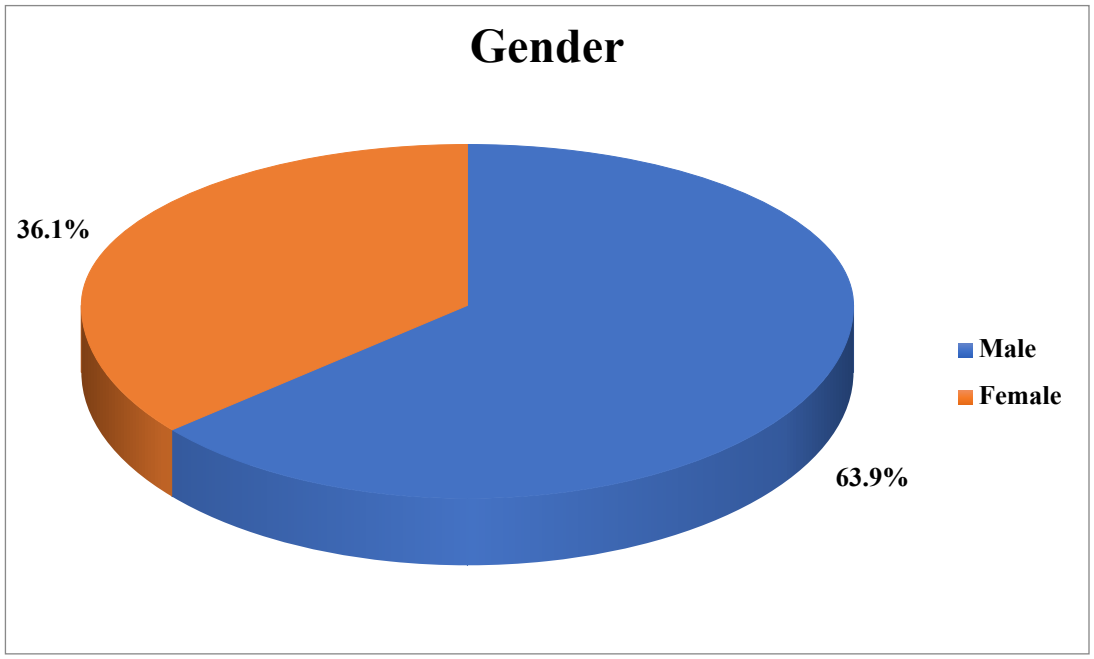
Deafness is characterised as the inability to primarily hear speech via one's ears, even with amplification. Since hearing loss hinders speech and language development, it has an impact on many areas of a child's overall development. Furthermore, it can obstruct and harm the child's social and financial interactions. Only when both ears are entirely deaf or when the better ear has a hearing loss of 70 dB or more, is then someone deemed to have hearing impairment. According to the Rehabilitation Council of India Act, 1992, a person with hearing levels between 61 and 70 dB is instantly disqualified from the hearing disability group.

Simple random sampling was used to screen the 638 hearing-impaired kids in four districts of North Karnataka. In a sample of 638 kids, 270 kids with syndromic hearing loss were disqualified from the study, leaving 368 kids with non-syndromic hearing loss overall (57.7%). 235 (63.5% of the total) and 133 (36% of the total) of the 368 young people that took part in the study were male and female (Table 5.2), respectively. In our study, there was no correlation between gender and the severity of

hearing loss [p value- 0.2] (Table 5.3). The same are represented in figure 5.1 and 5.2. Table two represents the distribution of cases according to gender. Table 5.3 shows the distribution of degree of hearing according to the gender.

Table 5.2: Distribution of Cases according to Gender

Gender	Number	Percent (%)
Male	235	63.9
Female	133	36.1
Total	368	100



** non-significant at 5% level of significance (p>0.05)

Figure 5.1: Degree of hearing loss according to Gender

Table 5.3: Distribution of degree of hearing loss according to the Gender

Degree of hearing loss	Male		Female		p value [Chi-square test]
	N	%	N	%	
Mild	9	3.8%	4	3.0%	0.2**
Moderate	14	6.0%	13	9.8%	
Severe	4	1.7%	6	4.5%	
Profound	208	88.5%	110	82.7%	
Total	235	100.0%	133	100.0%	

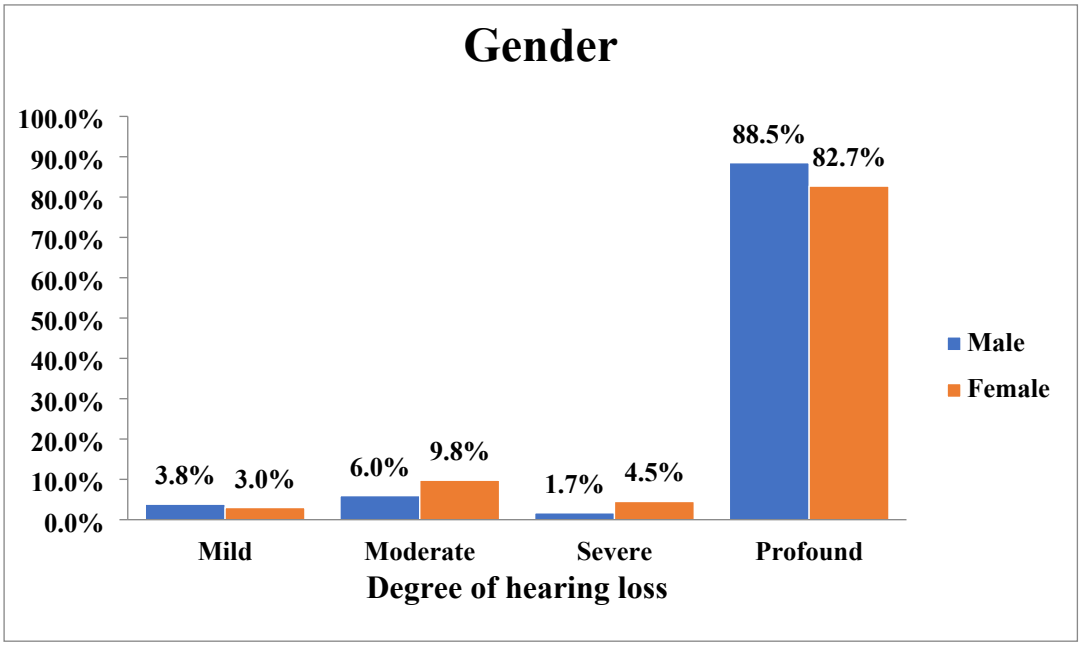


Figure 5.2: Degree of hearing loss according to Gender

Table 5.4 represents the distribution of cases according to the age group. The ages of the patients varied between 10 and 19 years, a lot of youngsters (80.7% vs. 19.3%, respectively). In terms of age, we discovered that the age group under 10 years had a significant frequency. The biggest number of young patients (94.6%) were prelingually deaf. (Table 4.4). In the study cohort, a significant correlation between hearing loss severity and age group was found [p value- 0.01] (Table 5.5). The same are represented in figure 5.3 and 5.4.

Table 5.4: Distribution of Cases according to Age

Age (yrs)	N	Percent
<10	71	19.3
10-19	297	80.7
Total	368	100

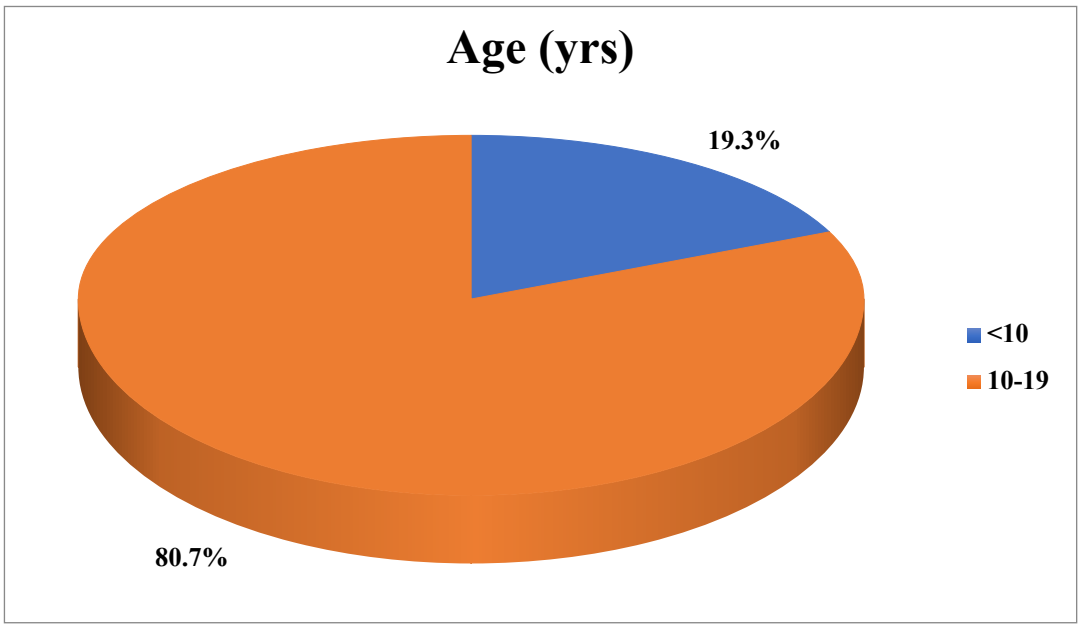


Figure 5.3: Distribution of Cases according to Age

Table 5.5: Degree of hearing loss according to Age,

Degree of hearing loss	Age<10yrs		Age 10-19yrs		p value [Chi-square test]
	N	%	N	%	
Mild	8	11.3%	5	1.7%	0.001*
Moderate	3	4.2%	24	8.1%	
Severe	2	2.8%	8	2.7%	
Profound	58	81.7%	260	87.5%	
Total	71	100.0%	297	100.0%	

Note: * significant at 5% level of significance (p<0.05)

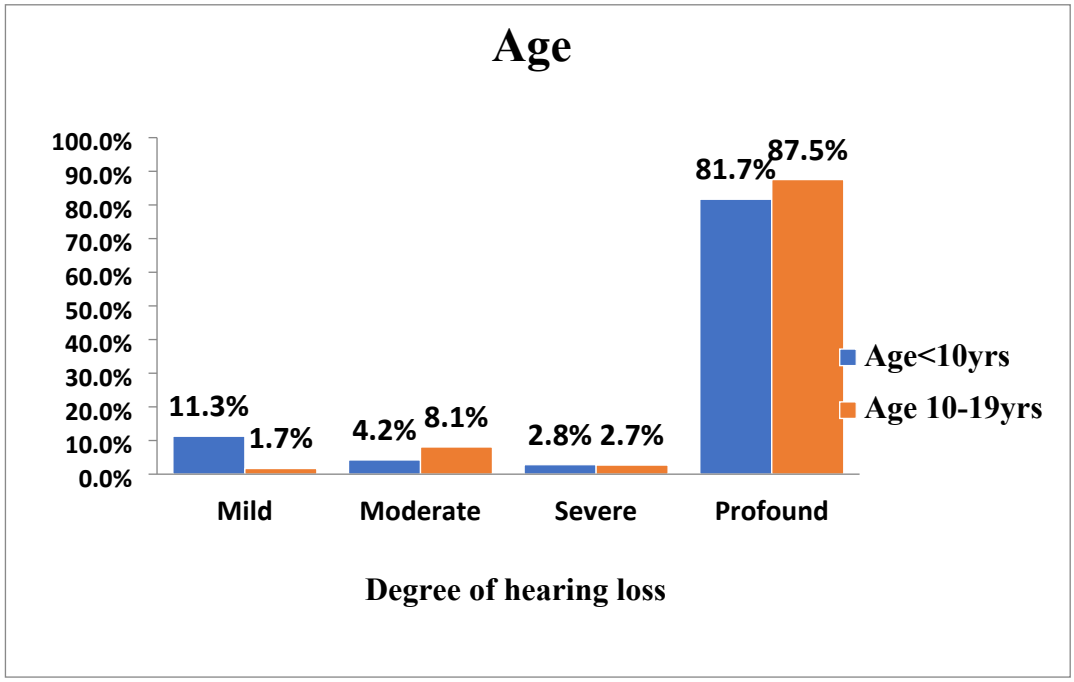


Figure 5.4: Degree of hearing loss according to Age

Table 5.6 represents the distribution of cases according to consanguineous marriage. Of the 155 [42%] deaf children now being examined; their parents were consanguineously married (Table 5.6). However, there was no correlation between hearing loss and consanguinity in the population being researched at this time [p value - 0.07] (Table 7). However, it is important to note that 139 (89.7%) of children with substantial hearing loss had consanguineous parents. The same are represented in figure 5.5

Table 5.6: Distribution of Cases according to Consanguineous Marriage

Consanguineous Marriage	N	Percent
Yes	155	42.1
No	213	57.9
Total	368	100

Table 5.7: Degree of hearing loss according to Consanguineous Marriage

Degree of hearing loss	With Consanguineous Marriage		Without Consanguineous Marriage		p-value [X^2 test]
	N	%	N	%	
Mild	7	4.5%	6	2.8%	0.071**
Moderate	8	5.2%	19	8.9%	
Severe	1	0.6%	9	4.2%	
Profound	139	89.7%	179	84.0%	
Total	155	100.0%	213	100.0%	

** non-significant at 5% level of significance (p>0.05)

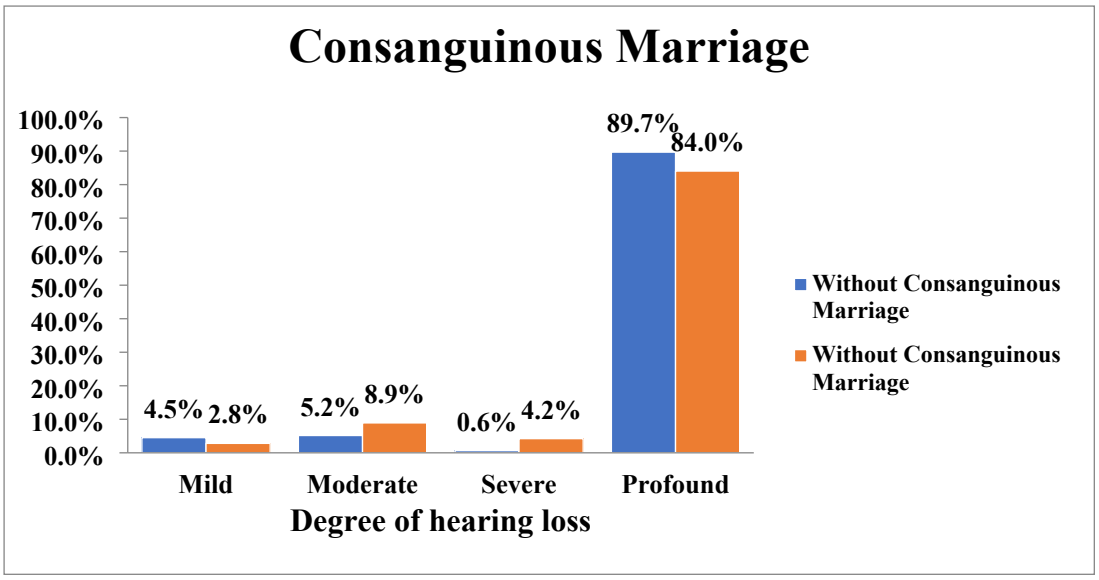


Figure 5.5: Degree of hearing loss according to Consanguineous Marriage

Table 5.8 shows the distribution of cases according to nature of deafness. Out of 368 study subjects 348 (94.6%) were prelingual and only 20 (5.4%) subjects were post lingual deaf. In that 293 (79.6%) showed stable for hearing loss, and progressive hearing loss was found in 75 (20.4%) study subjects. The same are represented in figure 5.6.

Table 5.8: Distribution of Cases according to Nature of Deafness

Nature of Deafness	N	Percent
Prelingual	348	94.6
Post lingual	20	5.4
Stable	293	79.6
Progressive	75	20.4

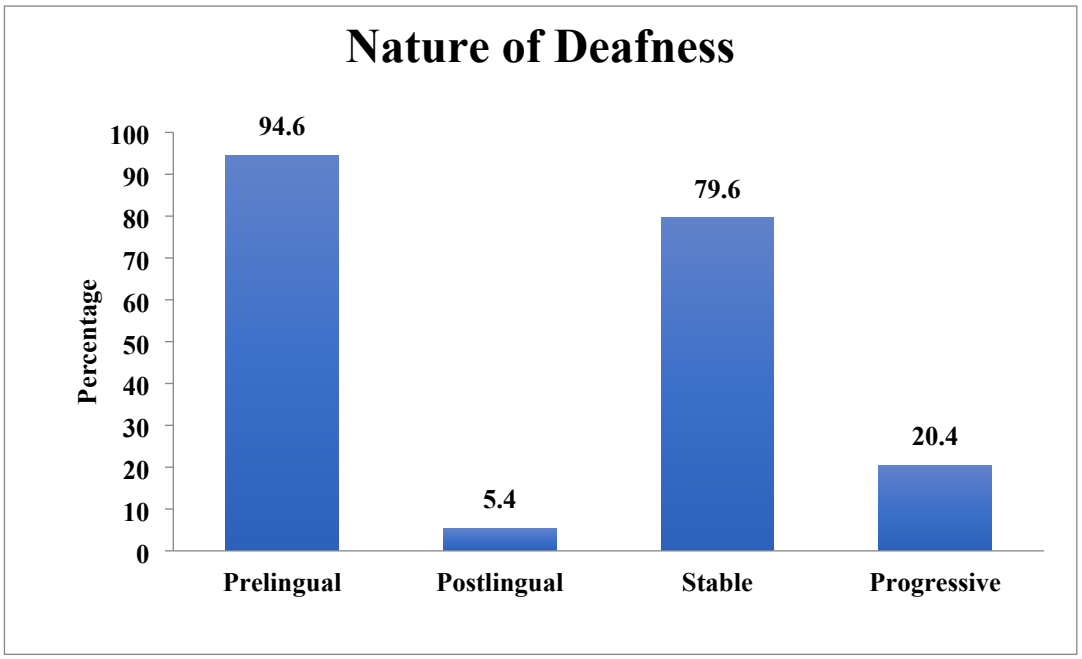


Figure 5.6: Distribution of Cases according to Nature of Deafness

Table 5.9 shows the cases according to pattern of deafness. In our study intriguingly, more kids [344; 93.5%] had bilateral deafness than unilateral deafness [24; 6.5%] (Table 5.9). So in pattern of deafness bilateral deafness is prominent. The same are represented in figure 5.7.

Table 5.9: Distribution of Cases according to Patten of Deafness

Patten of Deafness	**N**	**Percent**
Unilateral	24	6.5
Bilateral	344	93.5
Total	368	100

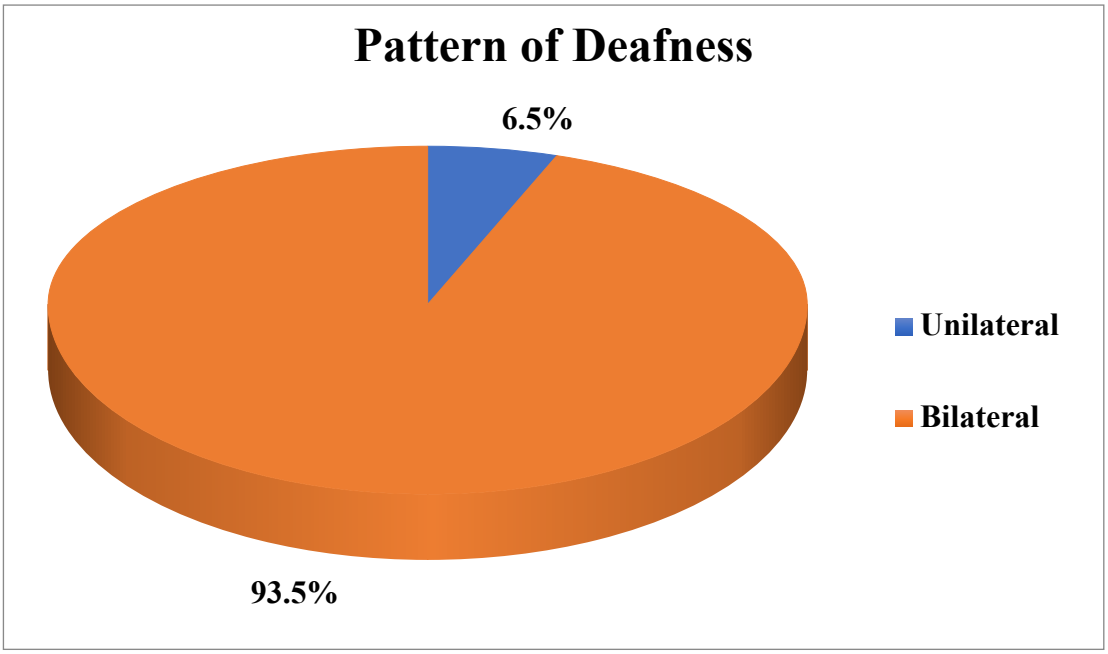


Figure 5.7: Distribution of Cases according to Patten of Deafness

Table 5.10 represents the distribution of cases according to hearing tone. 316 kids (85.9%) were found to have low hearing tones, followed by intermediate tones (8.7%) and high tones (5.4%). The same are represented in figure 5.8.

Table 5.10: Distribution of Cases according to Hearing tone

Hearing tone	N	Percent
High	20	5.4
Middle	32	8.7
Low	316	85.9
Total	**368**	**100**

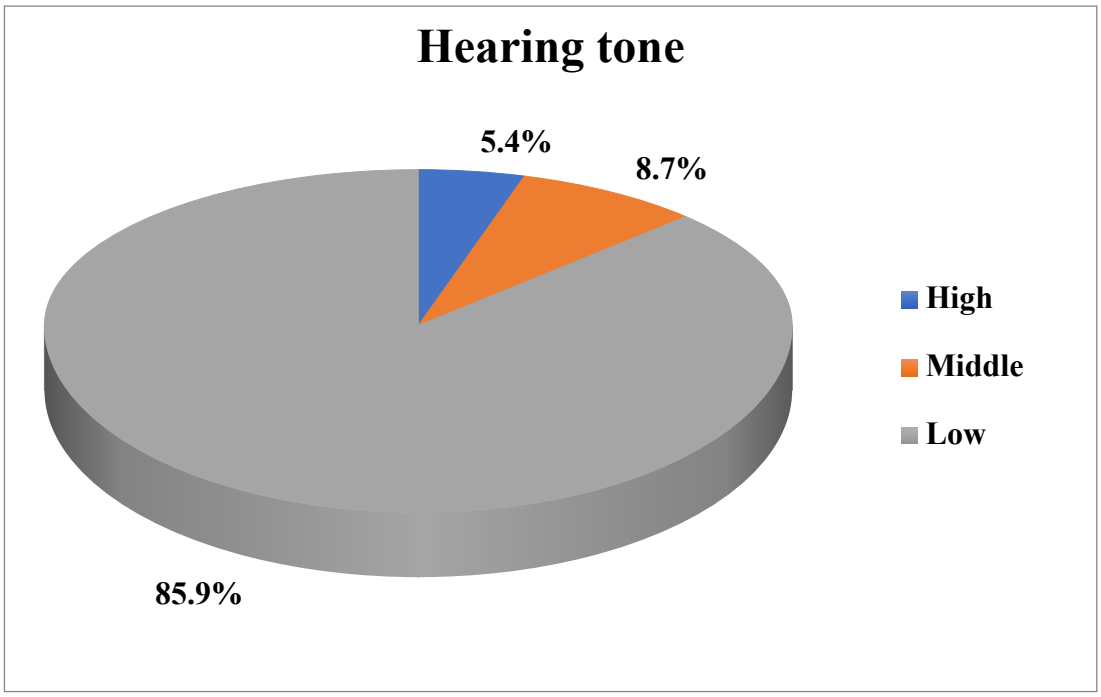


Figure 5.8: Distribution of Cases according to Hearing tone

The distribution of cases according to degree of hearing loss is presented in table 5.11. According this, 86.4% of the kids had profound hearing loss, which is followed by moderate [7.3%], mild [3.5%], and severe [2.7%] hearing loss (Table 5.11). We can say that profound hearing loss is high in the study subjects. The same are represented in figure 5.9.

Table 5.11: Distribution of Cases according to Degree of hearing loss

Degree of hearing loss	**N**	**Percent**
Mild	13	3.5
Moderate	27	7.3
Severe	10	2.7
Profound	318	86.4
Total	**368**	**100**

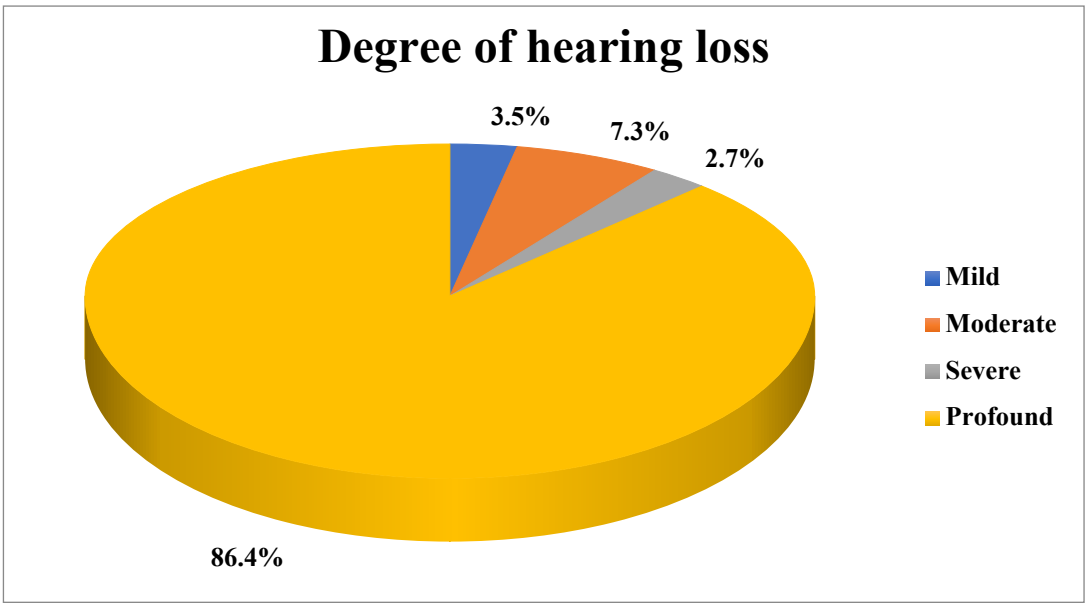


Figure 5.9: Distribution of Cases according to Degree of hearing loss

Our study shows according to the degree of hearing loss and degree of relation is not significantly associated with each other (p value- 0.761) (Table 5.12). The same are represented in figure 5.10.

Table 5.12: Degree of hearing loss according to Degree of Relation

Degree of hearing loss	Degree of Relation														p value
	3rd degree		4th degree		6th degree		First Cousin		Second Cousin		Undetermined		Nonrelatives		
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
Mild	4	6.8%	0	0.0%	0	0.0%	1	6.7%	0	0.0%	2	7.1%	6	2.8%	
Moderate	4	6.8%	1	2.5%	1	11.1%	1	6.7%	0	0.0%	1	3.6%	19	8.8%	
Severe	0	0.0%	1	2.5%	0	0.0%	0	0.0%	0	0.0%	0	0.0%	9	4.2%	0.761
Profound	51	86.4%	38	95.0%	8	88.9%	13	86.7%	2	100.0%	25	89.3%	181	84.2%	
Total	59	100.0%	40	100.0%	9	100.0%	15	100.0%	2	100.0%	28	100.0%	215	100.0%	

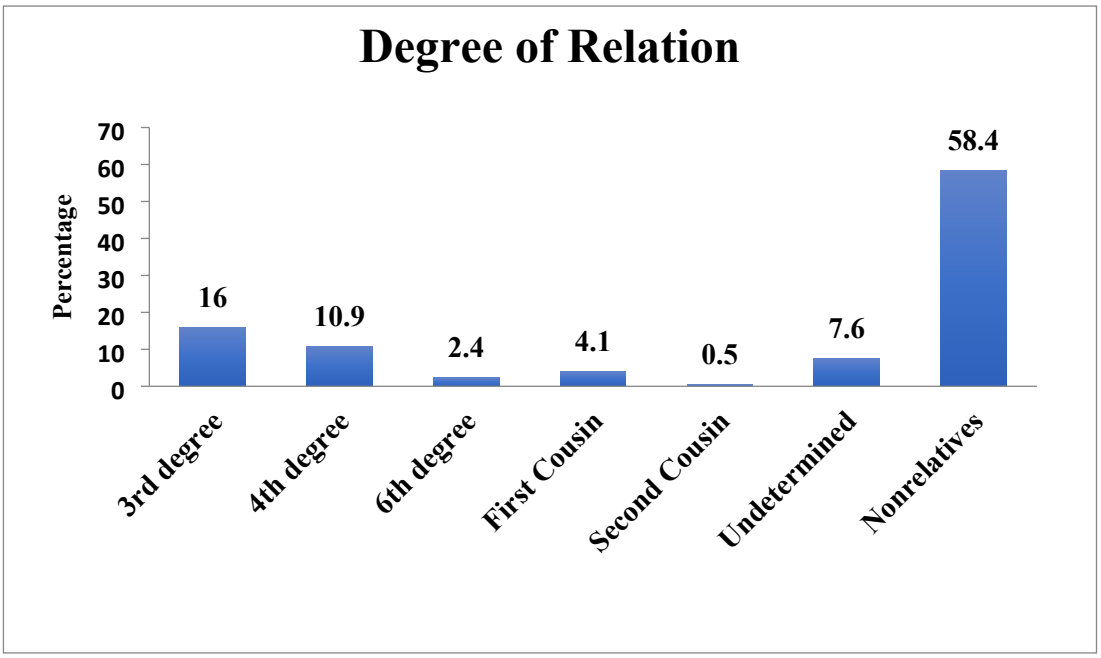


Figure 5.10: Distribution of Cases according to degree of Hearing

A family history investigation revealed that 62 children with hearing loss (16.8%) had a history of deafness. A total of 24 hearing-impaired youngsters had a deaf sibling. A statistical study revealed no correlation between the degree of relationship and hearing loss [p value- 0.76] (Table 5.13). Additionally, there was no conclusive link between the investigated children's deafness and their family history. Table 5.13 shows the degree of hearing loss according to family history. The same are represented in figure 5.11.

Table 5.13: Degree of hearing loss according to Family History

Degree of hearing loss	Family History of Deafness				p value [x^2- test]
	Yes		No		
	N	%	N	%	
Mild	1	1.6%	12	3.9%	
Moderate	3	4.8%	24	7.8%	
Severe	4	6.5%	6	2.0%	0.155**
Profound	54	87.1%	264	86.3%	
Total	62	100.0%	306	100.0%	

** non-significant at 5% level of significance (p>0.05).

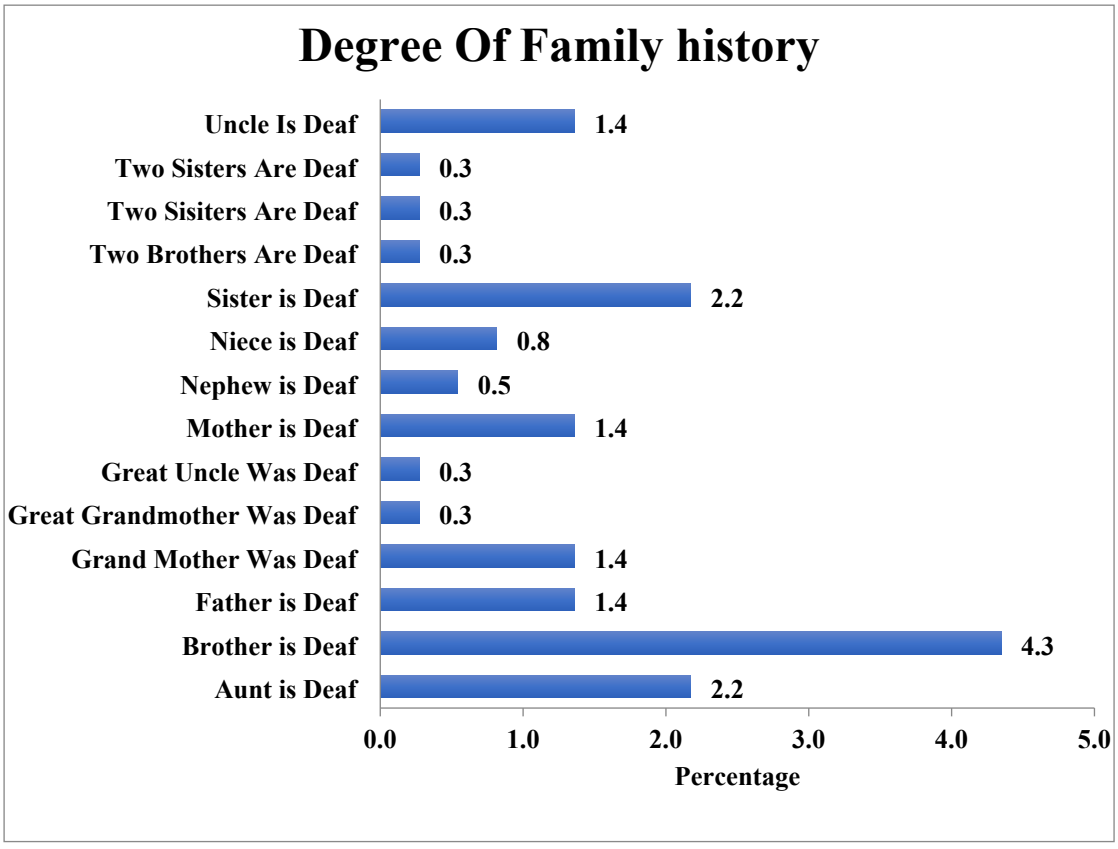


Figure 5.11: Distribution of Cases according to Family History of Deafness

Table 5.14 represents the clinicopathological features of study cohort. The whole analysis identified the epidemiological factors involved in the progression of deafness. Although it is still a challenging endeavour, preventing hearing loss is both doable and essential. In order to forecast future burdens and the resources required to prevent and control hearing loss, the burden of hearing loss must be continuously monitored.

Table 5.14: Clinicopathological features of study cohort

Clinic pathological condition	N	Percentage (%)
Age		
• <10	71	19.3%
• 10-19	297	80.7%
Gender		
• Male	235	63.9%
• Female	133	36.1%
Nature of Deafness		
• Prelingual	348	94.6%
• Post lingual	20	5.4%
Pattern of Deafness		
• Unilateral	24	6.5%
• Bilateral	244	93.5%
Degree of hearing loss		
• Mild	13	3.5%
• Moderate	27	7.3%
• Severe	10	2.7%
• Profound	318	86.4%
Family History of Deafness		
• Yes	62	16.8%
• No	306	83.2%
Consanguine marriage		
• Yes	155	42.1%
• No	213	57.9%

5.4 GENETIC ANALYSIS

5.4.1 QUALITY ANALYSIS OF GENOMIC DNA

The quality of extracted genomic DNA was checked by agarose gel electrophoresis. Figure 5.12 showing the good amount of DNA single intact bands without any damage in the samples DNA. DNA smear indicates the damaged DNA (Figure 5.12)

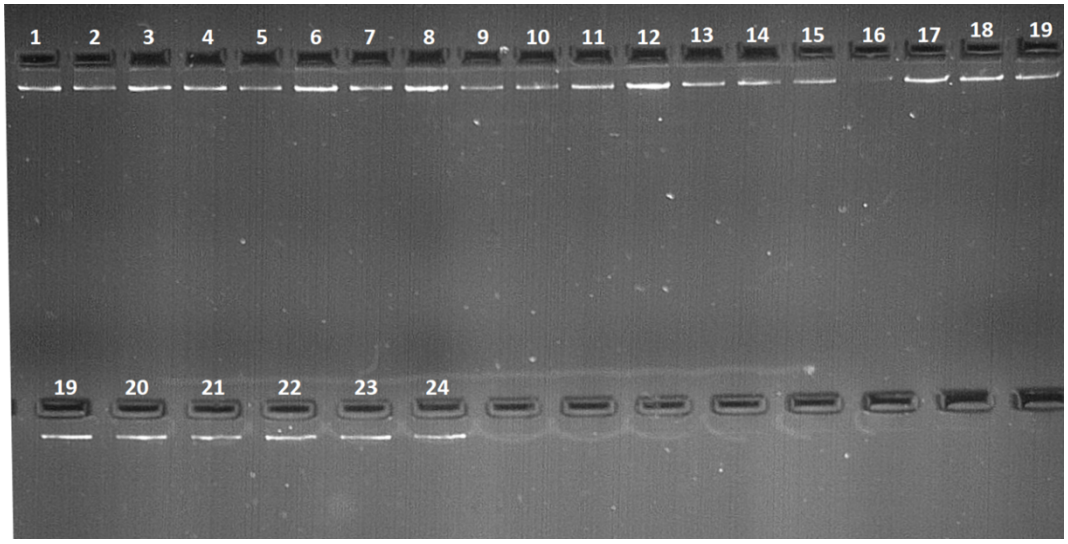


Figure 5.12: Quality analysis of Genomic DNA. Total 24 hearing impaired patient's DNA samples were shown in the gel image.

5.4.2 QUANTIFICATION OF GENOMIC DNA

DNA quantification was done using Nanodrop spectrophotometer and results were good. These samples were further used for the sequence analysis. Table 5.15 represents the quantification results of the isolated DNA samples.

Table 5.15: Quantification results of the hearing-impaired children DNA samples shown in the below table.

Sl. No	ID#	Sample type	A260	A280	260/280	ng/µl
1.	1.	dsDNA	0.285	0.182	1.57	14.2
2.	2.	dsDNA	0.245	0.145	1.69	12.3
3.	3.	dsDNA	0.987	0.834	1.18	49.3
4.	4.	dsDNA	0.365	0.265	1.38	18.3
5.	5.	dsDNA	0.278	0.177	1.57	13.9
6.	6.	dsDNA	0.495	0.355	1.39	24.7
7.	7.	dsDNA	0.245	0.127	1.94	12.3
8.	8.	dsDNA	0.352	0.186	1.89	17.6
9.	9.	dsDNA	0.186	0.099	1.88	19.3
10.	10.	dsDNA	0.265	0.154	1.72	13.2
11.	11.	dsDNA	0.291	0.163	1.79	14.6
12.	12.	dsDNA	0.419	0.223	1.88	21.0
13.	13.	dsDNA	0.278	0.154	1.81	13.9
14.	14.	dsDNA	0.258	0.140	1.84	12.9
15.	15.	dsDNA	0.252	0.163	1.54	12.6
16.	16.	dsDNA	0.272	0.140	1.94	13.6
17.	17.	dsDNA	0.379	0.205	1.85	18.9
18.	18.	dsDNA	0.433	0.289	1.50	21.7
19.	19.	dsDNA	0.258	0.127	2.04	12.9
20.	20.	dsDNA	0.318	0.168	1.90	15.9
21.	21.	dsDNA	0.285	0.136	2.10	14.2
22.	22.	DsDNA	0.272	0.131	2.07	13.6
23.	23.	DsDNA	0.285	0.127	2.25	14.2
24.	24.	DsDNA	0.298	0.145	2.06	14.9

5.4.3 PRIMER STANDERDISATION

Table 5.16 shows the list of primers used for the GJB2 Gene analysis in this study along with the sequence details, base pair size and annealing temperature in degree Celsius. Figure 5.13 to 5.18 showing the gradient PCR results of exonic region primers. Figure 5.19 to 5.25 showing the results of intronic region primers gradient PCR results.

Table 5.16: List of primers designed for the GJB2 gene analysis.

Sl No	Exon/ Intron	Primer ID (DM-EX)	Sequences	Base Pair (BP)	Temp in ^{O}C
1	Exon 1 (EX1)	Forward	CCCTCCGTAACTTTCCCAGT	363	59
		Reverse	CCAAGGACGTGTGTTGGTC		
2	Exon 2 (EX2)	EX2A-Forward	CCTGTTTTGGTGAGGTTGTG	532	60
		EX2A-Reverse	TGGGTTTTGATCTCCTCGAT		
3		EX2B-Forward	CTACTTCCCCATCTCCCACA	532	56.6
		EX2B-Reverse	CCTCATCCCTCTCATGCTGT		
4		EX2C-Forward	GTTTAACGCATTGCCCAGTT	504	50.2
		EX2C-Reverse	GGCACTGGTAACTTTGTCCA		
6		EX2D-Forward	CCAACTTTCCCCACGTTAAA	517	50.3
		EX2D-Reverse	TGGCTACCACAGTCATGGAA		
7		EX2E-Forward	GCACAGCTGAGAGGCTGTCT	439	56.0
		EX2E-Reverse	GCTGAAGGGGTAAGCAAACA		
8		EX2F-Forward	GGGGAGGGAGAAGTTTCTGT	548	57.0

		EX2F-Reverse	AATGGGGTCAGACACTCTGG		
9	Intron (IN)	IN1A-Forward	CTGGACCAACACACGTCCTT	503	59.0
		IN1A-Reverse	GGAAACAGACCCTCGTGAAG		
10		IN1B-Forward	CAGAGATTTGGGCGGAGTT	527	53
		IN1B-Reverse	TCACCAGGATCCAGAAAAGG		
11		IN1C-Forward	TGCACAGTCGGTCACAATTT	526	50.8
		IN1C-Reverse	CCAAACCCAGGTCATACACC		
12		IN1D-Forward	TCAGCTGATGGTAACTGGACA	510	60.0
		IN1D-Reverse	CACCAAGGTCAGGCAGAAAC		
13		IN1E-Forward	TGTTGTCTTTCCCAAGCTCA	549	52.6
		IN1E-Reverse	TCAACTCCCTCGGTTACTGG		
14		IN1F-Forward	CGCTTGCAGTAAGGAGTGTG	519	56.5
		IN1F-Reverse	AGGCTGAGAGGCCAAGTACA		
15		IN1G-Forward	CACTGCTACATGCCACGTCT	507	57.0
		IN1G-Rorward	TCTTCCTGAGCAAACACCAA		

I. DM-EX2A

Amplicon size: 532bp

Marker used (L): 100bp

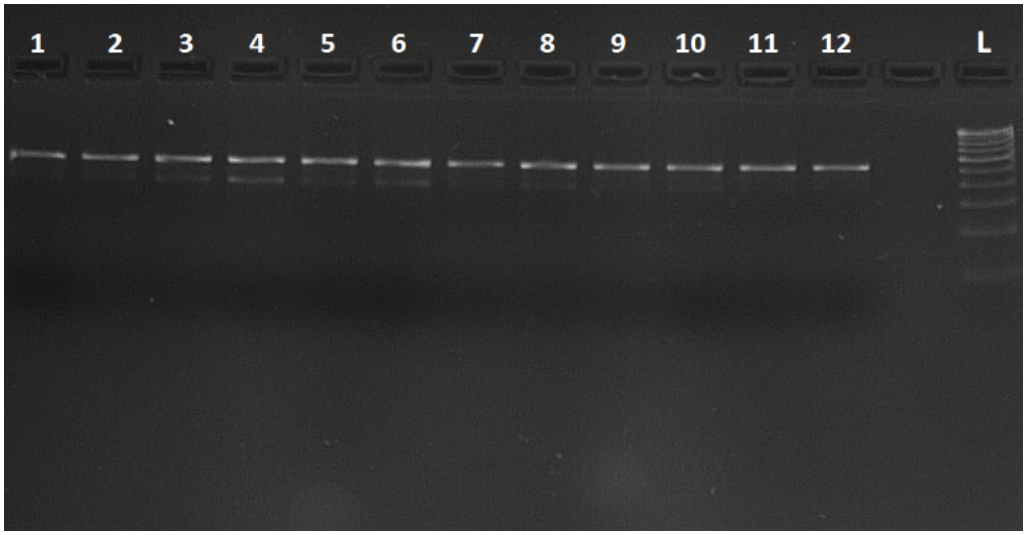


Figure 5.13: Agarose gel electrophoresis results of gradient PCR of DM-EX2A
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=61.0, G= ±4.0

Lane No.1- 59.0,	Lane No.2-59.1,	Lane No.3-59.3,	Lane No.4- 59.7,
Lane No.5- 60.0,	Lane No.6-60.8,	Lane No.7-61.2,	Lane No.8- 61.8,
Lane No.9- 62.3,	Lane No.10-62.7,	Lane No.11- 62.9,	Lane No.12- 63.0

II. DM-EX2B

Amplicon size: 532bp

Marker used (L): 100bp

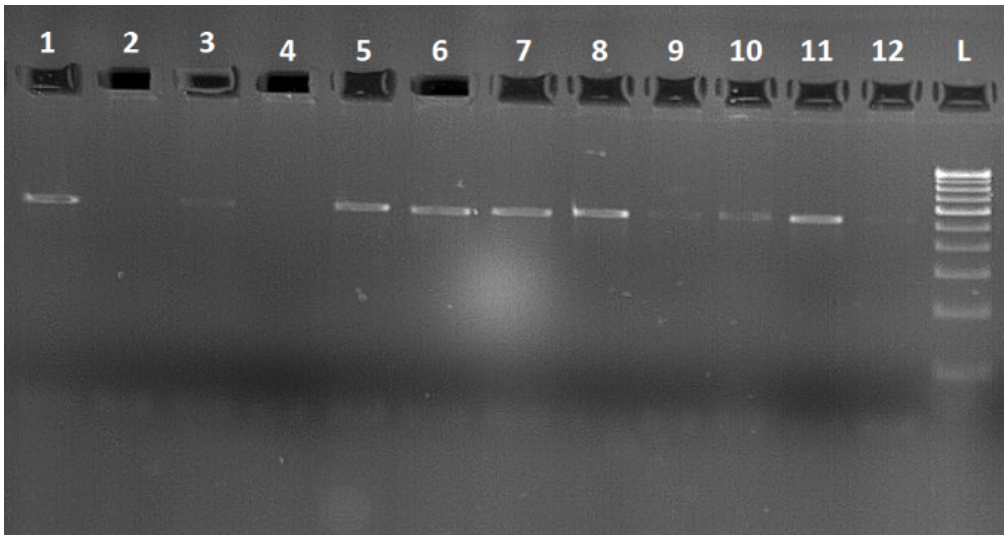


Figure 5.14: Agarose gel electrophoresis results of gradient PCR of DM-EX2B

L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=57.0, G= ±4.0

Lane No.1- 56.0,	Lane No.2-56.1,	Lane No.3-56.3,	Lane No.4- 56.4,
Lane No.5- 56.6,	Lane No.6-56.8,	Lane No.7-57.2,	Lane No.8- 57.8,
Lane No.9- 58.3,	Lane No.10-58.7,	Lane No.11- 58.9,	Lane No.12- 59.0

II. DM-EX2C

Amplicon size: 504bp

Marker used (L): 100bp

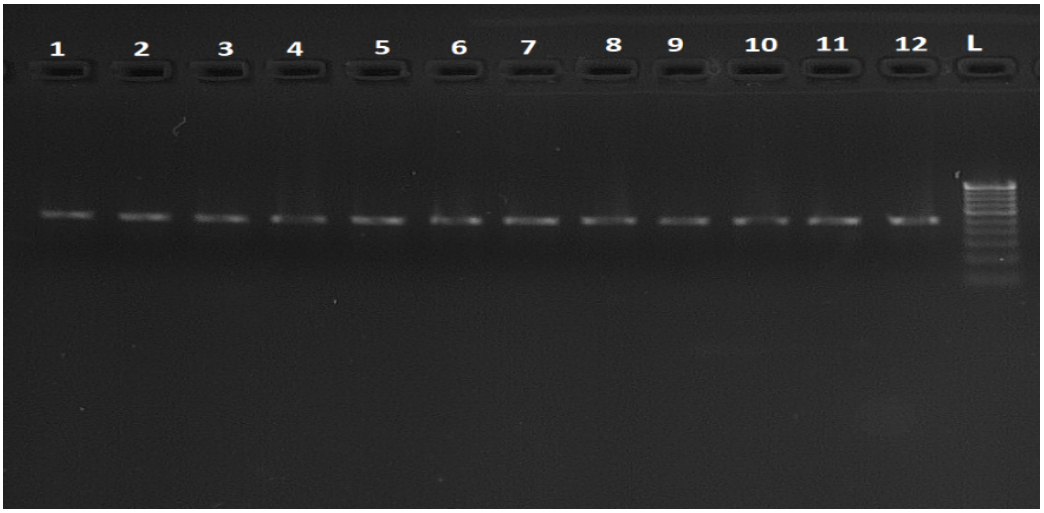


Figure 5.15: Agarose gel electrophoresis results of gradient PCR of DM-EX2C
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=49.9, G= ±0.1

Lane No.1- 49.1,	Lane No.2-49.5,	Lane No.3-49.6,	Lane No.4- 49.7,
Lane No.5- 49.8,	Lane No.6-49.9,	Lane No.7-50.1,	**Lane No.8- 50.2**,
Lane No.9- 50.3,	Lane No.10-50.4,	Lane No.11- 50.5,	Lane No.12- 50.6

II. DM-EX2D

Amplicon size: 517bp

Marker used (L): 100bp

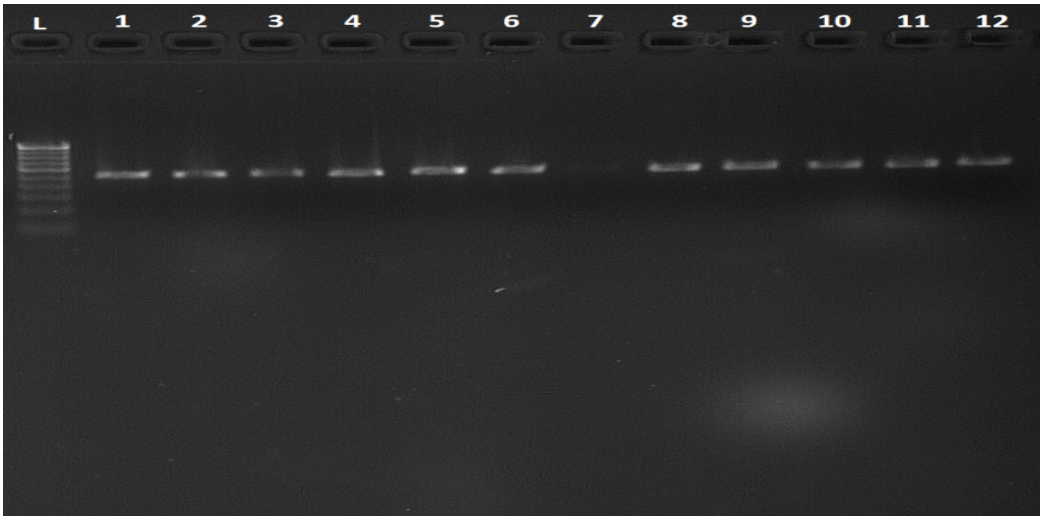


Figure 5.16: Agarose gel electrophoresis results of gradient PCR of DM-EX2D
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=49.9, G= ±0.1

Lane No.1- 49.1,	Lane No.2-49.5,	Lane No.3-49.6,	Lane No.4- 49.7,
Lane No.5- 49.8,	Lane No.6-49.9,	Lane No.7-50.1,	Lane No.8- 50.2,
Lane No.9- 50.3,	Lane No.10-50.4,	Lane No.11- 50.5,	Lane No.12- 50.6

II. DM-EX2E

Amplicon size: 493bp

Marker used (L): 100bp

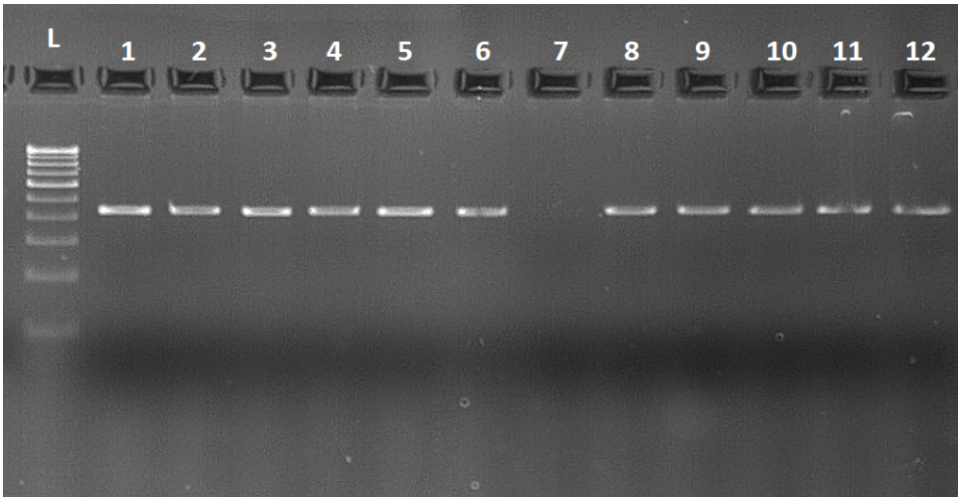


Figure 5.17: Agarose gel electrophoresis results of gradient PCR of DM-EX2E
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=56.7, G= ±4.0

Lane No.1- 56.0,	Lane No.2-56.1,	Lane No.3-56.2,	Lane No.4- 56.3,
Lane No.5- 56.4,	Lane No.6-56.5,	Lane No.7-56.6,	Lane No.8- 56.7,
Lane No.9- 56.8,	Lane No.10-56.9,	Lane No.11- 57.0,	Lane No.12- 57.1

II. DM-EX2F

Amplicon size: 538bp

Marker used (L): 100bp

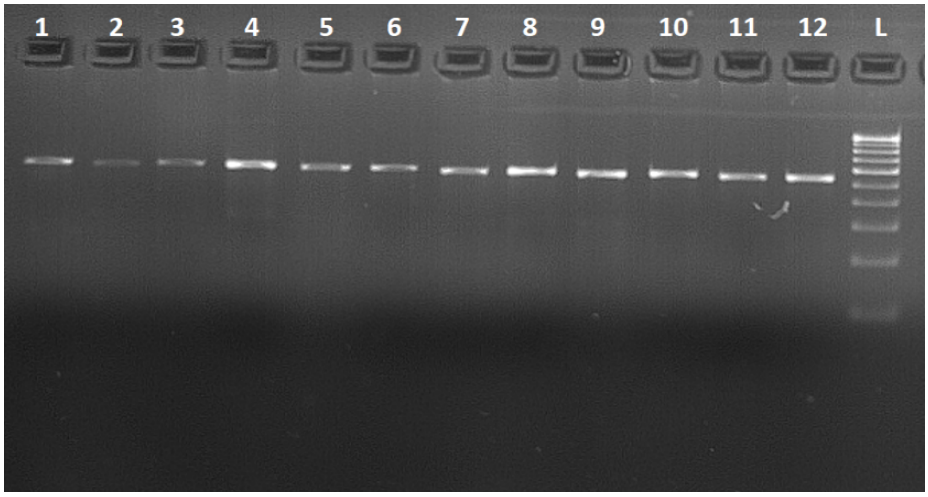


Figure 5.18: Agarose gel electrophoresis results of gradient PCR of DM-EX2F
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=57.0, G= ±4.0

Lane No.1- 56.0,	Lane No.2-56.1,	Lane No.3-56.3,	Lane No.4- 56.7,
Lane No.5- 57.0,	Lane No.6-57.1,	Lane No.7-57.2,	Lane No.8- 57.5,
Lane No.9- 57.7,	Lane No.10-57.8,	Lane No.11- 58.0,	Lane No.12- 58.2

GJB2 INTRON

I. DM-IN,1A

Amplicon size: 503bp

Marker used (L): 100bp

Figure 5.19: Agarose gel electrophoresis results of gradient PCR of DM-IN1A
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=61.0, G= ±4.0

Lane No.1- 59.0,	Lane No.2-59.1,	Lane No.3-59.3,	Lane No.4- 59.7,
Lane No.5- 60.2,	Lane No.6-60.8,	Lane No.7-61.2,	Lane No.8- 61.8,
Lane No.9- 62.3,	Lane No.10-62.7,	Lane No.11- 62.9,	Lane No.12- 63.0

II. DM-IN,1B

Amplicon size: 527bp

Marker used (L): 100bp

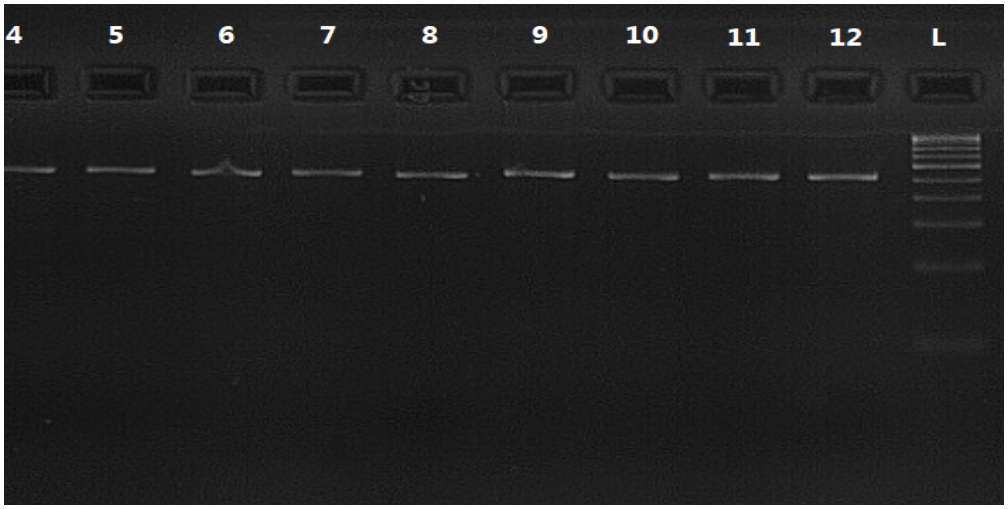


Figure 5.20: Agarose gel electrophoresis results of gradient PCR of DM-EX1B
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=52.0, G= ±4.0

Lane No.1- 50.5,	Lane No.2-50.6,	Lane No.3-50.8,	Lane No.4- 51.0,
Lane No.5- 51.4,	Lane No.6-51.8,	Lane No.7-52.2,	Lane No.8- 52.6,
Lane No.9- 53.0,	Lane No.10-53.2,	Lane No.11- 53.4,	Lane No.12- 53.5

III. DM-IN,1C

Amplicon size: 526bp

Marker used (L): 100bp

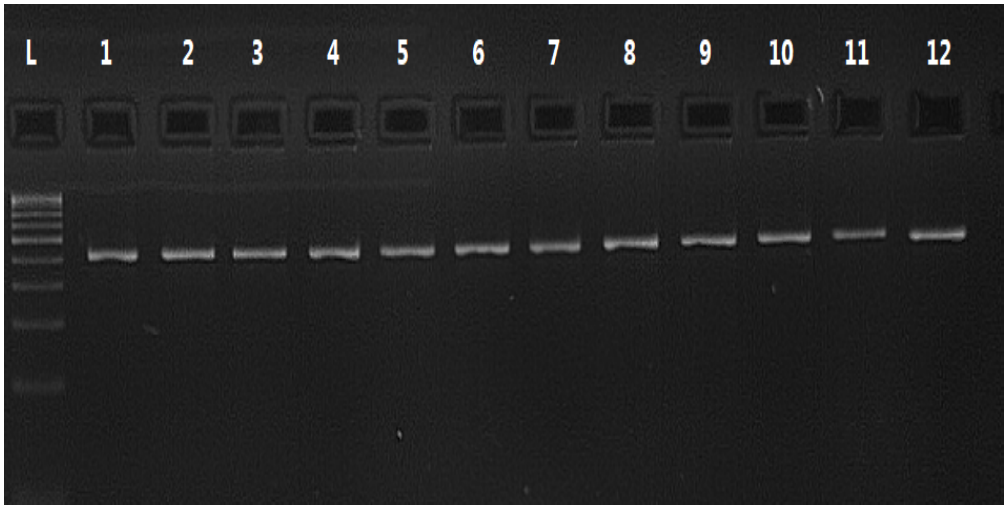


Figure 5.21: Agarose gel electrophoresis results of gradient PCR of DM-EX1C
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=52.0, G= ±4.0

Lane No.1- 50.5,	Lane No.2-50.6,	**Lane No.3-50.8**,	Lane No.4- 51.0,
Lane No.5- 51.4,	Lane No.6-51.8,	Lane No.7-52.2,	Lane No.8- 52.6,
Lane No.9- 53.0,	Lane No.10-53.2,	Lane No.11- 53.4,	Lane No.12- 53.5

IV. DM-IN,1D

Amplicon size: 510bp

Marker used (L): 100bp

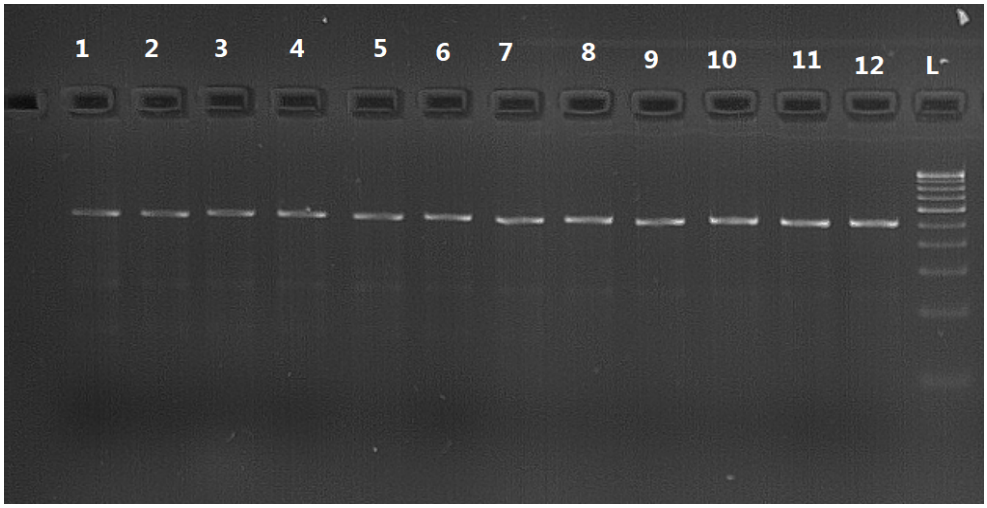


Figure 5.22: Agarose gel electrophoresis results of gradient PCR of DM-EX1D
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.5, G= ±4.0

Lane No.1- 54.0,	Lane No.2-54.1,	Lane No.3-54.3,	Lane No.4- 54.5,
Lane No.5- 54.9,	Lane No.6-55.3,	Lane No.7-55.7,	Lane No.8- 56.1,
Lane No.9- 56.5,	Lane No.10-56.7,	**Lane No.11- 56.9**,	Lane No.12- 57.0

V. DM-IN,1E

Amplicon size: 549bp

Marker used (L): 100bp

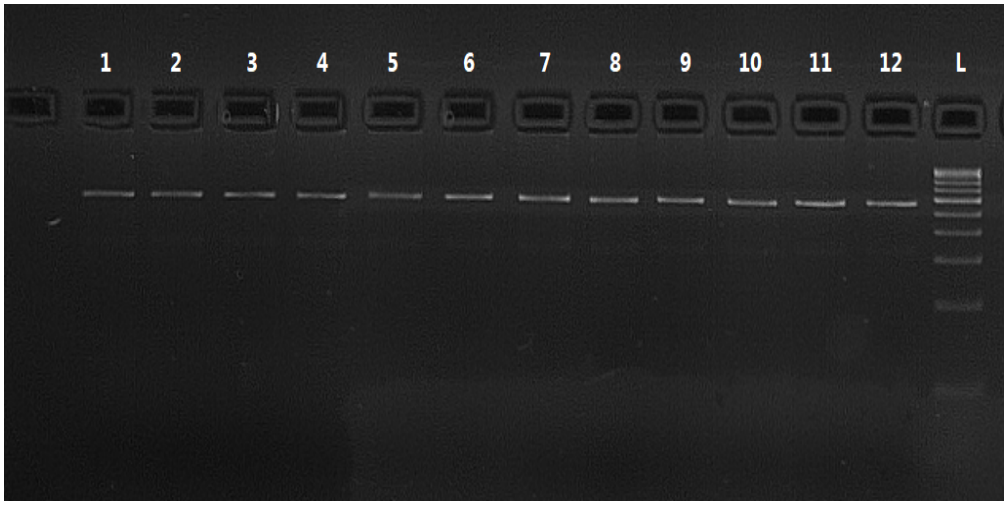


Figure 5.23: Agarose gel electrophoresis results of gradient PCR of DM-EX1E
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=52.0, G= ±4.0

Lane No.1- 50.5,	Lane No.2-50.6,	Lane No.3-50.8,	Lane No.4- 51.0,
Lane No.5- 51.4,	Lane No.6-51.8,	Lane No.7-52.2,	**Lane No.8- 52.6**,
Lane No.9- 53.0,	Lane No.10-53.2,	Lane No.11- 53.4,	Lane No.12- 53.5

VI. DM-IN,1F

Amplicon size: 519bp

Marker used (L): 100bp

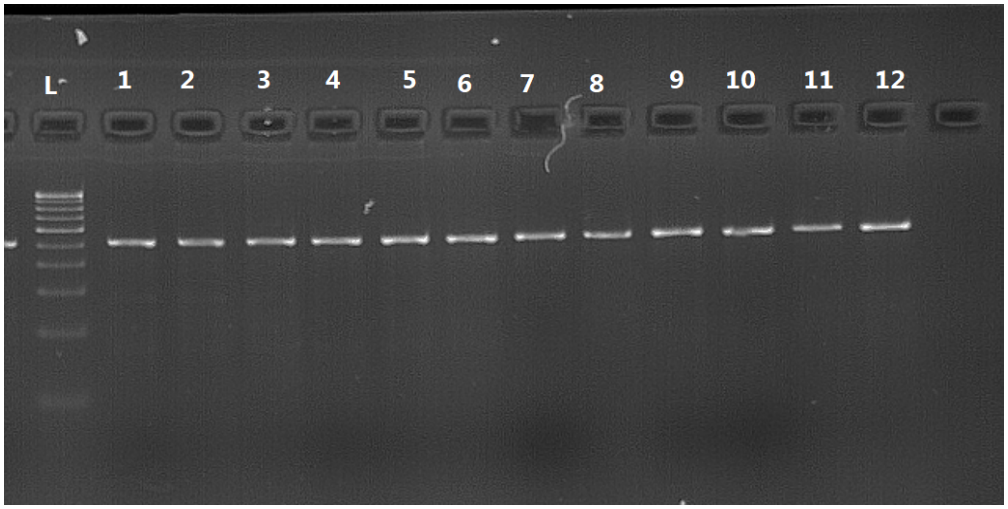


Figure 5.24: Agarose gel electrophoresis results of gradient PCR of DM-EXIF
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.5, G= ±4.0

Lane No.1- 54.0,	Lane No.2-54.1,	Lane No.3-54.3,	Lane No.4- 54.5,
Lane No.5- 54.9,	Lane No.6-55.3,	Lane No.7-55.7,	Lane No.8- 56.1,
Lane No.9- 56.5,	Lane No.10-56.7,	Lane No.11- 56.9,	Lane No.12- 57.0

VII. DM-IN,1G

Amplicon size: 5407bp

Marker used (L): 100bp

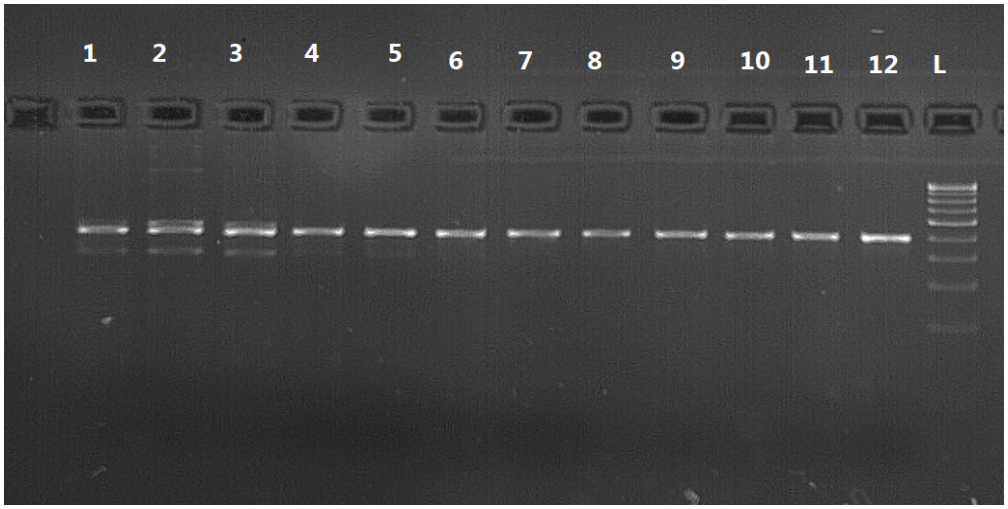


Figure 5.25: Agarose gel electrophoresis results of gradient PCR of DM-EX1G
L- 100bp Marker, Lane No. 1 – 12 PCR product from the different temperatures in degree Celsius.

T_m=55.5, G= ±4.0

Lane No.1- 54.0,	Lane No.2-54.1,	Lane No.3-54.3,	Lane No.4- 54.5,
Lane No.5- 54.9,	Lane No.6-55.3,	Lane No.7-55.7,	Lane No.8- 56.1,
Lane No.9- 56.5,	Lane No.10-56.7,	Lane No.11- 56.9,	**Lane No.12- 57.0**

5.4.4 MOLECULAR SCREENING

For this study, 613 NSRD children from the North Karnataka community were tested. 16.8% (62/368) of the 368 hearing-impaired kids we included (n_{male} = 235, n_{female} = 133) had a history of deafness. All the probands being students of deaf schools were essentially prelingually deaf, which was a pre-requisite for an admission in schools for the HI. Based on the audiological documentation results, 86.4% were bilaterally profound, 2.7% were severe and 7.3% were with moderate HL. In our study, 115 children's parents were married in the close relatives (115/368). We identified 18 mutations in the exonic (9 mutation) and intronic regions (9 variants) of the GJB2 gene. W24X, R127H, and W77X variants were the mutations that were discovered most often in this analysis. In our study, we also recorded 3 missense mutations, namely R127H, V153I, and I33T. Four 3'-UTR variants were identified (c.84T>C, c.1067G>T, c.1277T>C, c.1152G>A) (Table 5.17).

5.4.4.1 g.8465(c.71G>A) or p.W24X mutation

The G>A transition at nucleotide position 71 in the TM1 domain is what caused this nonsense mutation. When harmful mutations and polymorphisms in the connexin 26 gene are considered, the highest and most frequent mutation is p.W24X. variant Connexin 26's p.24 (W24X) stop codon, caused by a G>A transition at codon position c.71, results in a shortened protein that is one-tenth the length of the wild-type protein.

In the 788 chromosomes tested, the allele frequency of this harmful mutation is 15.99%. The GJB2 gene function is completely lost as a result of premature stop codons. W24X (25%) is one of the frequently occurring mutations found in the research group. For this mutation, 86 (23.3%) deaf children were homozygous and 6 (1.6%) were heterozygous. Figure 5.26 showing the e Electropherogram of p.W24X mutation recorded.

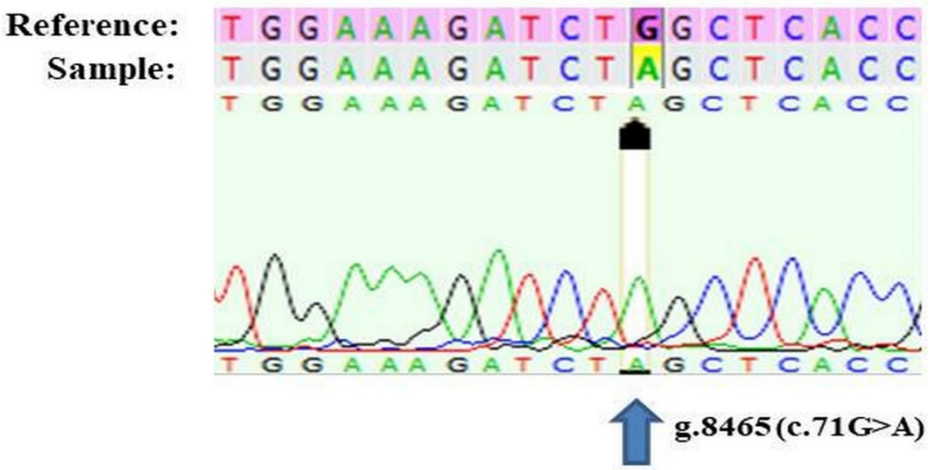


Figure 5.26: Electropherogram image of the p.W24X mutation

5.4.4.2 g.8625(c.231G>A) or p.W77X mutation

Due to a G>A transition at nucleotide position 231 in the TM2 domain, which results in a change from tryptophan to a stop codon in the protein, p.W77X is the second most frequent pathogenic, nonsense mutation. The frequency of the 788 examined chromosomes for the p.W77X allele is 0.63%. In all, 4.8% of the 368 HI that were examined had this mutation. One person (0.25%) has a compound heterozygous condition with another harmful mutation (p.W24X/p.W77X), while two people (0.51%) are homozygous. Figure 5.27 showing the Electropherogram of p.W77X mutation.

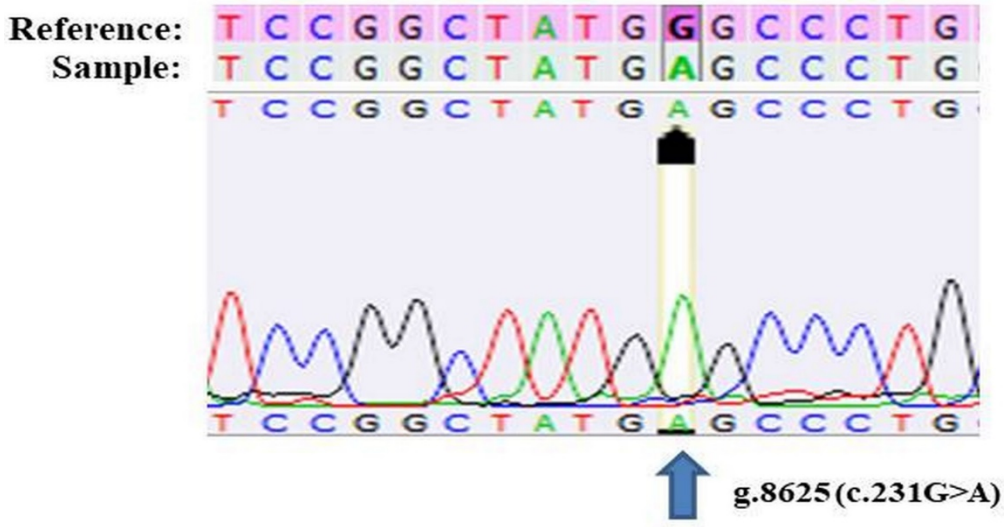


Figure 5.27: Electropherogram image of the p.W77X mutation

5.4.4.3 g.8774(c.380G>A) or p. R127H Polymorphism

At nucleotide position 380 in the IC2 domain, a G>A transition is responsible for this alteration. The second most frequent alteration in the connexin 26 gene among the HI is the p.R127H polymorphism, with an allele frequency of 14.21% in the 788 chromosomes examined. Additionally, we identified three missense mutations in our study: R127H, V153I, and I33T. Compared to the other two detected missense variants, the R127H mutation occurred at a higher frequency (14.9%) among deaf children. The remaining offspring were homozygous for the R127H polymorphism, whereas two of the children were heterozygous. This dominant mutation results from a change from G>A at position 224 in the protein's EC1 domain. This transformation causes Glutamine to replace Arginine in the 75th position in the protein chain. Figure 5.26 showing the Electropherogram of p.R127H mutation recorded.

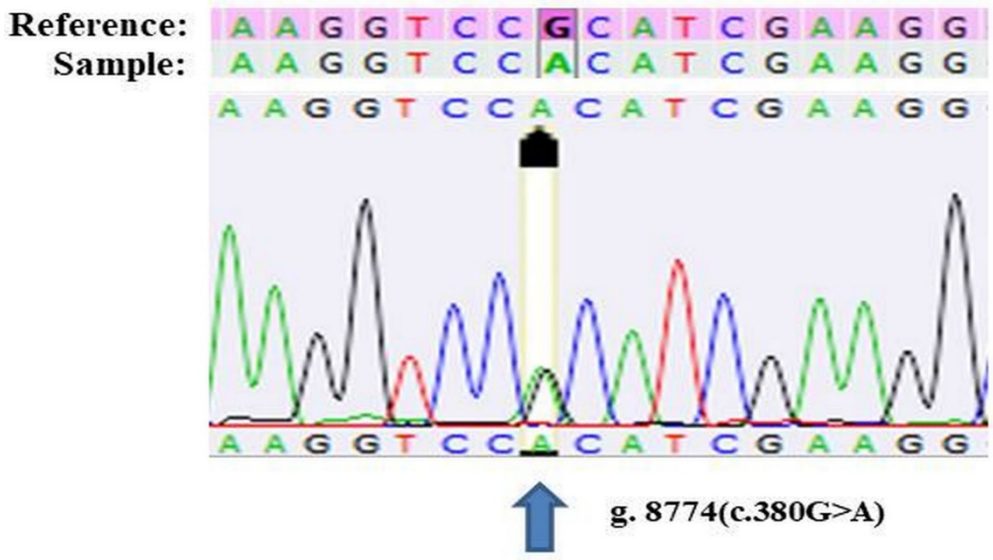


Figure 5.28: Electropherogram image of the p. R127H mutation

5.4.4.4 g.8851(c.457G>A) or p.I33T mutation

The missense pathogenic mutation p.I33T results from a change from T>C at position 98, which causes Isoleucine to convert to Threonine in the protein's TM1 domain. This mutation is present in a homozygous state in just one person. Figure 5.26 showing the Electropherogram of p.I33T mutation.

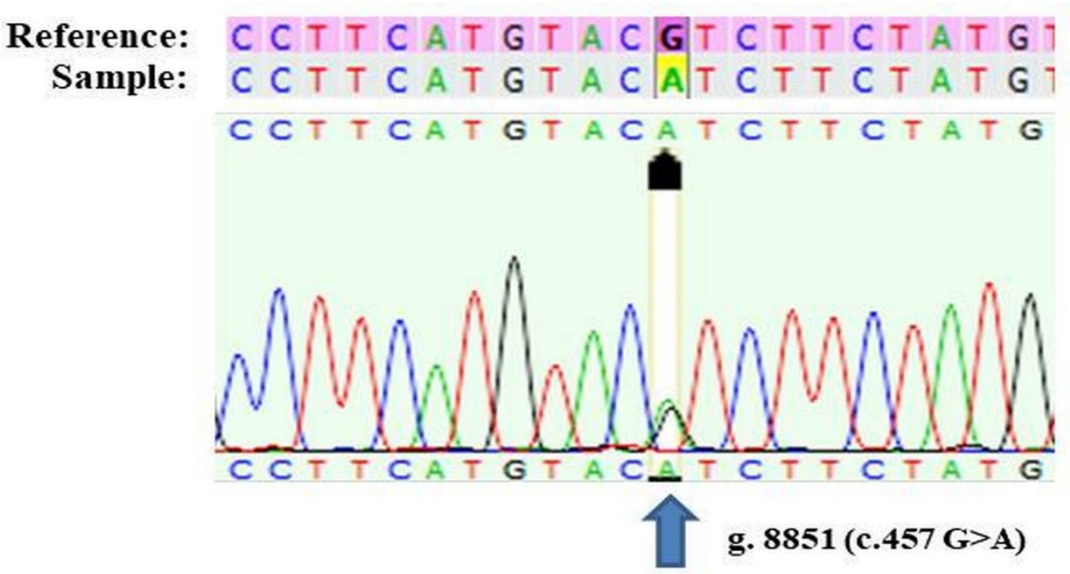


Figure 5.29: Electropherogram image of the p.I33T mutation

5.4.4.5 g.8492(c.98T>C) or p.V153I polymorphism

This results from a Valine to Isoleucine alteration in the connexin protein caused by the G>A transition at position 153 in the TM3 domain. This polymorphism occurs on 788 chromosomes with a frequency of 3.43%. Among all the HI probands tested, p.V153I accounts for around 4.9% of the third most frequent nucleotide alteration. For p.V153I, exclusive homozygosity is not present. 2.84% are compound heterozygous, while 3.55% are heterozygous. Figure 5.26 showing the Electropherogram of p.V153I mutation.

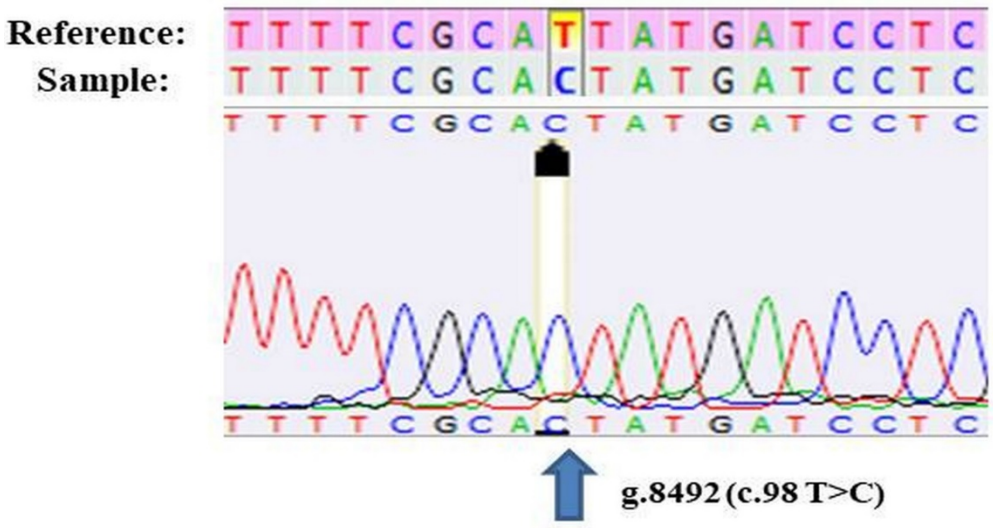


Figure 5.30: Electropherogram image of the p.V153I mutation

Figure 5.31 showing the graphical representation of the total mutations were recorded in our study cohort. Which is also showing the mutation record region on the GJB2 gene.

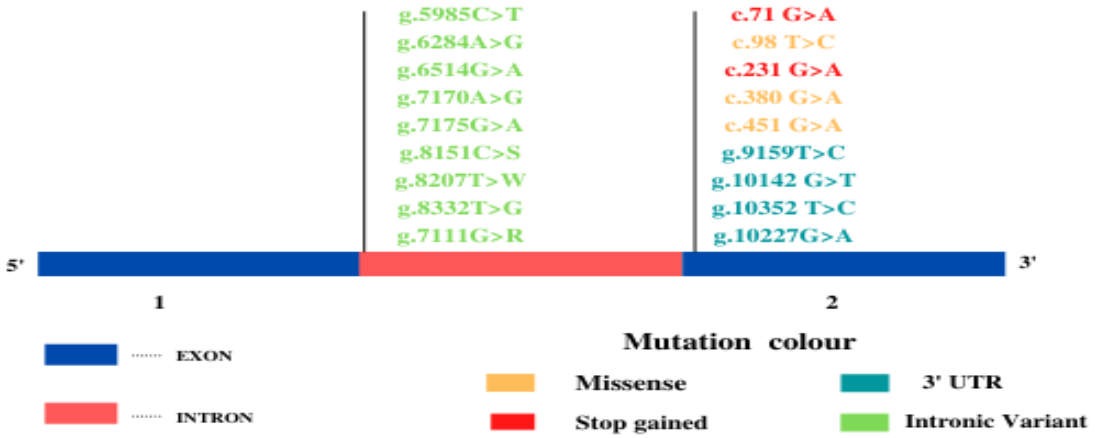


Figure 5.31: Graphical representation of mutations recorded in the *GJB2* gene of the present study cohort.

5.4.5 Distribution of the *GJB2* gene mutations among the structural domains of the Connexin protein

Table 5.17 displays each mutation's nucleotide and amino acid alterations, as well as its pathogenicity and the protein structural domain that was impacted in coding and non-coding region of GJB2 gene. In this study, 18 distinct nucleotide changes were discovered. Each variant is characterized by nucleotide substitutions. Four of the 18 alterations are deleterious, including three missense mutations and two non-sense mutations (P.W24X and p.W77X) (p.I33T, p.R127H, and p.V153I). The remaining 9 UTR region differences outnumber the remaining 4 regulatory area variations. Out of the 18 substitutions, four are transversions and nine are transitions (purine to purine or pyrimidine to pyrimidine) (purine to pyrimidine or vice-versa).

Table 5.17: List of mutations identified in the coding sequence of *GJB2* gene in the present study.

Sl. No	Nucleotide change	AA Change	Protein domain	Frequency	Phenotype	Description and type of effect
1	c.71 G>A	W24X	TM1	25%	Congenital profound hearing loss	Stop Gained
2	c.98 T>C	I33T	TM1	5.2%	Congenital profound hearing loss	Missense variant
3	c.231 G>A	W77X	TM2	4.8%	Congenital severe hearing loss	Stop gained
4	c.380 G>A	R127H	IC2	14.9%	Congenital severe hearing loss	Missense
5	c.451 G>A	V153I	TM3	4.9%	Congenital profound hearing loss	Missense
6	g.9159T>C	---------		100%	Congenital profound hearing loss	3'-UTR Variant
7	g.10142 G>T	---------		100%	Congenital severe hearing loss	3'-UTR Variant
8	g.10352 T>C	---------		100%	Congenital profound hearing loss	3'-UTR Variant
9	g.10227G>A	---------		5.1%	Congenital severe hearing loss	3'-UTR Variant

5.4.6 SCREENING OF NON CODING REGION (EXON 1) OF THE GJB2 GENE

All 368 HI children with the GJB2 gene were sequenced to screen for harmful mutations in exon 2 in trans condition in the non-coding region (exon 1). In this area, none of them had undergone any alterations.

5.4.6.1 Intronic variants

In addition to these exonic variants, we have also recorded 9 variants in the intronic region of the *GJB2* gene (Table 5.18), but these variants are unlikely to be causative.

Table 5.18: list of mutations identified in the intronic region of the *GJB2* gene in the present study.

Sl. No	Nucleotide change	Frequency	Phenotype	Description & type of effect
1	g.5985C>T	90%	Congenital profound HI	Regulatory region Variant
2	g.6284A>G	90%	Severe to profound HI	Regulatory region Variant
3	g.6514G>A	14.94%	Congenital profound HI	Regulatory region Variant
4	g.7170A>G	100%	Moderate HI	Regulatory region Variant
5	g.7175G>A	25%	Severe HI	Regulatory region Variant
6	g.8151C>S	4.9%	Severe HI	--------
7	g.8207T>W	14.94%	Congenital profound HI	--------
8	g.8332T>G	30%	Congenital Severe to profound HI	--------
9	g.7111G>R	14%	Congenital profound HI	---------

5.5 INSILICO ANALYSIS

To comprehend how mutations cause disease, it is crucial to understand how they alter structural and functional features. Knowing the molecular effects of GJB2 mutations on protein structure may also help us better understand the molecular origins of deafness as mutations in the GJB2 gene are the most frequent cause of hearing loss. Investigating the structural and functional implications of all known GJB2 missense mutations on the Cx26 protein using a variety of bioinformatics tools is one of the goals of this work.

5.5.1 Pathogenicity prediction

Insilco pathogenicity prediction of missense variants was predicted to evaluate the deleterious/harmful effect on the functions of GJB2 protein by PolyPhen2, PROVEAN, PANTHER, SNP&GO, PHD-SNP, DVD& CADD and SNAP2 (Table 5.19).

Table 5.19: Pathogenicity prediction of the missense variant by in silico tools

Variants	SNAP2	PolyPhen 2	PhD-SNP	SNPS & GO	DVD & CADD	PROVEAN	Panther
p.I33T	Effect Score:55	Possibly damaging Score:0.79	Disease P:0.548	Disease P: 0.548	Pathogenic 25.2	Deleterious Score: -3.72	Possibly damaging
p.R127H	Effect Score:1	Benign Score:0.01	Disease P:0.658	Disease P: 0.589	Benign 23.2	Neutral Score: -0.78	Possibly damaging
p.V153I	Neutral Score: 74	Benign Score:0.03	Neutral P:0.149	Neutral P: 0.083	Benign 23.4	Neutral Score: -0.20	Possibly damaging

Abbreviations: CADD, combined annotation dependent depletion.

- PROVEAN prediction tool predicted p.R127H and p.V153I variants have a neutral effect on the function of connexin 26 protein with a score of 0.78 and 0.20 respectively. p.I33T missense variant was predicted to cause a deleterious effect on the connexin 26 protein with the score of 3.72 (“Deleterious” if the prediction score was </ -2.5 and “Neutral” if the prediction score was >/-2.5).
- PHD-SNP pathogenicity prediction showed p.V153I missense variant has a neutral effect. p.I33T & p.R172H missense variants were predicted to cause deleterious effect on the connexin 26 protein with the score of 0.548 &0.658 respectively.
- SNP&GO prediction tool predicted, showed p.V153I missense variant has a neutral effect. p.I33T & p.R127H missense variants were predicted to cause deleterious effects on the connexin 26 protein with the score of 0.548 &0.589 respectively.
- Polyphen-2 predicted that only the p.I33T variant has a disease-causing effect with a score of 0.79 and remaining variants such as p.R127H and p.V153I have a neutral effect on the function of connexin 26 protein (Probably damaging” is the most disease-causing ability with a score near to 1. “Possibly damaging” is less disease-causing ability with a score of 0.5–0.8. “Benign” which does not alter protein functions with a score closer to zero).
- SNAP2 prediction tool also predicted that only the p.V153I variant had a neutral effect with a score of 74 and reaming variants such as p.I33T & p.R127H had a deleterious effect on the function of connexin 26 protein (“Neutral” if the score lays 0 to - 100. “Effect” if the score lays 0 to 100).
- PANTHER prediction tool predicted that all the 3 missense mutations have a possibly damaging role on connexin 26 protein. ("probably damaging" (time > 450my, corresponding to a false positive rate of ~0.2 as tested on HumVar), "possibly damaging" (450my > time > 200my, corresponding to a false positive rate of ~0.4) and "probably benign" (time < 200my).
- DVD& CADD prediction tool also predicted that only the p.I33T variant had a damaging effect with a score of 25.2 and reaming variants such as p.R127H & p.V153I has a neutral effect on the function of connexin 26 protein CADD predicts a continuous phred-like score that ranges from 1 to 99, higher values indicating more deleterious cases).

5.5.2 TRANSMEMBRANE STRUCTURE ANALYSIS

Nucleotide changes were seen in seven of the protein's nine structural domains. N- and C-terminal domains of the protein remain unaltered (Figure 5.32). Many mutations are found in the protein's TM1 (P.W24X, p.I33T), and EC2 (p.V153I) domains (two). Only one polymorphism (p.R127H) was discovered in the CL domain. Another damaging mutation (p.W77X) has been discovered in the TM2 domain. On the other hand, there were no alterations in the EC1, TM3, or TM4 domains.

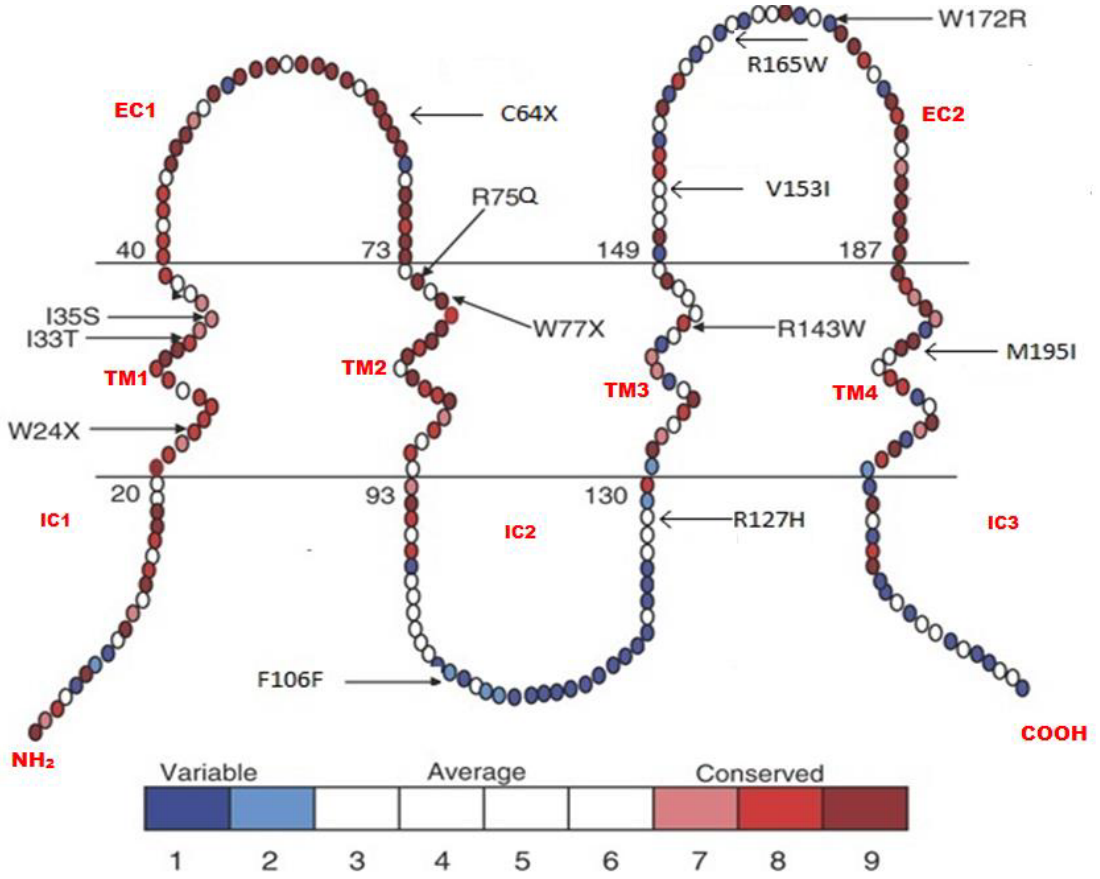


Figure 5.32: Red arrow marks on the Connexin 26 protein point to the locations of the mutations/variants discovered in this study. Colour coding indicates the degree of amino acid conservation. The blue-hued residues (1-2) are not preserved and are quickly evolving. White residues (3-6) have a fair amount of conservation, while red residues (7-9) are both well conserved and slowly evolving[2].

5.5.3 PROTEIN STABILITY PREDICTION

The effect of missense mutation on the stability of the Connexin 26 protein was predicted using the I-Mutant 3.0v tool. Prediction results showed the following results;

- Substitution of Arginine to Histidine at 127 residue (p.R127H) decreases the protein stability with DDG Value Prediction: 0.97 Kcal/mol (Figure 5.33)
- Substitution of Isoleucine to Threonine at 33 residue (p.I33T) decreases the protein stability with DDG Value Prediction: 1.99 Kcal/mol (Figure 5.34)
- Substitution of Valine to Isoleucine at 153 residue (p.V153I) decreases the protein stability with DDG Value Prediction: 0.76Kcal/mol (Table5. 20) (Figure 5.35)

Table 5.20: Connexin 26 Protein stability Prediction over missense mutations

Mutation	WT	New	pH	Temp	SVM2 Prediction Effect	RI	DDG Value
p.R127H	R	H	7.0	25	Decrease	7	0.97 Kcal/mol
p.I33T	I	T	7.0	25	Large decrease	8	1.99 Kcal/mol
p.V153I	V	I	7.0	25	Decrease	7	0.76 Kcal/mol

Note: DDG<-0.5: Large Decrease of Stability, DDG>0.5: Large Increase of Stability, 0.5<=DDG<=0.5: Neutral Stability RI: Reliability Index. WT: amino acid in Wild-Type Protein, NEW: New Amino acid after Mutation, Temp: Temperature in Celsius unit

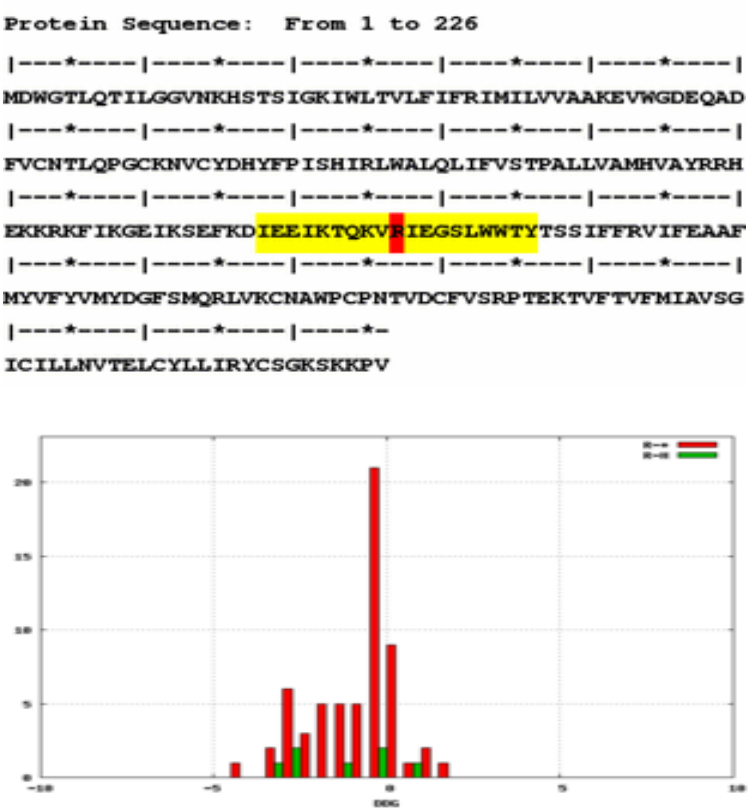


Figure 5.33: CONNEXIN 26 Protein stability prediction over p.R127H missense mutation

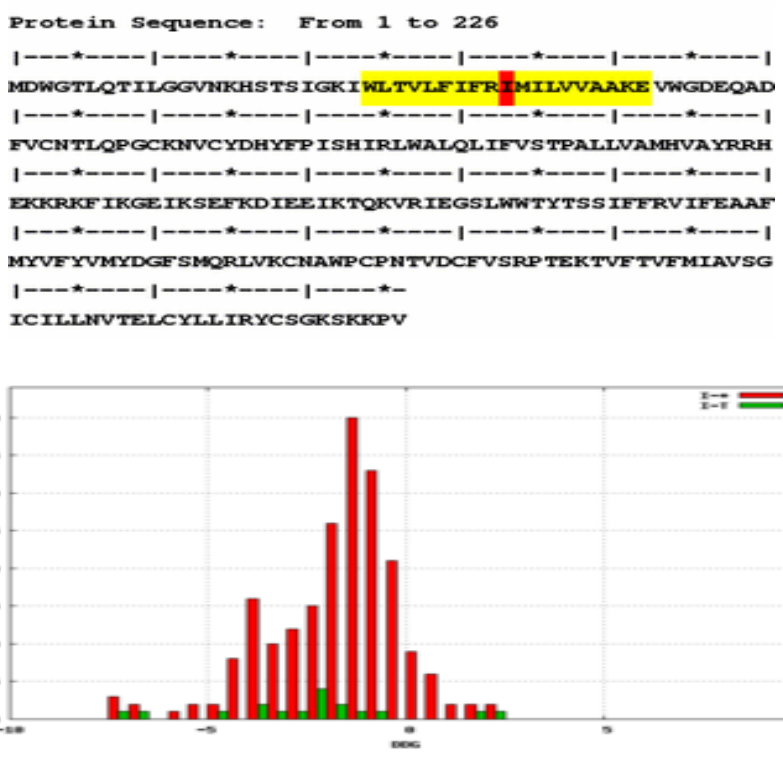


Figure 5.34: CONNEXIN 26 Protein stability prediction over p.I33T missense mutation

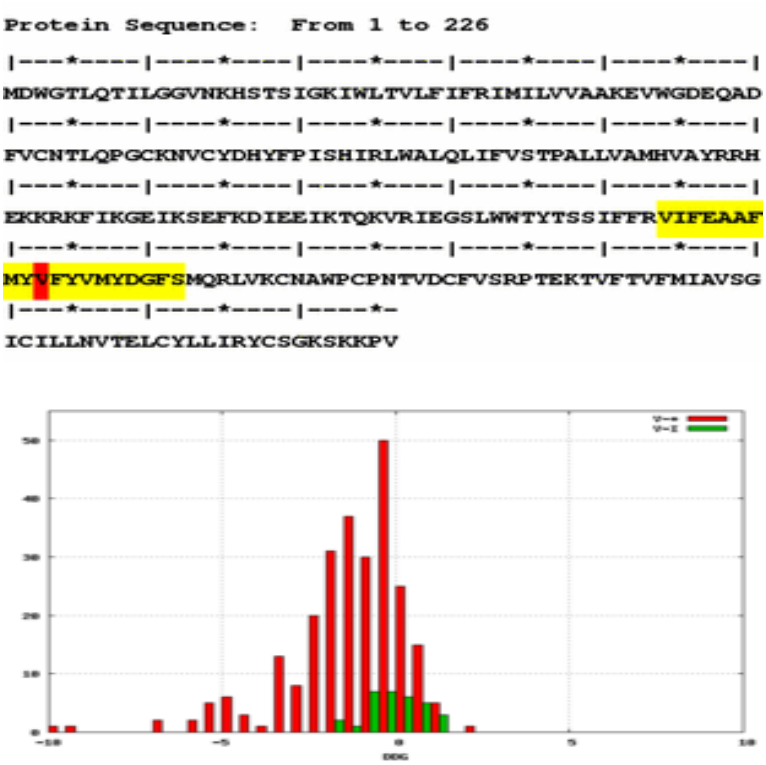


Figure 5.35: CONNEXIN 26 Protein stability prediction over p.V153I missense mutation

5.5.4 MULTIPLE SEQUENCE ALIGNMENT

Multiple sequence alignment analysis for connexin 26 protein was performed to find the sequence homology between the common ancestors, which also revealed whether they descended from the same/common ancestor[2]. Uniport accession numbers from different species were used for the analysis as follows- Xenla ***Q7ZYG3***, Mouse ***Q009777***, Rat ***P21994***, Sheep ***P46691***, Macau ***Q8MIT8***, Human ***P29033***, Congro ***Q8MHW5***, Calf ***J9NXR.*** Multiple sequence alignment of the connexin 26 protein was analysed using the Clustal omega. Mutation residue Isoleucine at 33 and Arginine at 127 were highly conserved over different species and mutation residue Valine 153 was semi-conserved. (Figure 5.36)

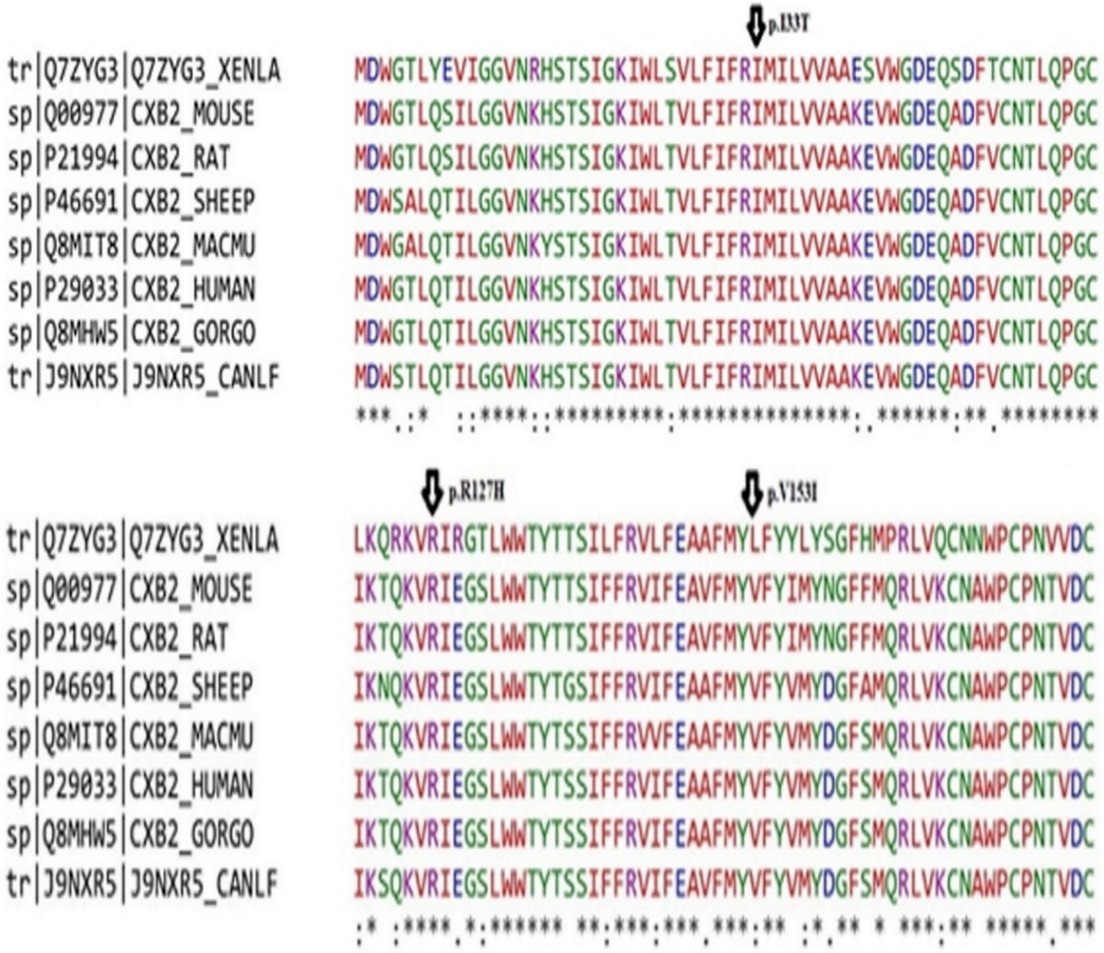
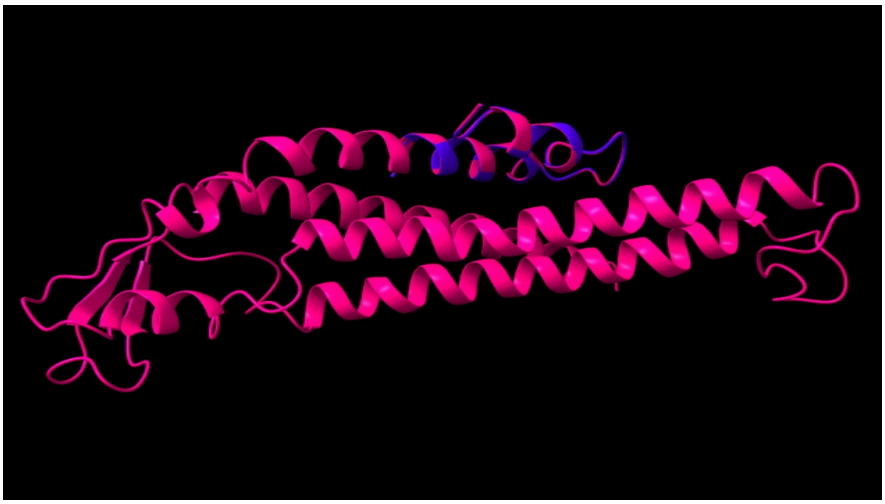


Figure 5.36: Multiple sequence alignment of connexin 26 protein A. first arrow (from left to right) showing I33T residue conservation B. R127H, and V153I residue conservation.

5.5.5 PROTEIN STRUCTURE PREDICTION

The 3D models for mutated Connexin 26 protein for two nonsense mutations (W24X and W77X) were generated using SWISS-MODEL. In the overlaid model, the majority of the connexin 26 protein sequences from both mutant proteins have been lost (Figure 5.37). The first transmembrane (S1) domain and second transmembrane (S2) domain of Connexin 26 both contain residues W24 and W77, respectively.

A



B

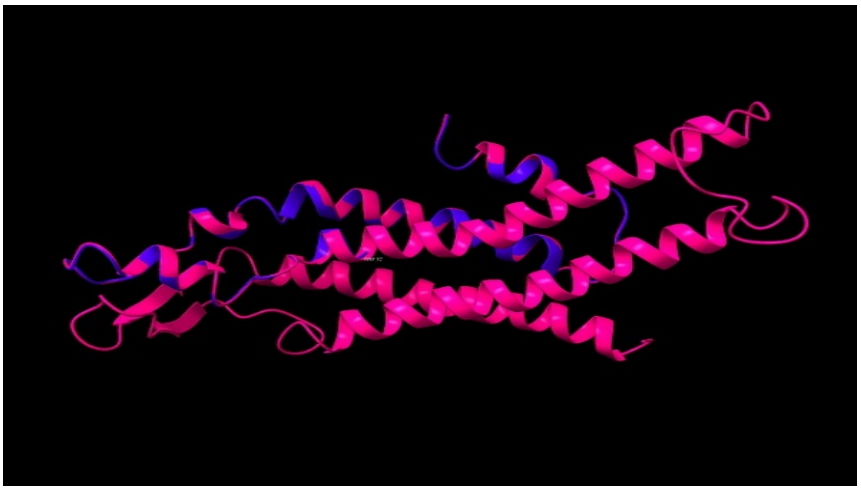


Figure 5.37: Connexin 26, the 3D structure of the protein. A. Superimposed connexin 26 protein model with wildtype and truncated protein (Blue- W24 truncated protein, Red- wild type protein), B. superimposed protein structure W77 truncated and wild type protein (Blue- W77 truncated protein, Red- wild type)

5.5.5.1 THE 3D MODELS FOR MUTATED CONNEXIN 26 PROTEINS FOR MISSENSE MUTATIONS

R12H

Every amino acid is unique in terms of its size, charge, and hydrophobicity. The characteristics of the original wild-type residue and the newly inserted mutant residue frequently diverge.

- When compared to the wild-type residue, the mutant residue is smaller.
- The mutant residue charge is NEUTRAL whereas the wild-type residue charge was POSITIVE. (Figure 5.38)

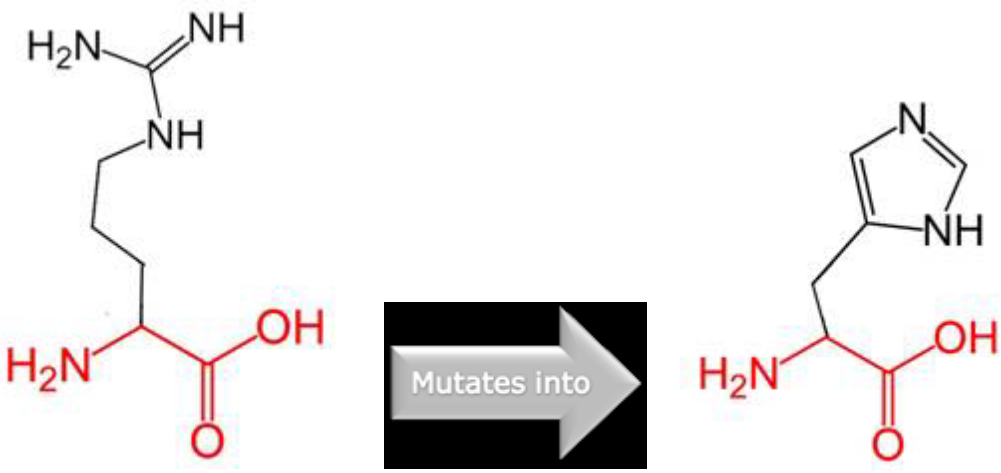
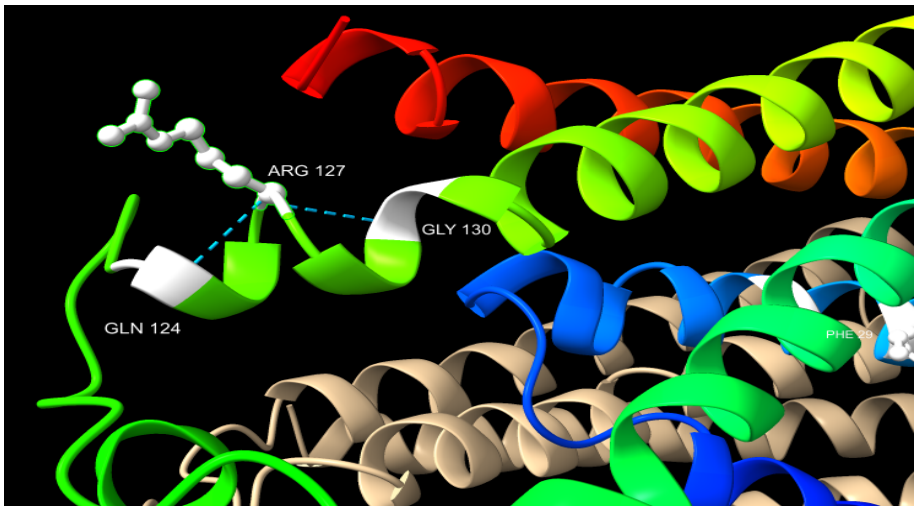


Figure 5.38: The figure shows the schematic structures of the original (left) and the mutant (right) amino acid. The backbone, which is the same foreach amino acid, is coloured red. The side chain, unique for each amino acid, is coloured black.

Figure 5.39 represents the 3D model of missense mutation R127H. A- Wild Type, B-Mutant. The mutant residue is smaller than the wild-type residue also wild-type residue charge was POSITIVE, and the mutant residue charge is NEUTRAL. This can cause loss of interactions with other molecules. This will cause a possible loss of external interactions.

A



B

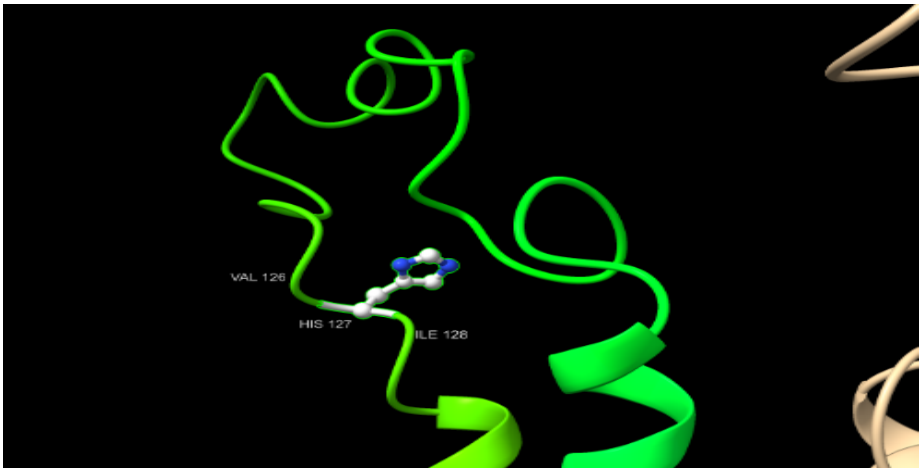


Figure 5.39: The 3D model of missense mutation R127H.

I33T

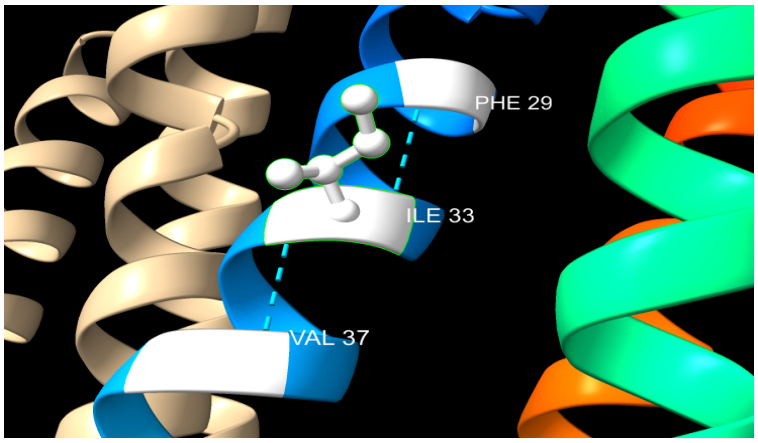
Every amino acid is unique in terms of its size, charge, and hydrophobicity. These characteristics frequently vary between the original wild-type residue and the newly inserted mutant residue. Protein Characteristics (Figure 5.40)

- The wild-type residue is more hydrophobic than the mutant residue, and the mutant residue is smaller.
- The sizes of the mutant and wild-type amino acids vary.
- When compared to the wild-type residue, the mutant residue is smaller.
- This might result in a decrease in interactions with the outside world.
- The hydrophobicity of the mutant and wild-type residues varies.
- The protein's surface hydrophobic contacts with other molecules may be lost as a result of the mutation.

Figure 5.40: The figure above shows the schematic structures of the original (left) and the mutant (right) amino acid. The backbone, which is the same foreach amino acid, is coloured red. The side chain, unique for each amino acid, is coloured black.

Figure 5.41 showing the 3D model of missense mutation I33T. A- Wild Type, B-Mutant. The mutant residue is smaller than the wild-type residue. This size difference can affect the contacts with the lipid-membrane. The wild-type residue is more hydrophobic than the mutant residue. This differences in hydrophobicity can affect the hydrophobic interactions with the membrane lipids.

A



B

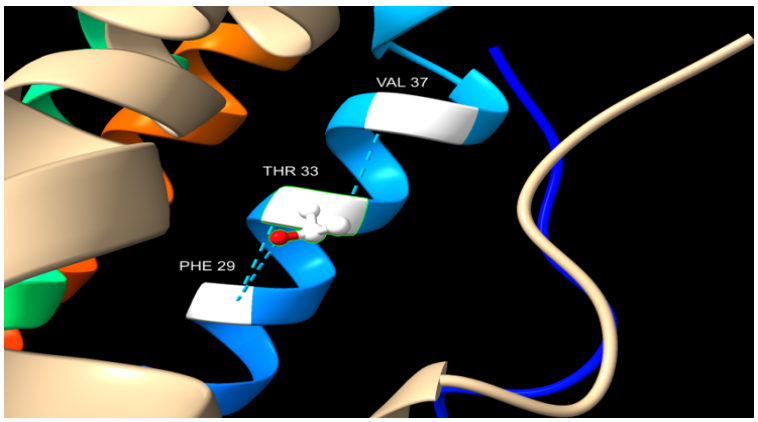


Figure 5.41: Figure showing the 3D model of missense mutation I33T.

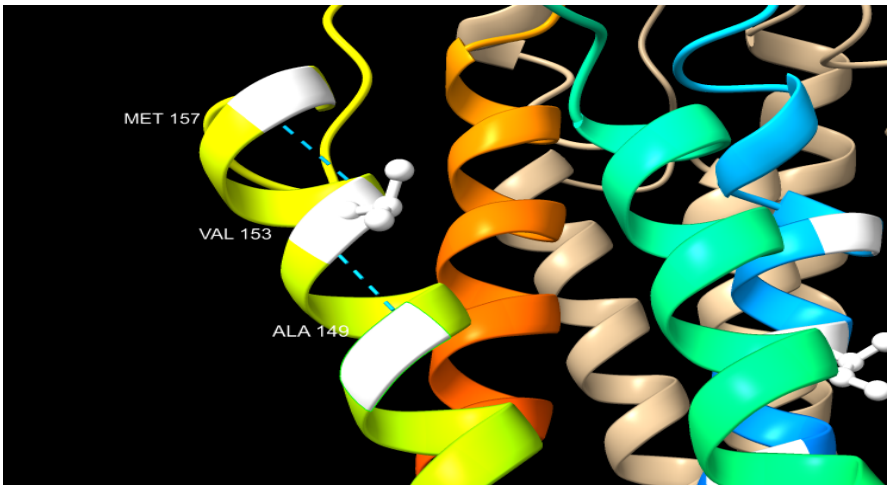
V153I

Every amino acid is unique in terms of its c size, charge, and hydrophobicity. These characteristics frequently vary between the original wild-type residue and the newly inserted mutant residue. In comparison to the wild-type residue, the mutant residue is larger. The mutation of this residue, which is positioned on the surface of the protein and is larger than the wild-type residue, might disrupt interactions with other molecules or other portions of protein. The sizes of mutant and wild-type amino acids are different (Figure 5.42).

Figure 5.42: The figure shows the schematic structures of the original (left) and the mutant (right) amino acid. The backbone, which is the same foreach amino acid, is coloured red. The side chain, unique for each amino acid, is coloured black.

Figure 5.43 showing the 3D model of missense mutation V153I. A- Wild Type, B-Mutant. This mutant residue is bigger than the wild type residue. This size difference can affect the contacts with the lipid-membrane also the residue is located on the surface of the protein, mutation of this residue can disturb interactions with other molecules or other parts of the protein.

A



B

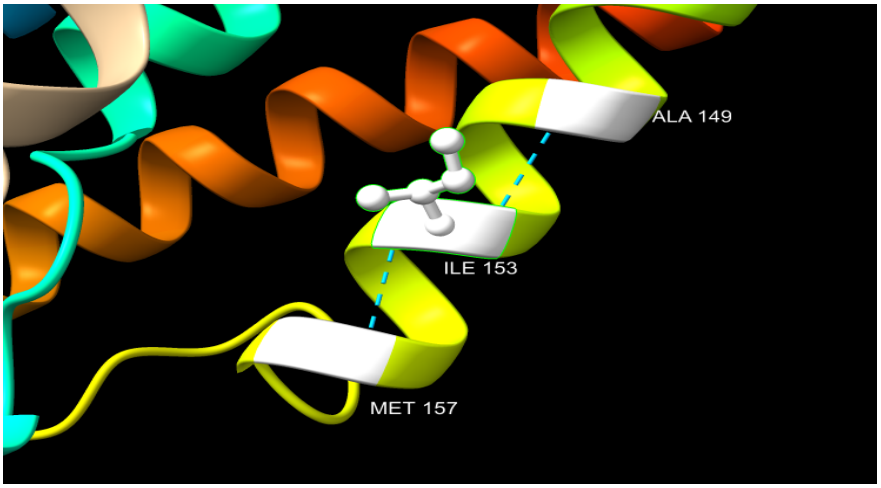


Figure 5.43: Figure showing the 3D model of missense mutation V153I.

5.5.6 GJB2 GENE MUTATION AMONG THE FAMILIES

Nine different variants were discovered in our research cohort (n_{family} = 20, $n_{pateints}$ = 35). There were two harmful nonsense variants, three missense variants, and four 3'-UTR variants. Nine out of 35 carriers of the c.71G>A nonsense mutation were affected (9/35). Nine of the affected individuals shared the heterozygous mutations c.71G>A and c.380G>A with one patient. For c.71G>A, eight people were discovered to be homozygous (p.Trp24Ter). Three missense variations—c.380G>A, c.457G>A, and c.98T>C—were discovered in five individuals. The other two were heterozygous for the variation c.380G>A, while two were heterozygous for the mutation c.457G>A (p.Val153Ile) (p.Arg127His) (Figure 5.44). Out of the others, one was homozygous. The five affected individuals also had four additional unique 3′-UTR mutations (Table 5.21).

Table 5.21: Clinical features of probands and family details (BN- Bilateral Normal, NK- Not Known, NSHL- Non Syndromic Hearing Loss, HL- Hearing Loss)

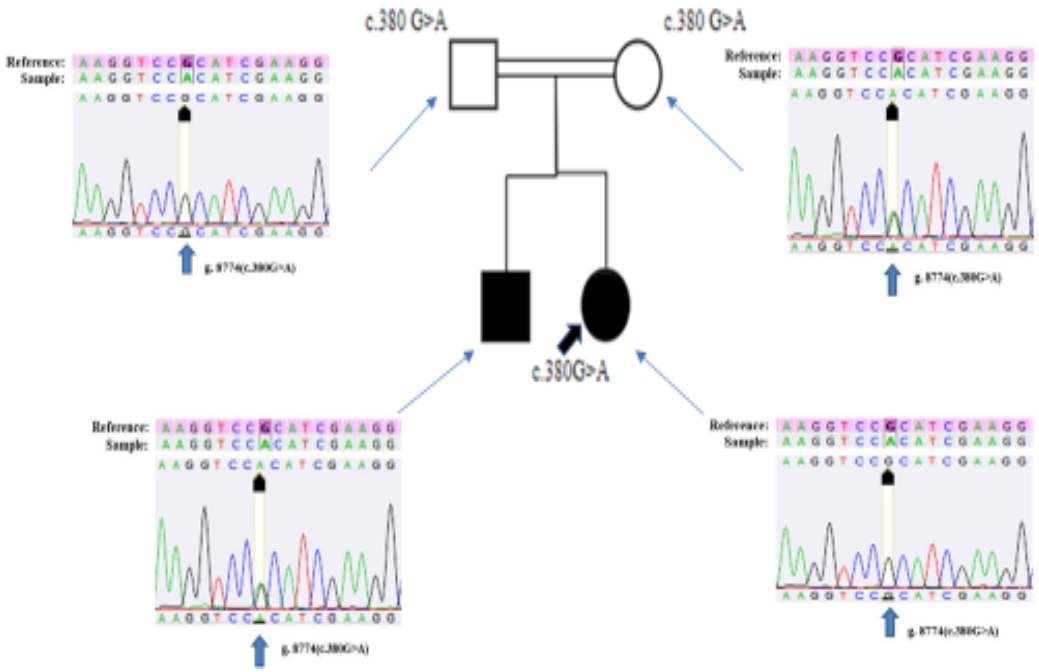
Patient Code	Sex/ Age	Clinical feature	Heavy medication history	HL level	HL type	Age onset	Variant finding
Father (DMF2)	M/45	Healthy	No	Normal	BN	NK	c.380G>A
Mother (DMF2)	F/30	Healthy	Yes (second pregnancy)	Normal	BN	NK	c.380G>A
Brother (DMF2)	M/13	NSHL	--	Moderate	Unilateral HL(right ear)	6Y	
Proband (DMF2)	F/10	NSHL	---	Profound	Bilateral sensorineural high frequency HL	By birth	c.380G>A
Father	M/35	Healthy	No	Normal	BN	-----	c.71G>A
Mother	F/30	Diabetic	Yes	Normal	BN	-----	c.380G>A
Brother	M/10	Healthy	---	Normal	BN	----	-----
Proband	F/7	NSHL	---	Profound	Bilateral sensorineural HL	By birth	c.71G>A
Grand father	M/70	Healthy	---	Normal	BN	----	----
Grand mother	F/60	Healthy	---	Normal	BN	----	---
Father	M/48	NSHL	----	Severe	Bilateral HL	7 years	c.71G>A
Mother	F/40	Healthy	NO	Normal	BN	----	---
Proband	M/15	NSHL	NO	Profound	Bilateral sensorineural HL	By birth	c.71G>A
Grand father	M/75	Healthy	-----	Normal	BN	----	No DNA available
Grand mother	F/61	Healthy	----	Normal	BN	----	No DNA available
Father	M/40	Healthy	NO	Mild	Unilateral HL (left ear)	NK	c.71G>A
Mother	F/36	Healthy	--	Normal	BN	---	---

Sister 1	F/18	Healthy	---	Normal	BN	----	----
Brother	M/15	Healthy	NO	Mild	Bilateral HL	NK	No DNA available
Sister 2	F/10	NSHL	NO	Severe	Bilateral sensorineural HL	8 years	c.71G>A
Proband	M/8	NSHL	NO	Profound	Bilateral sensorineural high frequency HL	By birth	c.71G>A
Father	M/50	Healthy	----	Normal	BN	---	c.71G>A
Mother	F/39	Healthy	---	Normal	BN	---	c.380G>A
Proband	M/6	NSHL	NO	Profound	Bilateral sensorineural high frequency HL	By birth	c.71G>A & c.380G>A

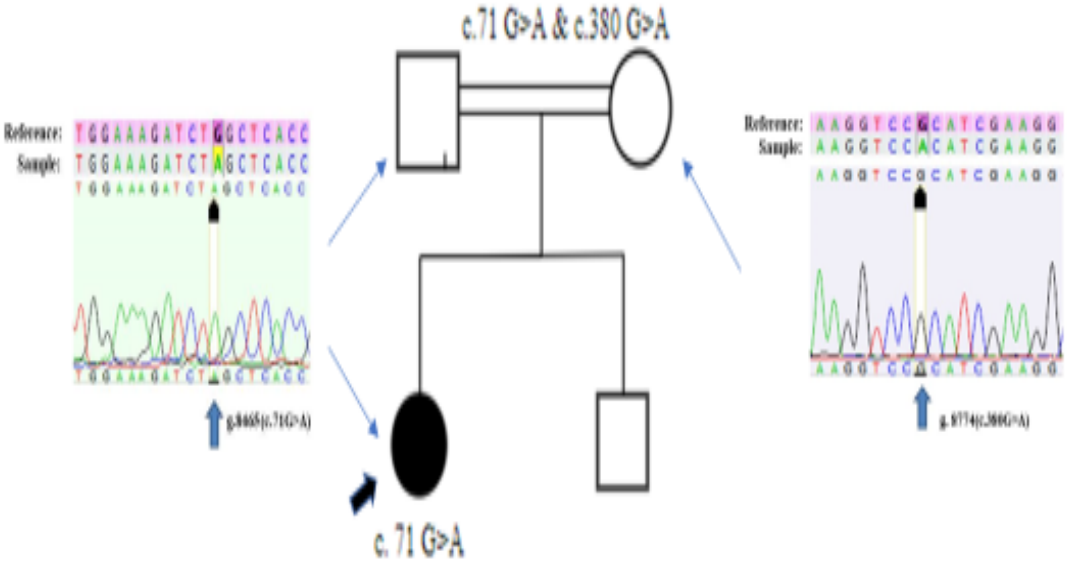
5.5.6.1 Family pedigree

Figure 5.44 showing the Five-family pedigree. The graphic depicts GJB2 gene mutations (A–E). (A) Pedigree and electropherograms of the missense mutation c.380G > A in Family 2. (B) A pedigree demonstrating the inheritance pattern of the nonsense mutation c.71G > A in Family 7. (C) A pedigree demonstrating the inheritance pattern of the nonsense mutation c.71G > A in Family 8. (D) Pedigree showing the pattern of inheritance of the nonsense mutation c.71G > A (p.Trp24Ter) in Family 19. (E) The pedigree of Family 20 demonstrates the inheritance pattern of a compound heterozygote of c.71G > A and c.380G > A, as well as electropherograms. (The pedigree was created with the visual paradigm online diagram tool[3]

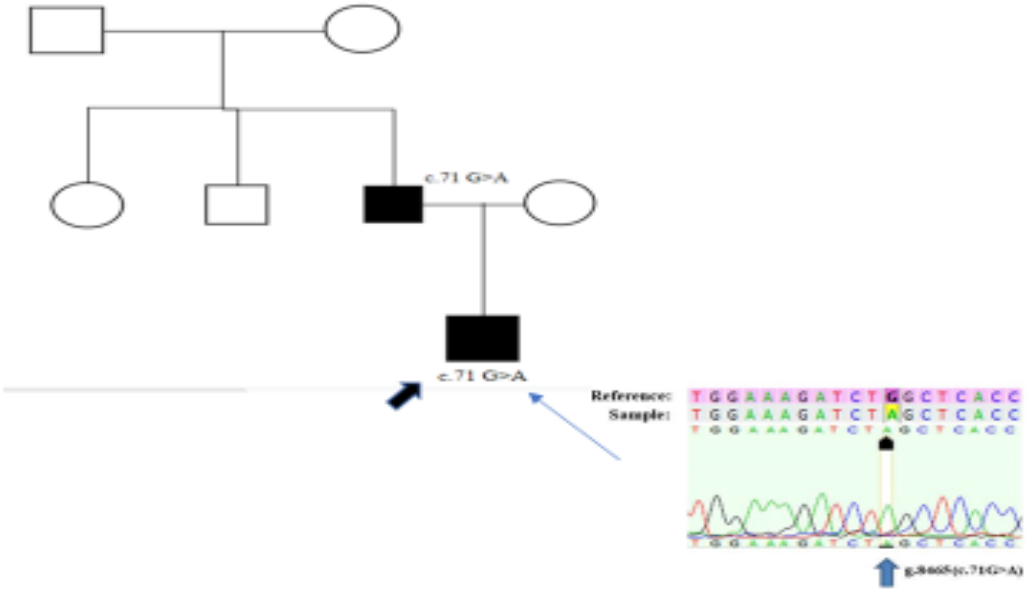
Family 2



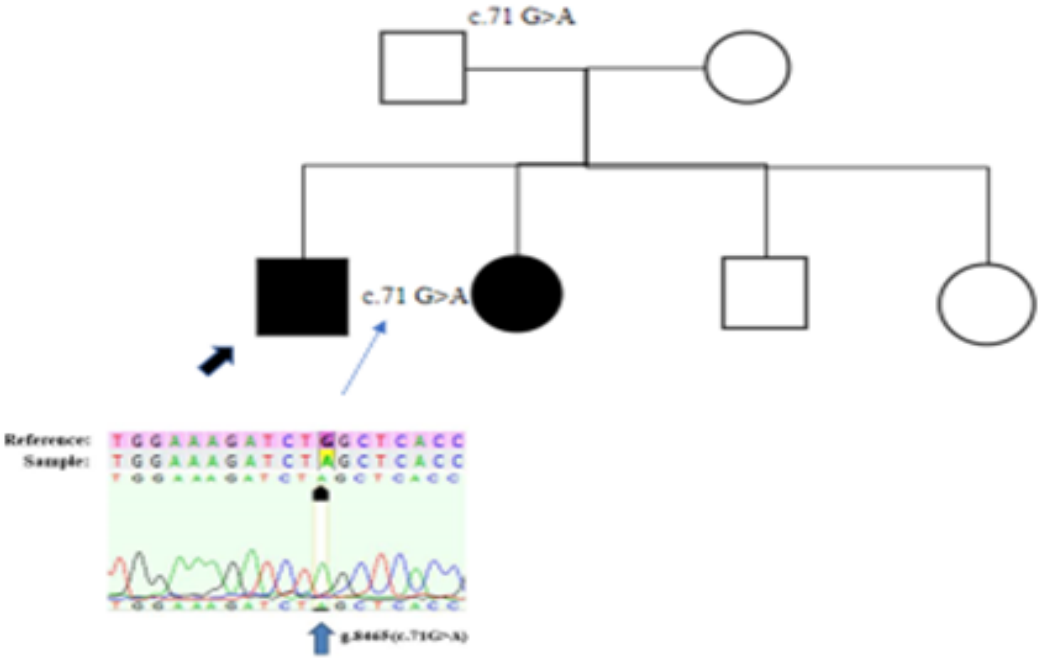
Family 7



Family 8



Family 19



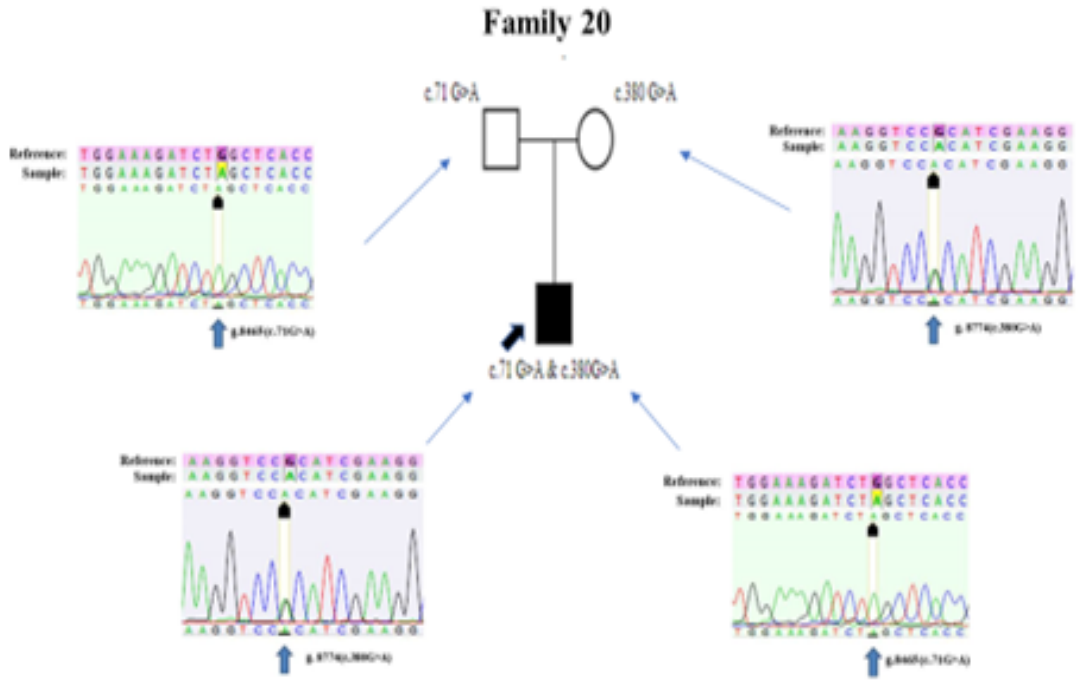


Figure 5.44: Family Pedigree. Pedigree of Family 2, Family 7, Family 8, Family 19, and Family 20. All mutations are mentioned in the figure from the *GJB2* gene.

5.6 REFERENCES

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2. Mani RS, Ganapathy A, Jalvi R, Srikumari Srisailapathy CR, Malhotra V, Chadha S, et al. Functional consequences of novel connexin 26 mutations associated with hereditary hearing loss. European Journal of Human Genetics. 2008;17(4):502–9.
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Chapter 6

DISCUSSION

The objective of the current study was to identify the genetic biomarker for North Karnataka communities of deaf-mute people by analysing the GJB2 gene. This is the first screening program of its sort carried out on the North Karnataka region's NSHL population. The main drivers behind this study were the heterogeneity of hereditary HL and the participation of various HL alleles in various groups.

The majority of children (80.7% vs. 19.3%, respectively) are between the ages of 10 and 19 years. In a 1997 conducted the study[1], on 1200 kids between the ages of 4 and 17 were included. They found that the frequency of children under 10 years old was higher (8.2%) than that of children over 10 years old (2.8%). The prevalence by age group does not match our findings, which shows that the age group under 10 years has a significant prevalence. Prelingual deafness affected the greatest percentage of children (94.6%). Out of the 368 youngsters that participated in the study, 235 (63.5% of the total) were male and 133 (36%) were female. Several earlier investigations conducted in the Indian population [1-4] showed a similar male preponderance. Of the 155 [42%] deaf children now being examined; their parents were consanguineously married. In 2019 [2], 5% of children with consanguineously married parents were documented. This figure is modest when compared to the results of our study. A family history investigation revealed that 62 children with hearing loss (16.8%) had a history of deafness. Previous study records [2] showed that 4% of young children ages 0 to 3 had a deafness genealogical link. Additionally, investigation conducted in 1988 [5] found that 10% of children with a history of deafness also had a hearing impairment.

The majority of deaf children had hearing-impaired second and third-degree relatives. A total of 24 hearing-impaired children had a deaf sibling. A statistical investigation revealed no correlation between the degree of connection and hearing loss (p-value = 0.76). Additionally, there was no conclusive link between the investigated children's deafness and their family history. In our study, 316 kids (85.9%) were found to have low hearing tones, followed by intermediate tones (32.8%) and high tones (20.54%).

It is interesting to see that more children [344; 93.5%] have bilateral deafness than unilateral deafness [24; 6.5%]. Our findings differ from those of study conducted in 2019,[4] because more instances of unilateral hearing loss were noted in that research. A further finding from the study shows that 86.4% of the kids had profound hearing loss, which is followed by moderate [7.3%], mild [3.5%], and severe [2.7%] hearing

loss. According to study [6], profound hearing loss occurs more frequently than in severe, moderate, and mild instances. The majority of people with mild hearing loss were discovered, followed by those with moderate, severe, and profound hearing loss, according to a 2017 study [3] on the population of north India. This is completely different from what we found. The whole analysis identified the epidemiological factors involved in the progression of deafness. Although it is still a challenging endeavour, preventing hearing loss is both doable and essential. To predict future burdens and the resources required to prevent and control hearing loss, the burden of hearing loss must be continuously monitored.

6.1 MOLECULAR ASPECTS OF DEAFNESS

The most prominent mutations causing congenital non-syndromic hearing loss in the North Karnataka population were examined for frequency in the current study. We looked at nuclear gene mutations, including those in the frequently reported GJB2 gene (Connexin 26). The worldwide hearing-impaired children's population's [7,8] have the highest frequency of GJB2 gene mutations. We carefully evaluate the contribution of the GJB2 gene mutations in this study. Till today genes linked to non-syndromic hearing loss are found in around 102 chromosomal sites. The first locus implicated with autosomal recessive deafness was DFNB1, and two genes, GJB2 and GJB6, which encode the gap-junction proteins Connexin 26 and Connex 30, have been linked to this locus. Despite this variation, GJB2 mutations have been shown to be responsible for up to 50% of prelingual recessive non-syndromic deafness worldwide [7,8]. Connexin 26 is a member of the family of transmembrane proteins with a similar structure that come together to create a gap junction [9]. These gap junction channels let both ions and tiny metabolites to pass through them [10]. The foundation of junction ionic selectivity and gating ability is built by over 120 distinct connexin isoforms [10]. The GJB2 mutation's impaired sorting and failure to trigger the development of homotypic gap junctions are the most significant causes of hearing loss [11,12]. In addition, in 2005 [13] reported that the root cause for hearing loss is the gap junction's inability to permit the passage of inositol triphosphate (IP3), because IP3 signalling is directly connected to calcium signalling, which is essential for the propagation of Ca2- waves in cochlear supporting cells. The GJB2 gene has more than 220 known mutations, polymorphisms, and unclassified variations as of this date[14]. The GJB2 gene's most prevalent mutation is c.35delG, which accounts for 30–63% of mutations

in Caucasian populations and has a carrier frequency of 1:31 in Mediterranean cultures[7,15]. c.235delC is most prevalent in the Japanese population (carrier rate: 1% to 2%)[16,17]. c.167delT is most prevalent in the Ashkenazi Jewish population (carrier rate: 7.5%); and p.V37I is most prevalent in Thailand (carrier rate: 11.6%); the c.35delG mutation is present in various ethnic groups at a lower prevalence compared to Caucasian population [18].

The main goal of this study was to provide an overview of the mutation spectrum of the connexin 26 genes in 368 cohorts with hearing loss. Comparatively to other deafness-related genes, the GJB2 is frequently mutated gene, according to a global analysis on the genes. High rates of the mutations W24X (25%) R127H (14.9%) and W77X (5.2%) were found in our research cohort. W24X and W77X were the nonsense mutations and R127H was the missense variation. This coding region (exon 2) mutations, previously been discovered in a small number of individuals from the Indian subcontinent, particularly India[2]. Results from analyses of protein stability, protein structure, and conservation indicates that mutations in the GJB2 gene (W24X, W77X, and R127H) seen in our community are the primary genetic cause of hearing loss. W24X and W77X were two significant mutations that affect the structure of the Connexin 26 protein. This alteration will disrupt the gap junction permeability (Figure 6.1), cause K+ ions to build up and hair cell degeneration, finally resulting in hearing loss. Connexins, which are membrane proteins, will assemble into hexameric complexes to produce the channels connecting nearby cells since they are membrane proteins. They perform the role of a small molecule transporters, including for K+ ions. The CX26 protein is necessary to maintain the K+ content in the inner ear's endolymph. A problem with the hearing procedure exists if Connexin 26 is lacking (Figure 6.2).

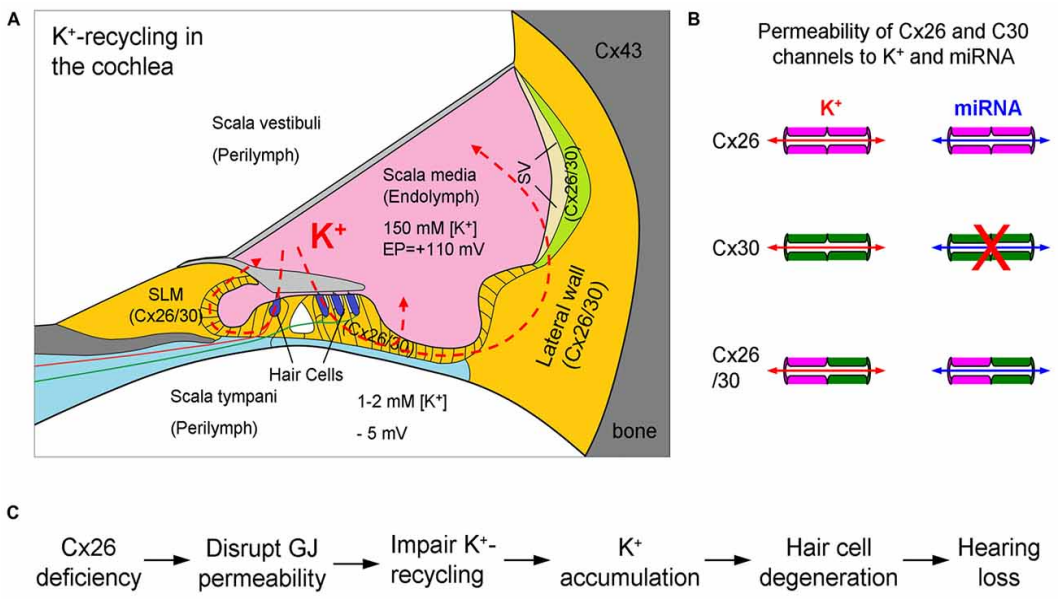

Figure 6.1: K^+-RECYCLING In the cochlea and hypothesized deafness mechanism of cx26 deficiency.

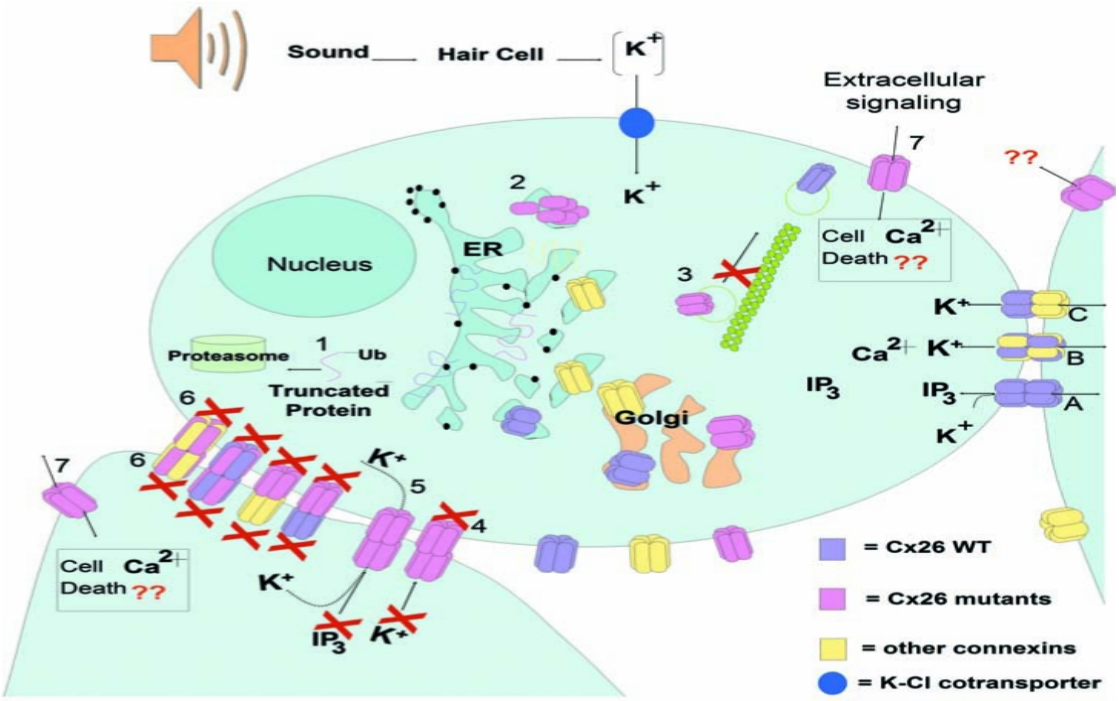

Figure 6.2: Pathogenic mechanism of deafness-associated Cx26 mutations. Wild-type connexins oligomerize in the ER/Golgi. Hemi channels traffic to the plasma membrane through the secretory pathway by a cytoskeletal-dependent mechanism. Epithelial and supporting cells in the cochlea express both Cx26 and Cx30. (A) Cx26 homomeric GJCh is permeable to ions, like K^+, and bigger molecules, like IP_3. Cx30 homomeric GJCh has a high permeability to K^+ but lower permeability to IP_3. (B) Heteromeric Cx26–Cx30 GJCh. (C) Heterotypic channels. Deafness-associated Cx26 mutations may produce.

I33T (5.2%) and V153I (4.9%), two missense variants, were also present in our cohorts along with four 3'UTR variants. The W24X mutation in our population found in heterozygous condition in six individuals, and two samples also included the R127H and V153I polymorphisms. In East Asian lineages, C.235delC is the primary mutation that results in hearing loss[19]. Ancestors of European and African heritage noticed the 35delG and C.167delT mutations as a cause of NSHL[20]. In south Iran, GJB2 mutations were present in about 11.5% of deaf families, with c.35delC being the most often found variant[21]. Our study deviates from the findings of previous works because the 35delG and C.167delT variations were not found in our investigation. The main deafness-causing gene variations 35delG and c.71G>A (W24X) have been identified in a few studies from India[7,22]. A limited number of people from the Indian subcontinent, mainly India, have previously been shown to carry the GJB2(exon 2) coding region mutations (W24X, W77X) described in this paper[5]. In southern Europe and the United States, congenital deafness is associated with biallelic GJB2 mutations (between 30-35%)[23-25]. Additionally, in studies on the Chinese Hans population, 25.65% of hearing-impaired people had biallelic mutations in the GJB2 gene[26]. Contrarily, only 10–20% of Indian cohorts of congenitally deaf people exhibit biallelic GJB2 mutations. We thus hypothesise that there may be more genes that are common in India in addition to the deafness-causing genes already found. We used the Clustal Omega multiple sequence alignment software to assess the evolutionary conservation of each amino acid position. The polymorphisms R127H, I33T, and V153I have been identified to be highly conserved and semi-conserved, respectively (Figure 3). The R127H polymorphism has been transfected into Hela cells in two trials. The connexon 26 protein with the R127H mutation functions normally in the first analysis[27]. The gap junction is generated in the second study, but the gap junction's activity is reduced[5,28]. Further analysis of the mutations observed in our study using bioinformatics revealed that the R127H polymorphism is not detrimental (Table 3). Despite being often seen in many Indian groups, the R127H mutation clearly suggests that it is not a causative polymorphism for hearing loss. Additionally, six of the hearing-impaired patients carried the W24X mutation, and two samples were also in heterozygous condition for R127H and V153I missense variants. The individuals' hearing loss might have been caused by a variety of different factors. The primary possibility may be the digenic origin, a result of another connexin gene (GJB3 or GJB6). We have restricted our investigation to one gene since the GJB2

gene predominates in hearing loss. Connexin's home page mentions 200 or more GJB2 gene mutations[14]. The GJB2 gene's detrimental mutation frequency (W24X) is prevalent in our study population.

6.2 PREVALENCE OF P.W24X MUTATION AMONG DIFFERENT POPULATIONS IN THE WORLD

We can identify different trends in various groups by looking at the prevalence of the W24X mutation globally. W24X was initially identified in an Indian family in 1998[29]. In this study, seven families with ARNSHL, including four Indian, one Caucasian, one Dominican-Puerto Rican, and one Israeli Bedouin, had their Cx26 gene's coding region analysed for mutations. The W24X mutation was shown to be homozygous in the Indian family, while it was discovered to be compound heterozygous with the W77X mutation in another affected individual. Both of the parents of this compound heterozygous individual had different mutations. Study conducted in 2003[30] evaluated 45 families from three distinct Indian states, namely Karnataka, Tamilnadu[5], and Delhi, with non-syndromic hearing loss. There were four homes with W24X homozygotes, or around 8.8% of the total population. Two families had afflicted members who were compound heterozygous for W24X; one had R143W and the other 35delG. The mutant allele that is found most often in this investigation is W24X. Additionally, isolated Karnataka families from Bangalore and Mysore displayed this mutant gene.

W24X was found to be the most prevalent mutation in research by previous study, who looked at 215 people from southern and western India in 2003[31]. Six heterozygotes and 36 homozygotes were found among the probands for W24X. The heterozygotes' non-coding exon of the Cx26 gene was also checked for changes. One more mutation was only present in NS l(+l) G+ A. Around 18% of the chromosomes tested, or 72/78, were W24X chromosomes, the majority of which were homozygous as in the present research. 20% of the probands overall were impacted by this mutation. The bulk of the individuals in their research were consanguineous and came from good families. Cases having a potential non-genetic aetiology have also been removed. The people for the current experiment were chosen without regard to any of these criteria. Therefore, it is possible to consider the frequency of W24X discovered in the current study to be a "real" estimate among children who require special education because of their hearing loss. Study looked at 196 unrelated Pakistani families in 2005[32] that had autosomal recessive nonsyndromic hearing loss to see if

there were any Cx26 gene variations (ARNSHI). They reported four individuals who were homozygous for the W24X mutation. This means that the mutation, which was present in 2.3% of the chromosomes analysed, was present in just around two percent of them. The low prevalence of W24X homozygotes in Pakistan, according to the researchers, is due to the country's high level of consanguinity.

In 2001, examined 51 British families including families of Asian heritage, for mutations in the Cx26 gene. 75%, or 38 of these homes, had a predominance of Caucasians[33]. Originating in Bangladesh, Sri Lanka, Pakistan, and India, the Asian families W24X was discovered to be heterozygous (W24X/+) in one Indian family. It was discovered that another Indian family was homozygous for Q124X. This mutation was not discovered in any of the Indian research. It has been discovered that one person of "Caucasian" descent has W24X compound heterozygotes with 35delG.

It is not just the Indian subcontinent that has the W24X mutation. It has also been noted in a few communities in Europe. Except for Slovakia, the countries from which W24X occurrence was documented were Slovakia, Czech, Hungary, Greece, Germany, and Middle Eastern Asian nations including Turkey and Iran, albeit at a very low frequency (range: 0.4 to 4.1% of the mutant chromosomes)[34-40]. W24X was found in 39% of the mutant chromosomes (25/64) among Slovak Roms from eastern Slovakia, according to 2003 research[36], the reason for this high frequency is that Slovak Rorns, commonly known as "Gypsies," who number 8 million and are dispersed over the whole European peninsula, have their origins in the Indian subcontinent. They reportedly came in Europe roughly a thousand years ago, according to historical reports. Similar to this, the Cx26 gene's coding region was studied in a big cohort of 156 unrelated Czech patients (including children and adults) with NSHI, including seven with Gypsy ancestry[38]. 35delG and W24X were the two mutations that were found most often in this group. Six out of 156 probands, or 4.5 percent of those studied, were homozygous for W24X, while one was compound heterozygous (71156). When stated in terms of mutant chromosomes, the frequency of this mutant allele is thought to be 9.7 percent (131134) of the mutant alleles. It is significant that W24X homozygotes were Gypsies and that the patient who was compound heterozygous had a Gypsy grandparent. The diagnosis of "Pseudodominance" was made for a vertical transmission that looked to be dominant in one family of Gypsy heritage, with the affected mother and child being homozygous for W24X and the unaffected father being heterozygous. In 2004,[39]

examined the Cx26 gene's coding region in 194 ARNSHL patients from the northeaster region of Hungary, including 102 familial and 92 sporadic cases. In this study, W24X was discovered to be homozygous in two individuals and heterozygous in eight. Eight heterozygotes were discovered, four of which were compound heterozygotes for the 35delG mutation and originated from a single family. The low frequency of W24X in these patients and the possibility that the mutation W24X emerged independently in their population may be attributed to the patrilateral pattern of cousin marriages and the high carrier rate (61430) among north-eastern Hungarians (a geographically constrained territory with inhabitants). Similar in Germany, two individuals with 35delG were found to be compound heterozygous for the W24X mutant allele among the 228 hearing-impaired patients investigated[35].

Two random persons with 35delG were found to have W24X in 2002, Greece[34] when they tested 210 people with NSHI. Another study reported in 2001[41] the W24X mutation in compound heterozygous form with the novel mutation 135s in an Australian study. The W24X mutation was found at varying frequency in Iran and Turkey. 2003 study[37]examined the coding and non-coding regions of the Cx26 gene in 60 Turkish families with non-syndromic sensorineural hearing loss. 8% of the mutant chromosomes had the W24X allele, which was discovered (3138). One of the two carriers of this mutation is a compound heterozygote, whilst the other is a homozygote. In 2005[42] evaluated 93 ARNSHL patients in northeast Turkey for a different study. The mutation W24X was discovered in approximately 10% of the mutant chromosomes (6158) with a Cx26 gene mutation. The coding part was the sole subject of this investigation. They were all homozygous individuals. By comparing the prevalence of this mutation in the Indian subcontinent, the researchers concluded that Turkey's population may have migrated from middle Asia around 1000 years ago. After screening 168 Iranians from 83 families for non-syndromic hearing loss, 2002 study discovered one homozygous and one compound heterozygous individual for the W24X allele[40]. The presence of Cx26 mutations in this population indicates that Iran which is located along the Silk Road, already has protracted battles with other nations, and has experienced substantial immigration from its neighbouring countries. Iran's inherent geographic boundaries have helped to maintain its racial homogeneity. Another Iranian study analysed 664 familial and 35 sporadic ARNSHL patients from diverse ethnic groups[40]. (Persian-296, Turk-147, Kurd-103, Gilaki and Mazandarani-3 6, Lur-33, Arab- 1 5, Ballochi-33 and Turkmen- 1). The presence of W24X was

detected in seven individuals, including two 35delG compound heterozygotes, two homozygotes, and one IVS 1 + 1 G-r A compound heterozygote. The non-coding area included the later mutation. As a result, the W24X mutant allele made up around 3.7 percent of the mutant alleles (11/297), or about 0.8 percent of the total alleles (1111328). The researchers discovered that southeast Iran has strong ethnic links to neighbouring Pakistan by examining the varied rates of Cx26 deafness from northwest to southeast areas. Three were heterozygotes, one was compound heterozygote with V91M, and thirty-four were homozygous for the W24X mutation in the current study's total of thirty-eight carriers of the mutation (38/366). Q8OQ and T186M are the extra mutations present in each of the two homozygous individuals. Because it is a silent mutation, the first one is of no functional relevance. The threonine at position 186' is highly conserved across connexins from various species, and the other mutation is a new mutation that was discovered for the first time in this study (Fig-15). As the protein is shortened in the TMl domain itself, this mutation, which is localised in EC2, could not have clinical consequence. The 'new' mutation V91M, which was discovered in the compound heterozygous condition, is situated in the TM2 domain. The oligomerization of connexon hemi channels requires this domain[43]. Hearing loss might be attributed to the conversion of highly preserved valine to methionine. It was unable to identify the clinical relevance of the heterozygous individuals found in the current investigation. The majority of the hearing loss is recessive, necessitating homozygosity for the mutant alleles. Numerous studies have documented such instances. Regarding the clinical importance of W24X heterozygotes, one might infer a number of things. It seems sense to anticipate a second mutation, either in a connexin gene or a non-connexin gene that contributes to the development of gap junction channels. The real importance of this harmful mutant allele in the heterozygous form can only be revealed by further specialised investigation.

A nonsense mutation p.W24X results in an early termination at the 24th amino acid rather than the 226th amino acid[44]. A stop codon is added to the GJB2 gene, resulting in the production of a protein that is only one-tenth as long as the wild-type protein[45]. Cells in the homozygous condition lack any functional Cx26 monomers. This affects the recycling of K+ to endolymph, and there is little or no physiological response to sound stimuli[44]. In 12 investigations from 7 nations (Bangladesh, India, Pakistan, Iran, Czech Republic, Turkey, and Spain), the p.W24X allele was shown to be a significant allele in 224 of 1424 (15.7%) hearing loss probands, and in these

participants, the p.W24X allele accounted for 47.3% of all GJB2 mutations[46].The high prevalence of p.W24X in several Caucasian communities suggests that these Romanies, often known as "Gypsies," originated in the Indian subcontinent around a thousand years ago. These populations include the Slovak Romany and the Spanish Romany [46] (Table-7). After being discovered in multiple Asian families, the p.W24X (c.71G>A) mutation was originally identified in a Pakistani family[17,29,33,40,45]. When examining seven families from four different nations, in 1998[29] discovered the p.W24X mutation for the first time in a south Indian Tamil family. Recent statistics show that the DFNB1 locus is the most common one in India [30,31,47,48]. The discovery of a high prevalence of the p.W24X mutation in Slovak and Spanish gipsies as well as in India links the mutation's origins to the Indian subcontinent and raises the possibility that this GJB2 mutant allele is the most prevalent in other European-Romanian communities[44]. The p.W24X mutation has been recorded in various nations, most of which are in the Mediterranean area and a few of which are oriental, in addition to the Indian subcontinent. On an epidemiological scale, the research from France, Greece, Italy, Germany, and other countries were examined. However, only a very low frequency of this mutation (0.07) was detected in the French population[21]. According to 2003 research from Turkey[37], 2.5% of the population has the mutation p.W24X homozygote, which is also present in the deaf community. The p.W24X mutation caused deafness in the north-eastern Hungarian population at a frequency of 4.3%, although the carrier frequency in the same population was discovered to be substantially lower at 1.4%[39]. p.W24X mutation frequency in the Indian population. The most frequent mutation discovered in the Indian population, according to a haplotype analysis of markers surrounding the GJB2 gene, is p.W24X, which most likely results from a founder effect[31]. The frequency of the p.W24X mutation was reported to be 6.5% among the 200 probands examined in 2005[48] and 8.6% among the 303 probands screened in two investigations carried out in Andhra Pradesh (2009)[49,50]. Of the 530 Indian patients with NSHL, 128 (24%) had GJB2 mutations, according to Mani et al. 112 individuals[50], or around 21%, exhibited biallelic mutations. With an allelic frequency of 16.4%, the p.W24X mutation was the most prevalent one. More recent research in 2011[51] found that the p.W24X mutation was present in seven out of twenty-seven (26%) unrelated Indian individuals with congenital non-syndromic SNHL who were recommended for cochlear implantation. According to Bhalla et al., while this mutation has been substantial in some Indian studies, particularly in the

south Indian and western Indian populations, it has not been in the north Indian population (2009)[52]. The most frequent mutation was c.35delG, which is mostly seen in Caucasian people and was initially discovered by them in the north Indian population. It was followed by p.W24X mutation (3.8%). Found a similar discovery in 2009[53] that suggested there could be variation in the incidence of GJB2 mutations in various parts of India. The high prevalence of heterozygosity in GJB2 mutations among their patients, which suggests the involvement of other moderating factors such nuclear-mitochondrial gene interactions, was another intriguing discovery in their study. family research Numerous researches based on GJB2 mutations have been conducted on families, according to various organisations in India. Out of 45 families from Karnataka, Tamil Nadu, and Delhi that had two or more people with ARNSHI, in 2003[30] found that four families were homozygous and two families were compound heterozygous for the p.W24X mutation. In different research done in 2004[48], showed each afflicted individual in six families out of the 13 families identified from Andhra Pradesh was homozygous for the p.W24X mutation. Twenty of the 59 families in a study from Kerala[54], that included 59 households with at least one sick person were found to be positive for the p.W24X mutation (33.9%). 288 unrelated families from western and southern India were tested for GJB2 gene variants in more recent research in 2010[55]. The most frequent mutation, accounting for 56 out of 288 (19.4%), was p.W24X. Pavithra et al. (2014) screened eight assortative mating hearing-impaired families from Kerala for GJB2 mutations. They discovered five distinct GJB2 gene variants (p.W24X, p.W77X, p.Q124X, c.IVS1+1G>A, and p.I35S) in seven of eight families, illuminating the high prevalence of this mutation in this area. Indian population's p.W24X mutation carrier frequency Numerous groups have sought to research the carrier status of the p.W24X mutation in the Indian community of people with normal hearing after observing a high incidence of this mutation in the Indian HI population. When 205 hearing patients were tested for this mutation in 2003[31], the carrier frequency was discovered to be 0.024. A carrier rate of 0.0357 was also observed in 2009[54], which was similar to the earlier finding. The carrier frequencies of other prevalent GJB2 mutations like c.35delG and c.167delT in various populations are comparable to the carrier rates of the p.W24X mutation in the Indian population. This shows that the p.W24X mutation occurs often in our group and may be unique to the Indian population.

6.3 R127H

The first R127H mutation in a single hearing-impaired Spanish person was reported in 1998[15]. They assert that, in contrast to Cx43, Cx40, and Cx32, Arg at this position is preserved in Cx26, Cx38, Cx46, Cx37, and Cx50 connexins. The amino acid histidine is replaced in the final three connexins. In 50 unrelated control people that were examined, they failed to find this mutation. Study conducted in 2005[56] revealed that among the 196 afflicted families examined in Pakistan, R127H was present in twenty-one cases (homozygote-1, compound heterozygote-2, and heterozygote-1/8). Additionally, two heterozygotes carried the V153I mutation. In one of these two families, the mother passes on the R127H mutation, whereas in the other, both parents had the mutations. The scientists classified this mutation as a benign polymorphism based on evolutionary conservation. Study done in 2003,[31] calculated that among 215 hearing-impaired people in India, the frequency of this variant allele was 12.3%. (10 homozygotes and 33 heterozygotes). The frequency in the control samples (n=60) was calculated to be 1 7.5 percent (3 homozygotes and 15 heterozygotes). R127H is not racially exclusive. This mutation was discovered to be the most common one in the current investigation. Most investigations found that one or two chromosomes had this mutation[10,25,57,58,59]. Study conducted in 2004[39] identified R127H in a single family where four members with normal hearing (including the parents) were found to be heterozygous and three members with hearing impairment were found to be homozygous in a study of 194 families from north-eastern Hungary. On the other hand, parents of two unrelated families with normal hearing were discovered to be homozygous for R127H in 2004th. Both the afflicted and unaffected offspring shared the genotype R127WM34T or R127HIW24X. Similar to the current study, Slovak Romanies (Gypsies) were also found to have a high prevalence of the R127H mutation. This population's estimated frequency was 19.4%[36]. Their shared ancestry with the various Indian subcontinental populations may be the cause of the high frequency found. Functional research on R127H is still inconclusive. R127H transfection in HeLa cells had no impact on the development of channels[60]. However, in 2003[61], studied the expression of the R127H mutant in N2A cells (a cell line with a fault in communication) found that the creation of defective gap junctional channels in the cell membranes and the junctional conductance were both significantly reduced. It is obvious from this that the R127H mutation's contribution to hearing loss is unclear. It could play a part in situations

when it does so in a compound heterozygous form, at least when causal mutations are present, or when particular environmental factors or modifier genes are present in a heterozygous state[21]. Investigation is still needed to determine if or whether the R127H allele has any modest effects.

6.4 W77X

W77X distribution: The W77X (c.231- A) mutation was found in 4.8% in our study cohort. In a family of Pakistani descent, first detected this mutation[45]. The afflicted people were found to be homozygous, and their parents were carriers. In 2001, conducted more research on this mutation in two British Asian families[33]. One of them is of Pakistani descent, and the other is of Bangladeshi descent. The parents were carriers in both situations. The homozygous status of this mutation was also observed in one of the 215 afflicted families from South India [31]. According 2005[th] study[56], the Pakistani population had the greatest frequency of W77X, which was around 2.5 percent. The most frequent variation found to cause hearing loss in their investigation was W77X. They contend that Tryptophan is a highly conserved residue at position 77, and that a mutation there leads in a malfunction. Pakistan has a two-fold lower rate of Cx26 gene mutations contributing to hearing loss than India, while patients there had five times more W77X mutations. Another harmful mutation, W77R (Tg Arg; c.229T+ C), had been found at this location [41,62,63]. Connexons that go to the target location in the plasma membrane are affected by this mutation [11]. One of the two W77X homozygous individuals identified in this investigation also carries the heterozygous mutation F83L. According to reports, this mutation is a polymorphic variation.

6.5 V153I

Significance of the V153I mutation: The V153I (c.457-A) mutation was found in 4.9 percent in the current study. Both heterozygous and homozygous condition of the mutation were present (with R12H, and W24X). None of the people had compound heterozygous traits. Among a previous study from South India[31], as well as in Slovak Romanies, a comparable allele frequency among the hearing impaired was documented (Gypsies)[36]. In these two investigations, neither homozygous nor compound heterozygous individuals were among those who underwent screening. The present study's mutant allele frequency is the highest of the two studies' mutant allele frequencies. It is interesting to note that in 2003, calculated a relatively high frequency

(0.058 or 5.8 percent)[31] for this mutant allele in those with normal hearing (n-60). In addition, they noted two homozygotes. On the other hand, in research on 196 unrelated hearing-impaired families, found one homozygote and twelve heterozygotes for the V153I mutation[56]. In four families, they also noticed compound heterozygosity. The trans-arrangement was present in the two families that were found to be compound heterozygous for the V153I and R127H mutations. V1531 and R165W mutations were discovered to be haplotypes in the two more families. The haplotype was homozygous in one of these two families. Based on the family analysis, it was deduced that the haplotype nature existed in the other family. A big Sri Lankan family with this specific haplotype (V153I/R165W) has also been documented[33]. One of the five deaf people in Malaysian research who was heterozygous for V153I was identified, but not among the 100 participants with normal hearing who were examined [65]. A significant frequency of V153I has also been noted in the Iranian population, which is consistent with these results [40]. Most frequently, V153I was found to be heterozygous or compound heterozygous in both afflicted and normal hearing individuals at a relatively low frequency. For instance, in a sample of 156 hearing-impaired individuals, found four heterozygotes and two compound heterozygotes in Czech research[38]. The compound heterozygotes were 35delGN1531 and M34TN 153I. Several investigations have found the V1531 mutation to be compound heterozygous with T8M[57,66,67]. This compound heterozygote was shown to be connected to a variety of traits, including mild to late-onset hearing loss (after the first decade). Although these particular mutant combinations (T8MN1531) were determined to be pathogenic, the pathogenicity of the V1531 mutation is up for debate because it was also detected in healthy individuals[31,33,66]. According to 2004,'s[68] investigation, however, showed that V153I entirely lost its capacity to produce functional channels and that their conductance is lower than the control values. Based on their genotype-phenotype connection for several mutations of the Cx26 gene, another study done in 2004, also inferred a comparable result (mild mutation) in research[69].

6.6 PREDICTION OF PATHOGENIC EFFECTS

A transmembrane protein with the same protein architecture is encoded by each of the twenty-one distinct connexin genes that have been identified in humans till date[70]. Each connexin protein comprises two extracellular loops (EL1 and EL2) linking the TM1-TM2 and TM3-TM4 domains, four α-helical transmembrane

domains (TM1-TM4), and a cytoplasmic loop (CL) between TM2 and TM3. Additionally, the cytoplasmic amino-terminal (NT) and carboxy-terminal (CT) domains protrude outward[71-72].

The GJB2 gene has 1347 known sequence variations, 182 of which are classified as missense or nonsynonymous variants that result in a different amino acid sequence, according to the Ensembl (version 74) database[73]. Because they are linked to disorders like hearing loss, some of the changes are thought to be harmful missense mutations. Experimental confirmation of the potential harmful consequences utilising in vivo functional protein studies is one of the extensive and precise techniques to understanding the molecular basis of illnesses caused by point mutations[74]. However, it takes a lot of work and time to experimentally evaluate the consequences of protein mutations on their functional and structural properties. Using bioinformatics tools, on the other hand, is an additional strategy that may be used to quickly acquire important data on the effects of mutations[75]. In this context, several single nucleotide polymorphisms or mutations linked to illnesses have previously been assessed using in silico techniques, with impressive findings. Two unique missense mutations (M34V and L205V) were found in earlier work, in which the whole coding region of the GJB2 gene was directly sequenced, but they were unable to demonstrate their functional effect experimentally[76]. This work serves as yet another illustration of how bioinformatic techniques are useful tools for determining the effects of mutations on protein structure and function. We examined the evolutionary conservation of each amino acid site using the Clustal Omega multiple sequence alignment programme. R127H and I33T polymorphisms are shown to be highly conserved, whereas V153I polymorphism is found to be semi-conserved (Figure5.36). Additionally, a bioinformatics examination of the mutations found in our study showed that the R127H polymorphism is not harmful (Table 5.19). Despite being observed often, the R127H mutation clearly shows that this is not a causal polymorphism for hearing loss in other Indian communities. There may be a variety of causes for the deafness in such subjects. The digenic origin, which is an implication of another connexin gene, may be the major candidate (GJB3 or GJB6). We can categorically state from this study that GJB2 is the causal gene for hearing loss in our study population because of the prevalence of the pathogenic mutations (W24X & W77X) in the GJB2 gene.

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Chapter 7

CONCLUSION

1. The present study aims to evaluate the contribution of Cx26 gene (DFNB 1; 13q11–12) mutations to childhood hearing impairment with reference to North Karnataka, India. The study group consisted of 368 school children who attended special schools for the hearing impaired located in North Karnataka. The age of the subjects ranged from 6 to 19 years, with a mean age of 13.5 years. Audiological information was obtained from the school records, and all patients had severe to profound sensorineural hearing impairments. After obtaining institutional ethical committee approval and the consent of the subject's parents and school authorities, approximately 2 ml of blood was obtained from each subject via venipuncture. DNA was extracted from the blood samples using the Qiagen kit method.
2. Sequencing was carried out to analyze the coding and non-coding regions (the whole gene) of the GJB2 gene using the ABI 3500 Genetic Analyzer (Applied Biosystems).
3. 613 NSRD children from North Karnataka were screened, and 368 (n = 235, n = 133) were chosen. 16.8% (62/368) of the 368 hearing-impaired children included in the study had a history of deafness in their families.
4. Based on the audiological documentation results, 86.4% were bilaterally profound, 2.7% were severe and 7.3% were with moderate HL. In our study, 115 children's parents were married in the close relatives (115/368). We identified 18 mutations in the exonic (9 mutation) and intronic regions (9 variants) of the GJB2 gene. W24X, R127H, and W77X variants were the mutations that were discovered most often in this analysis. In our study, we also recorded 3 missense mutations, namely R127H, V153I, and I33T. Four 3'-UTR variants were identified (c.84T>C, c.1067G>T, c.1277T>C, c.1152G>A).
5. In total, 18 mutations were found. Three of the mutations were missense mutations, and four were 3' UTR variants.
6. W24X (c.7-1 G-A) was the most common mutation. This mutation was observed in about 25 percent. R127H (c.380G-r A) was the second most common mutation. This mutation was observed in about 5.2 percent. W77X (c.231G.A.) was another causative mutation observed in about 4.8 percent of the chromosomes analyzed.

7. A 20-family study showed nine of the affected individuals shared the heterozygous mutations c.71G>A and c.380G>A with one patient. For c.71G>A, eight people were discovered to be homozygous (p.Trp24Ter). Three missense variations—c.380G>A, c.457G>A, and c.98T>C—were discovered in five individuals. The other two were heterozygous for the variation c.380G>A, while two were heterozygous for the mutation c.457G>A (p.Val153Ile) (p.Arg127His). Out of the others, one was homozygous. The five affected individuals also had four additional unique 3′-UTR mutations.
8. The high frequency of heterozygosity (about 25 percent of the affected) of Cx26 gene mutations among the hearing impaired indicates that the mechanism of hearing may be complex, in the sense that childhood hearing impairment could be caused not only by the homozygosity of the individual genes but also by the interaction of mutations at more than one locus.
9. Analysis of Cx26 gene mutations based on ethnic populations may be helpful not only in understanding the mechanism of hearing but also in genetic counseling.
10. When it comes to the Karnataka state's North Karnataka area, there are high rates of consanguineous marriages. In many Indian households, consanguinity is practiced. Common genetic disorders seen in children of consanguineous marriages include thalassemia, cystic fibrosis, Down's syndrome, infantile cerebral palsy, and hearing and visual disabilities. All of the aforementioned disorders are very common in the northern Karnataka region, as well as in many other parts of India where consanguinity is still widely practiced. In our study, findings also supported the notion that consanguineous marriages are associated with an increased risk for congenital malformations and autosomal recessive diseases like hearing defects.
11. The entire analysis exposed the epidemiological factors involved in the onset of deafness. In order to provide high-risk couples with the necessary genetic counselling and prevent them from having such children, extensive research on the epidemiology of deafness and the identification of genetically inherited disorders is required.

12. This study may lead us to believe that identifying this mutation in babies facilitates the early identification of hearing loss. Therefore, it is possible to implement remedies for the handicap early on. Restriction fragment analysis or sequencing techniques can be used to quickly find assay mutations. These methods can be easily adopted in India due to the high levels of consanguinity and ethnicity, which help with genetic counseling. The early rehabilitation of children with congenital hearing loss will benefit from this.

CLINICAL IMPLEMENTATION

Our findings from this study imply that detecting the GJB2 gene mutation in babies aids in the early diagnosis of hearing loss. So, it is conceivable to undertake early intervention measures including prenatal diagnosis, abortion, cochlear implantation, and gene therapy.

Mutations can easily be identified using DNA sequencing techniques or restriction fragment analysis assays. Due to the significant amount of consanguinity and ethnic diversity in a nation like India, these approaches may be easily used, aiding in genetic counselling.

The treatment of children with cochlear implants greatly benefits from the early diagnosis of genetic abnormalities within the first year of life. GJB2 gene mutations have been found to be related to improved post-implantation results. The age at which a cochlear implant was placed affected the result. Young recipients who get implantation before age 3.5 years have the link between mutations and cochlear implantation outcome, whereas older recipients do not. Last but not least, this discovery is significant because it might aid in the creation of a genetic screening tool to identify individuals who are sensitive and could lower the prevalence of hereditary deafness.

MANAGEMENT STRATEGIES

Early detection, diagnosis, and treatment of ear issues that cause hearing loss and deafness can help us to prevent preventable hearing loss due to illness or accident and also provide medical rehabilitation for deaf people of all ages by enhancing the already-existing cross-sectoral connections to ensure the continuity of the rehabilitation programme for the deaf. By providing funding for supplies and people training, we can build institutional capacity for hearing care services.

Chapter 8

ANNEXURES

INFORMED CONSENT FORM AND DATA SHEET

Govt of Karnataka
Karnataka Institute for DNA Research
Dharwad, Karnataka

Informed consent form-cum-Patient details for Genetic Analysis of GJB2 gene Mutation in Deaf Mutism

a) I am giving blood sample with my own knowing fully well about the purpose of collection of the sample.
b) I consent to the test(s), which I understand will be based on DNA/RNA.
c) I agree to the request to use the blood sample for genetic studies, which may lead to discovery of new techniques or improving the existing one. Furthermore, I also investigators of Karnataka Institute for DNA Research to use the blood samples for research purpose that may facilitate better understanding of human genome and diseases provided confidentiality of the identity of the sample is maintained.
d) I also allow investigators to publish the data obtained from the aforementioned studies.
e) I agree to have no financial claims out of the study.

1. Name of the subject :
2. Hospital case NO. :
3. Clinical diagnosis :
4. Date of Birth : 5.Gender :
6. Blood group of Patient:
7. Permanent Address :

Street: Taluk:

Dist.: Pin code:

8. Phone number a) Mobile :

b) Land Line with code :

Signature of the subject or Thumb impression (with two witnesses) OR
Signature of Parent/Guardian (If the subject is under 16 years of age)
:

*Name of the Hospital :

*Blood collected by :

*Blood sample relates to

Data sheet for Genetic analysis of GJB2 gene mutation in Deaf Mutism

The data obtained from the patient will be kept as secret, the data will be coded and utilized for our work.

Name: _____

Age: _____ Sex: _____

Occupation: _____ Height:_____Weight:_____

Cast (GM/OBC/SC/ST):_____Religion:_____

Place of living:

a. Present :_____
b. Past five years :_____
c. Past ten years:_____

Deafness by Prelingual: _____ OR Post lingual_____

Stable: _____ OR Progressive: _____

Criteria:

1. Hearing impairment:

Mild:_____ Profound:_____ Unilateral:_____ Bilateral:_____

2.Fever:_____ 3.Dizziness:_____

3.Blindness:_____

4.Other:_____ 5.- Audiometric results_____

Hearing tone:

High:_____ Middle:_____ Low:_____

Food habitats:

Veg/Non veg/Both:_____ Frequency:_____

Family history of subject : _____

Note: Pedigree chat (If required)

ETHICAL CLEARANCE CERTIFICATE

BLDE (DEEMED TO BE UNIVERSITY)

[Declared as Deemed-to-be-University u/s 3 of UGC Act, 1956 vide Government of India Notification No.F.9-37/2007-U.3(A)]

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

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The ethical Committee of this University met on21st December 2018 at 11 a.m.to scrutinizes the Synopsis/ Research Projects of Post Graduate Student / Under Graduate Student Faculty members of this University / College from ethical clearance point of view. After scrutiny, the following original/ corrected and revised version Synopsys of the thesis/ research projects has been accorded ethical clearance.

Title. Genetic and molecular profiling of gjb2 gene in deaf mute population of north karnataka

Name of the Faculty member /PhD/PG/UG student. SmitaHegde

Name of the Guide; Dr. R.S. BULGOUDAMD Associate Professor, Dept of Anatomy

Dr. Sharada Metgud
Chair person
IEC, BLDE (DU),
VIJAYAPURA

Dr.G.V.Kulkarni
Member Secretary
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MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University)
Vijayapura-586103, Karnataka

Note:-Kindly send Quarterly progress report to the Member Secretary

Following documents were placed before ethical committee for Scrutinization.

- Copy of Synopsis/Research Projects
- Copy of inform consent form
  Any other relevant documents

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PLAGARISM CERTIFICATE

BLDE (DEEMED TO BE UNIVERSITY)

PLAGIARISM VERIFICATION CERTIFICATE

1. Name of the Student: **Mrs. Smita Hegde** Reg No: **17PHD007**
2. Title of the Thesis: **Genetic and Molecular Profiling of *GJB2* Gene in Deaf Mute Population of North Karnataka**
3. Department: **Anatomy**
4. Name of the Guide & Designation: **Prof. Rudragouda S Bulagouda**, MD, Professor & HOD, Department of Anatomy, BLDE (Deemed to be University), Vijayapura.
5. Name of the Co Guide & Designation: **Prof. Pramod B Gai**, PhD, Director, Karnataka Institute for DNA Research [KIDNAR], Dharwad

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PUBLICATIONS

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Original Article

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Molecular alteration in the Gap Junction Beta 2 (*GJB2*) gene associated with non-syndromic sensorineural hearing impairment

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SUMMARY Non-syndromic sensory neural hearing defect is one of the genetic diseases inherited from parents to offerings. The autosomal recessive form affects a large population worldwide and has become a major concern in the social and professional lives of many people. There are many factors and genes which are involved in hearing loss but the Gap Junction Beta 2 (*GJB2*) gene which encodes the connexin 26 protein, is a major cause of non-syndromic recessive deafness (NSRD). This study aims to record and analyze *GJB2* gene mutations in the hearing-impaired population of North Karnataka, India. In this study, we included 368 congenitally hearing-impaired children from North Karnataka, India, under 18 years of age. After thorough clinical examinations, patient's history and proper audiological results, peripheral blood samples were collected and subjected to genetic analysis. We recorded that 54.8% of the NSRD cases have an autosomal recessive mutation in the coding region of the *GJB2* gene. The frequency of W24X (25%) mutation was found to be high in the present study population. From this study we can suggest that, identifying this mutation in new-borns definitely helps in the early diagnosis of hearing loss.

Keywords autosomal recessive, non-syndromic recessive deafness, connexin 26, deaf mute, India

1. Introduction

Hearing loss or deafness is one of the most common global health issues, where patients who lose the ability to hear may be permanent or oscillating. Deafness is also one of the most prevalent inherited sensory disorders in most parts of the world (*1*). Approximately 1 in 1,000 new-borns suffer from severe to profound hearing loss. In that > 50% of cases are due to genetic factors (*2*). Environmental factors also play a major role in inducing deafness. For example, congenital hyperbilirubinemia, ototoxic medication, neonatal hypoxia, viral infections, and meningitis are some non-genetic factors inducing congenital hearing loss (*3*). 70-80% of non-syndromic genetic hearing loss is inherited as an autosomal recessive form (DFNB), whereas autosomal dominant forms (DFNA) make up about 10-20% (Dominant forms are designated with the suffix 'A' and recessive forms with suffix 'B') and the X linked form (DFN) accounts for about 1-2% (*4*). DFNB1 gene locus, which is responsible for non-syndromic deafness is present on the 13q11 chromosome locus is most prevalent all over the world (*5*). 13q11 chromosome region includes two major genes viz, connexin 26(*GJB2*) and connexin 30(*GJB6*), which show high involvement in deafness. Connexin 26 (*GJB2*) is the major gene responsible for hearing loss all over the world (*5*). Epidemiological data evaluation of deafness in different populations of the world also revealed that connexin 26 is the single most cause of inherited deafness (*5*). Syndromic forms of deafness which account for more than 500 types can be easily diagnosed whereas non-syndromic forms of deafness can only be resolved by genetic analysis (*6*). The genetic cause of deafness is heterogenous. Until now, more than 100 mapped loci associated with non-syndromic hearing loss have been described (*https://hereditaryhearingloss.org/*). According to the Human Gene Mutation Database (HGMD) the total number of genes involved in hearing loss (HL) is 316, in that 105 genes are related to NSRD. 444 mutations are recorded worldwide specific to the *GJB2* gene (Disease causing mutations-355, non-syndromic HL mutations-51) (*http://*

www.hgmd.cf.ac.uk). W24X mutation in the *GJB2* gene is known to be common in the Indian population. Still, the full spectrum of mutation of this gene occurring in India is not known (*7*).

Connexin is a membrane protein with four transmembrane domains, which are called connexins. These connexin proteins form a hexamer by combining six molecules, called a connexon. A gap junction is formed by the hexamers forming a cell-to-cell channel to the adjacent cells. Small molecules and ions move through this junction to the adjacent cells. Various forms of connexin proteins form different types of hexamers that determine the permeability of different molecules or ions through them (*8*).

2. Materials and Methods

2.1. Subjects

After screening 613 bilateral sensory neural congenital hearing loss children under 18 years of age (median age 12), a total of 368 children (n_{male} = 235, n_{female} = 133) were considered for the study. Children belonging to unrelated families, and having a family history of hearing loss or born to consanguineous marriages were included in the study. Children who had a syndromic hearing loss or were affected by environmental factors and above the age of 18 were excluded from the study. To confirm that the hearing loss occurred because of non-genetic causes (viral and bacterial infections, intake of ototoxic drug during pregnancy, and premature birth), a detailed clinical history and data were collected from each family. After proper physical examination pure tone audiometry (250 to 8,000Hz) was obtained from each child. Clinical samples were collected after obtaining written informed consent from each child. Ethical approval for the study was obtained from the Institutional Ethical Committee of, Shri B. M. Patil Medical College, hospital & research centre, BLDE (Deemed to be University), Vijayapura (Ref no-BLDE(DU)/ IEC/335/2018-19).

2.2. Mutation Analysis

DNA was isolated from peripheral blood using DNeasy blood and tissue kit (QIAGEN, Germany) according to the standard procedures. This was followed by the polymerase chain reaction (New England Biolab, USA). *GJB2* gene (NG_008358) was amplified using specific primers and to check for the proper amplification, PCR products were analyzed by agarose gel electrophoresis. Purified PCR products of the *GJB2* gene were sequenced using forward primer and big dye terminator cycle sequencing kit V3.1 (Applied biosystem, USA) using an ABI 3500 Sanger sequencer. A comparison of the *GJB2* reference sequence to the individual DNA sequence was made to determine the *GJB2* sequence variation.

2.3. Bioinformatics analysis

Pathogenic effects of missense variants were predicted using the following Bioinformatics tools, Protein variation effect analyzer (PROVEN) (*http://provean.jcvi.org/index.php*), Protein Analysis Through Evolutionary Relationships (PANTHER) (*http://www.pantherdb.org*) SNAP2 (*https://www.rostlab.org/services/snap*), Polymorphism Phenotyping2 (PolyPhen-2) (*http://genetics.bwh.harvard.edu/pph2*), Predictor of human Deleterious Single Nucleotide Polymorphisms (PhD-SNP) (*https://snps.biofold.org/phd-snp/phd-snp.html*), SNPs & GO (*https://snps.biofold.org/snps-and-go/snps-and-go.html*) and Deafness Variation Database (DVD) (*https://deafnessvariationdatabase.org/gene/GJB2*). These Bioinformatic tools were used to predict the pathogenic effects of the variant on the functions of the proteins (*9*).

Multiple sequence alignment analyses for connexin 26 protein were performed to find the sequence homology from a common ancestor, which also revealed whether they descended from the same/common ancestor (*10*). Uniport accession numbers from different species were used for the analysis as follows- Xenla *Q7ZYG3*, Mouse *Q009777*, Rat *P21994*, Sheep *P46691*, Macmu *Q8MIT8*, Human *P29033*, Corgo *Q8MHW5*, Canlf *J9NXR*. Homology modelling of the mutated protein was predicted using a Swiss-model server and predicted protein model was visualized, and analyzed on a UCSF chimera program.

3. Results

In this study, we have screened 613 NSRD children from the North Karnataka population. We have included 368 unrelated children with hearing loss (n_{male} = 235, n_{female} = 133) out of that 16.8% (62/368) children had a family history of deafness. Frequency of consanguineous marriage was 42.1% (115/368) in our study. We recorded 18 mutations in exonic and intronic regions of the *GJB2* gene. W24X, and W77X variants were the common mutations identified in this study. A G>A transition at c.71 results in a stop codon at p.24 (W24X) of connexin 26 that produces a truncated protein which is one-tenth the length of the wild type protein and a G>A transition at c.231 results in a stop codon at p.77 (W77X) of connexin 26 protein that also produces truncated protein. Both these premature stop codons result in complete loss of the *GJB2* gene function. W24X (25%) is one of the common mutations observed in the study cohort. 86 (23.3%) deaf children were homozygous and 6 (1.6%) deaf children were heterozygous for this mutation and the W77X mutation was also found to be homozygous (Figure 1).

In our study, we also recorded 3 missense mutations, namely R127H, V153I, and I33T. The frequency of R127H (14.9%) mutation among deaf children was

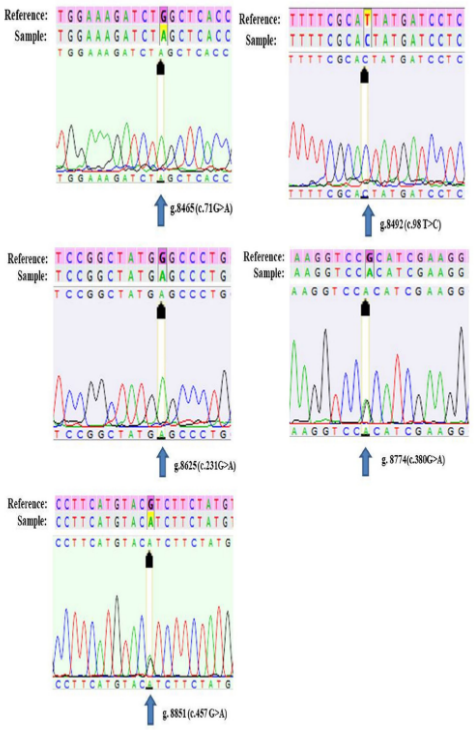

Figure 1. Sequence electropherograms of mutations identified in the present study. W24X (c.71G>A), I33T (c.98T>C), and W77X (c.231G>A) mutations were recorded as homozygous. Whereas R127H (c.380G>A) and V153I (c.457G>A) mutations recorded as heterozygous.

high as compared to the other two identified missense mutations. Four 31-UTR variants were identified (c.84T>C, c.1067G>T, c.1277T>C, c.1152G>A) (Table 1). There were no novel mutations observed in our study population. In addition to these exonic variants, we have also recorded 9 variants in the intronic region of the *GJB2* gene (Table 2), but these variants are unlikely to be causative. The top and middle electropherograms show the homozygous mutations. The position of mutation is indicated by the arrowhead. Heterozygous mutations are in the bottom c.380 G>A and c.451 G>A (Figure 1).

Pathogenicity prediction of the I33T mutation showed the damaging/deleterious effect on the functions of connexin 26 protein. V153I mutations showed a neutral effect, but R127H mutation showed a harmful effect by PhD-SNP, SNP & GO, and SNAP2 (Table 3). DVD database classified these variants, that shows I33T mutation as pathogenic and the other two mutations V153I and R127H as benign (Table 3). Multiple sequence alignment of the connexin 26 protein was analyzed using the Clustal omega. Mutation I33T and R127H were highly conserved over different species and mutation V153I was semi-conserved (Figure 2). The 3D models for mutated Connexin 26 protein (W24X and W77X) were generated using SWISS-MODEL. The superimposed model shows the loss of the majority of the connexin 26 protein sequences from both mutated proteins (Figure 3). Residue W24 and W77 are present in the first transmembrane (S1) domain and second transmembrane (S2) domain of Connexin 26 respectively.

Table 1. List of mutations identified in the coding sequence of *GJB2* gene in the present study

Sl. No.	Nucleotide change	AA change	Frequency	Phenotype	Description and type of effect
1	c.71 G>A	W24X	25%	Congenital profound hearing loss	Stop Gained
2	c.98 T>C	I33T	5.2%	Congenital profound hearing loss	Missense variant
3	c.231 G>A	W77X	4.8%	Congenital severe hearing loss	Stop gained
4	c.380 G>A	R127H	14.9%	Congenital severe hearing loss	Missense
5	c.451 G>A	V153I	4.9%	Congenital profound hearing loss	Missense
6	g.9159T>C	----------	100%	Congenital profound hearing loss	3'-UTR Variant
7	g.10142 G>T	----------	100%	Congenital severe hearing loss	3'-UTR Variant
8	g.10352 T>C	----------	100%	Congenital profound hearing loss	3'-UTR Variant
9	g.10227G>A	----------	5.1%	Congenital severe hearing loss	3'-UTR Variant

Table 2. Mutations were identified in the intronic region of the *GJB2* gene in the present study

Sl. No.	Nucleotide change	Frequency	Phenotype	Description & type of effect
1	g.5985C>T	90%	Congenital profound HI	Regulatory region Variant
2	g.6284A>G	90%	Severe to profound HI	Regulatory region Variant
3	g.6514G>A	14.94%	Congenital profound HI	Regulatory region Variant
4	g.7170A>G	100%	Moderate HI	Regulatory region Variant
5	g.7175G>A	25%	Severe HI	Regulatory region Variant
6	g.8151C>S	4.9%	Severe HI	--------
7	g.8207T>W	14.94%	Congenital profound HI	--------
8	g.8332T>G	30%	Congenital Severe to profound HI	--------
9	g.7111G>R	14%	Congenital profound HI	---------

Table 3. Pathogenicity of the missense variant by insilico tools

Variants	SNAP2	PolyPhen 2	PhD-SNP	SNPS & GO	DVD & CADD	PROVEAN	PANTHER
I33T	Effect Score:55	Possibly damaging Score:0.792	Disease P: 0.548	Disease P: 0.548	Pathogenic 25.2	Deleterious Score: -3.722	Possibly damaging
R127H	Effect Score:1	Benign Score:0.001	Disease P: 0.658	Disease P: 0.589	Benign 23.2	Neutral Score: -0.786	Possibly damaging
V153I	Neutral Score: 74	Benign Score:0.003	Neutral P: 0.149	Neutral P: 0.083	Benign 23.4	Neutral Score: -0.205	Possibly damaging

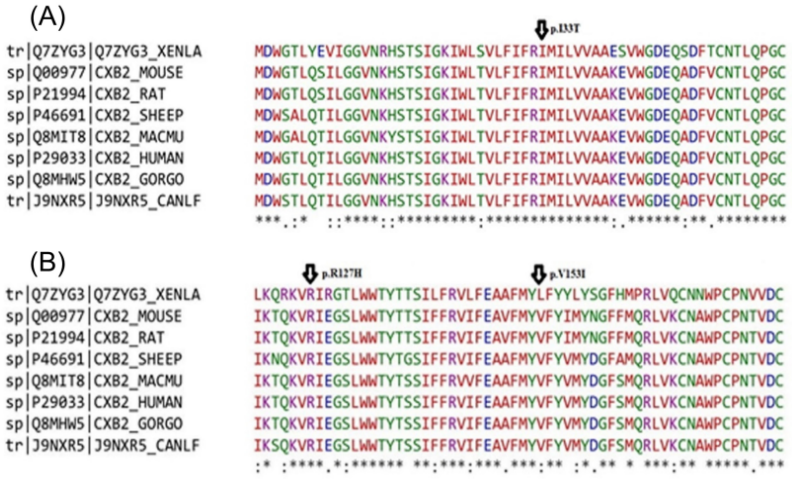

Figure 2. Multiple sequence alignment of connexin 26 protein (A). arrow showing I33T residue conservation (B). first arrow (from left to right) showing the R127H, and second arrow showing the V153I residue conservation.

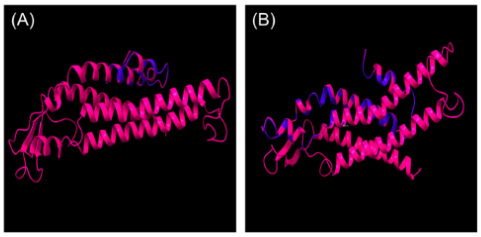

Figure 3. Connexin 26, the 3D structure of the protein. (A), Superimposed connexin 26 protein model with wildtype and truncated protein (Blue- W24 truncated protein, Red- wild type protein); **(B)**, superimposed protein structure W77 truncated and wild type protein (Blue- W77 truncated protein, Red- wild type).

4. Discussion

An overview of the mutation spectrum of the connexin 26 gene of 368 cohorts with hearing loss is presented in this study. A worldwide study on the GJB2 gene shows that it is a frequently mutated gene compared to other deafness related gene loci (*11*). W24X (25%), R127H (14.9%), W77X (5.2%) mutation frequencies were found to be higher in our study. 2 missense variations were also recorded in our cohorts viz, I33T (4.9%), and V153I (4.9%). In East Asian lineages, c.235delC is the major mutation that causes hearing loss (*12*). European and African ancestors recorded the 35delG and C.167delT mutations as a causative factor for NSHL (*11*). *GJB2* mutations were found in ~11.5% of deaf families of south Iran and c.35delC was the common mutation identified (*13*). In our study, we didn't find 35delG and C.167delT mutations. Few studies done in India show 35delG and c.71G>A (W24X) as major deafness causing gene mutations (*6,14*). Mutations recorded in the *GJB2*(exon 2) coding region (W24X, W77X), described in this study, have been found previously in a few subjects from Indian subcontinents, including India (*5*). In southern Europe and the United States, congenital deaf cases have biallelic *GJB2* mutations (between 30-35%) (*15-17*). A study on the Chinese Hans population has also recorded 25.65% of Hearing-Impaired patients had biallelic mutations in the *GJB2* gene (*18*). Whereas only 10-20% of congenital deaf cohorts in India have biallelic GJB2 mutations. Therefore, we predict that, there may be additional deafness causing genes that are common in India, other than those already found. We

used the Clustal Omega multiple sequence alignment tool, to analyze evolutionary conservation in each amino acid position. It is observed that R127H, and I33T polymorphisms are highly conserved, and polymorphism V153I is semi conserved (Figure 3). Two different studies transfecting the R127H polymorphism to Hela cells have been conducted. In the first study the R127H mutation acts like a normal connexon 26 protein (*19*). In the second study, it forms the gap junction but there is a reduction in the activity of the gap junction (*5,20*). Bioinformatics analysis on mutations observed in our study also revealed that the R127H polymorphism is non-pathogenic (Table 3). In other Indian populations the R127H mutation strongly suggests that, this is not a causative polymorphism for hearing loss even though its recorded at high frequency. W24X mutation in our cohort shows a heterozygous condition in six subjects with hearing loss and also R127H and V153I polymorphisms were heterozygous in 2 samples. The cause of deafness in those subjects could be because of many factors. The major possibility may be the digenic origin, which is an implication of another connexin gene (*GJB3* or *GJB6*). We have restricted our study to a single gene because of the major involvement of the *GJB2* gene in hearing loss.

Over 200 or more GJB2 gene mutations are recorded on the home page of connexin (*http://davinci.crg.es/deafness*), so it's necessary to sequence the complete coding region of this gene. The frequency of the pathogenic mutation (W24X) in the *GJB2* gene is high in our study population. From this study we can suggest that identifying this mutation in new-borns definitely helps in the early diagnosis of hearing loss. So, implementation of strategies to overcome the disability at an early stage is possible. Using sequencing techniques or restriction analysis, assay mutations can be easily identified. In a country like India, there is a high level of consanguinity and ethnicity and as such these techniques can be easily included, which also help in genetic counselling. This will be beneficial in the early rehabilitation of congenital hearing-impaired children. There is a need for expanding screening in other deafness genes to further resolve *GJB2* mutations. This is the first study to see the effect of genetic variation in deafness in the north Karnataka population. In north Karnataka, out of 12 districts only 4 districts have been included for the genetic analysis of the single targeted gene (GJB2) in hearing impaired children was the major limitation of our study.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Original Article

Analysis of Genetic Variations in Connexin 26 (*GJB2*) Gene among Nonsyndromic Hearing Impairment: Familial Study

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Glob Med Genet

Abstract

Objective The goal of this research was to investigate the gap junction beta 2 (*GJB2*) gene mutations associated with nonsyndromic hearing loss individuals in North Karnataka, India.

Materials and Methods For this study, patients with sensorineural genetic hearing abnormalities and a family history of deafness were included. A total of 35 patients from 20 families have been included in the study. The patient's DNA was isolated from peripheral blood samples. The *GJB2* gene coding region was analyzed through Sanger sequencing.

Results There is no changes in the first exon of the *GJB2* gene. Nine different variants were recorded in second exon of the targeted gene. W24X and W77X are two nonsense mutations and three polymorphisms viz. R127H, V153I, and I33T were reported along with four 3′-UTR variants. A total (9/20) of 45% of families have been identified with mutations in the targeted gene.

Conclusion *GJB2* mutations were identified in 19 deaf-mute patients (19/35), and 13 patients were homozygous for the mutations identified in our study cohort. In our study, W24X mutation was found to be the pathogenic with a high percentage, prompting further evaluation of the other genes, along with the study of additional genetic or external causes in the families, which is essential.

Keywords
- variation
- familial
- nonsyndromic hearing Impairment
- *GJB2*
- connexin 26

Introduction

One in 1,000 newborns has been documented congenital hearing loss (HL), of which half are attributed to a genetic origin. In inherited deafness, the gap junction beta 2 (*GJB2*) gene (NG_008358) mutations are one of the single most frequent causes.[1,2] And worldwide recessive mutations in *GJB2* genes are commonly recorded in genetic HL.[3,4] Identification of genes and mutations by genetic analysis in deaf-mute

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children may reveal the unique behavior of several mutant alleles. Worldwide study on deaf-mute populations also recorded the *GJB2* gene involvement in causing the HL. Many of those studies involved subjects from the Indian population.[5,6] Though they included the Indian subjects, those studies failed to show the prevalence of connexin 26 mutation in the Indian cohorts. HL, though not life threatening, can become a major burden in social and professional life, and also the *GJB2* gene has emerged as the predominant cause of deafness worldwide.[3] Connexin protein contains different domains (cytoplasmic domain [CL], extracellular domains [E1–2], and transmembrane [TM1–4] domains) and all are connected in the membrane to form connexon or channels.[7] *GJB2* gene is present on chromosome 13q12, which codes for connexin 26 and it is present on DFNB1 locus.[8,9]

GJB2 is a small gene, and as such, analysis and checking for mutations is easy. For nonsyndromic congenital deaf patients, *GJB2* gene analysis gives a good starting position for mutation study.[8] Nonsyndromic hearing loss (NSHL) is a riddle that can be resolved through genetic tests, and genetic diagnosis always gives the better knowledge of abnormal and normal sensory processes.[1] To date, ~180 genes have been identified which are associated with HL. In that, 124 genes are specifically involved in the NSHL (https://hereditary-hearingloss.org/). Among these genes, *GJB2* is a major etiological cause of nonsyndromic hearing impairment.[2] A total of 444 mutations related to HL are recorded to date, in those, 355 disease-causing mutations and 51 mutations come under NSHL (http://www.hgmd.cf.ac.uk/ac/index.php), and they also exhibit a variety of audiometric phenotypes mild to profound. A detailed report has already been made on connexin 26 protein expression patterns correlated with the audiometric phenotypes form and in the transfected cell.[10] A mouse model study on *GJB2* gene-related deafness also showed that there are drastic changes in gap junction and decreased intercellular communication in the cochlea.[2,10] A connexin 26 protein is major protein required for normal working cochlea during the hearing. In nonsyndromic deafness (DFN), DFNB1 is the first mapped region, suffix B for the autosomal recessive inheritance.[6] *GJB2* gene mutations causing DFNB1 HL identification helps reveal a high frequency of *GJB2* mutation among NSHL patients. Since then, worldwide, most of the population demonstrated DFNB1 HL.[9,11] So, the *GJB2* gene became one of the major targets for mutation searching by the genetic diagnosis of nonsyndromic sensorineural HL. However, the mutation caused by the *GJB2* gene was majorly recorded to follow recessive inheritance, and results occurred in DFNB1 nonsyndromic hearing impairment followed homozygous or compound heterozygous pattern of inheritance.[12]

Materials and Methods

Clinical and Audiometric Analysis

In this study, a molecular analysis of the *GJB2* gene was performed on 35 affected individuals from 20 families, who showed NSHL. All the probands were examined for HL and also for any physical illness apart from HL. Before going to the audiometric test, all the individual's information was obtained by personal interview to know clinical history, family history of HL, or any other disorders running in the family and also about consanguinity. This information was obtained after taking the informed consent from each patient.

Pure tone audiometry was done on each individual and a hearing grade was obtained based on the results. Only NSHL probands without any acquired (associated) etiology were included for the study. Probands having the sign and symptoms other than HL or any infections history such as rubella, meningitis, and history of ototoxic drugs intake during pregnancy were excluded from the study. Two to 3 mL of peripheral blood was collected in an EDTA vacutainer (BD, United States) along with the written consent and family pedigree from the patients.

Statement of Ethics

In 2018 to 2019, ethical permission for this study was obtained by Shri B.M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura (Ref no. BLDE(DU)/IEC/335/2018–19), and Karnataka Institute for DNA Research (KIDNAR), Dharwad (Ref no. KIDNAR/2016/07/05).

Mutation Analysis

A DNeasy Blood and Tissue Kit was used to extract DNA from the patient's blood sample (QIAGEN, Germany). The extracted DNA's purity and amount were confirmed using a nanodrop spectrophotometer (Quawell, Q3000 UV spectrophotometer) and % gel electrophoresis, respectively. Sequencing was performed with the help of big dye terminator cycle sequencing kit V3.1 (Applied biosystem, United States) on the ABI 3500 Sanger sequencing platform. The complete coding region (exons 1 and 2) of the *GJB2* gene was sequenced. Before sequencing, the coding region was amplified with the help of a polymerase chain reaction (PCR) technique. Only purified PCR products were subjected to sequencing. Results were compared with the standard reference sequence in the NCBI database (NG_008358) to confirm the changes in the patient's nucleotide sequence. The list of primers used was tabularized in ►**Table 1**.

Insilico Analysis for *GJB2* Gene

We used bioinformatics-based methods to predict the effect of nucleotide changes recorded. dbSNP, 1000 Genome, ExAc, and ClinVar databases were used to identify the variations which are already recorded by different studies. Pathogenicity of the recorded mutations was checked by the different insilico analysis tools such as PolyPhen-2,[13] PANTHER, PROVEAN, PhD-SNP, SNPs&GO, and SNAP2. Evolutionary conservation of Connexin 26 protein sequence analyzed using the ConSerf server.

Results

Clinical and Audiometric Results

The patient's history and physical examinations did not show any environmental factors influencing deafness, confirming

Table 1 The list of primers used for the study

Exon	Primer ID	Nucleotide sequence
Exon 1	DM-EX1-F	CCCTCCGTAACTTTCCCAGT
	DM-EX1-R	CCAAGGACGTGTGTTGGTC
Exon 2	DM-EX2A-F	CCTGTTTTGGTGAGGTTGTG
	DM-EX2A-R	TGGGTTTTGATCTCCTCGAT
	DM-EX2B-F	CTACTTCCCCATCTCCCACA
	DM-EX2B-R	CCTCATCCCTCTCATGCTGT
	DM-EX2C-F	GTTTAACGCATTGCCCAGTT
	DM-EX2C-R	GGCACTGGTAACTTTGTCCA
	DM-EX2D-F	CCAACTTTCCCCACGTTAAA
	DM-EX2D-R	TGGCTACCACAGTCATGGAA
	DM-EX2E-F	GCACAGCTGAGAGGCTGTCT
	DM-EX2E-R	GCTGAAGGGGTAAGCAAACA
	DM-EX2F-F	GGGGAGGGAGAAGTTTCTGT
	DM-EX2F-R	AATGGGGTCAGACACTCTGG
Intron	DM-IN1A-F	CTGGACCAACACACGTCCTT
	DM-IN1A-R	GGAAACAGACCCTCGTGAAG
	DM-IN1B-F	CAGAGATTTGGGCGGAGTT
	DM-IN1B-R	TCACCAGGATCCAGAAAAGG
	DM-IN1C-F	TGCACAGTCGGTCACAATTT
	DM-IN1C-R	CCAAACCCAGGTCATACACC
	DM-IN1D-F	TCAGCTGATGGTAACTGGACA
	DM-IN1D-R	CACCAAGGTCAGGCAGAAAC
	DM-IN1E-F	TGTTGTCTTTCCCAAGCTCA
	DM-IN1E-R	TCAACTCCCTCGGTTACTGG
	DM-IN1F-F	CGCTTGCAGTAAGGAGTGTG
	DM-IN1F-R	AGGCTGAGAGGCCAAGTACA
	DM-IN1G-F	CACTGCTACATGCCACGTCT
	DM-IN1G-R	TCTTCCTGAGCAAACACCAA

Note: The coding and noncoding regions of the *GJB2* gene (complete gene) were amplified with the help of 14 sets of primers.

the nonsyndromic form of deafness. All patient's hearing tones, severity, and types were revealed by the audiological outcome (200–8,000 Hz). The hearing level and degree of HL were defined, according to the mean. Up to 25 dB (hearing level measured in decibels [dB]) Normal hearing is defined as 26 to 40 dB; mild hearing is defined as 41 to 70 dB; moderate hearing is defined as 71 to 90 dB; severe hearing is defined as >90 dB; and profound hearing is defined as >90 dB.[14]

Molecular Study Results

Nine different variants ($n_{family} = 20$, $n_{pateints} = 35$) were identified in our study cohort. They included two pathogenic nonsense variations, three missense variations, and four 3′-UTR variations (shown in ►**Table 2**). c.71G > A nonsense pathogenic variants recorded in nine affected individuals (9/35). In nine affected individuals, one patient was heterozygous for c.71G > A and c.380G > A variants. Remaining eight were identified as homozygous for c.71G > A (p.Trp24Ter). Three missense variations viz. c.380G > A, c.457G > A, and c.98T > C were identified in five individuals. Two individuals were occurred in heterozygous state for c.457G > A (p.Val153Ile), and other two were heterozygous for c.380G > A (p.Arg127His) variant. Remaining one was homozygous. Addition to this four, different 3′-UTR variants were identified in the five affected individuals.

Family Pedigree Analysis

To explore the pattern of inheritance of coding region mutations c.71G > A, c.231G > A, and 380G > A, all members of five families (Family 2, Family 7, Family 8, Family 19, and Family 20) were submitted to Sanger sequencing, and their pedigrees are illustrated in ►**Fig. 1**. The potentially damaging c.71G > A and c.231G > A genotypes are passed down as a homozygous recessive manner. In Family 2, the proband was homozygous for c.380G > A, and his impacted sibling was heterozygous (as indicated in ►**Table 3**), but their unaffected father was homozygous and their unaffected mother was heterozygous for the same variation. The mutation c.380G > A is not a pathogenic variant, thus the causative gene in this family must be distinct from the chosen gene. In Family 7, the proband was homozygous, while parents were heterozygous for c.71G > A variant. In the instance of Family 8, the proband was homozygous for c.71G > A, and his father, who also had NSHL, did not reveal any pathogenic variations in the genes included for this analysis, indicating that other genes may be involved in HL. The proband and both of his afflicted siblings were determined to be homozygous for the c.71G > A pathogenic mutation in Family 19. And his other two siblings were perfectly normal. Because of newborn screening and subsequent treatments, the proband can converse vocally. Family 20 is the perfect example of a compound heterozygous inheritance pattern. The proband is compound heterozygous for both the c.71G > A and the c.380G > A alleles. Each heterozygous parent passed on these variations to the proband. In our cohort, the most prevalent mutations in the *GJB2* gene were c.71G > A and c.380G > A. Because of the great diversity of autosomal recessive NSHL, epidemiological investigations across a diverse range of ethnic groups are required to determine the prevalence of GJB2 mutations as a cause of hearing impairment.

Discussion

We conducted this research to discover the gene variants that cause NSHL in the North Karnataka population of India. This is the first kind of screening program conducted on the NSHL population of the North Karnataka region. Heterogenicity in genetic HL and also the involvement of different alleles in HL in different populations were major reasons for conducting this study.[15–20] In our study group, pathogenic variants specific to the *GJB2* gene were identified in 9 individuals out of 35 affected individuals (28%). Various genetic research on HL have also revealed that the c.71G > A (W24X) mutation is the most common pathogenic variation causing NSHL in

Table 2 Genetic variation recorded in our study cohort

Gene	dbSNP	Nucleotide sequence variant	Effect on protein	Protein domain	Function consequences	Clinical significant	N/R
GJB2	rs104894396	c.71G > A	W24X	TM1	p.Trp24Ter (nonsense mutation)	Pathogenic	R
	rs80338944	c.231G > A	W77X	TM2	p.Trp77Ter (nonsense mutation)	Pathogenic	R
	rs575453513	c.98T > C	I33T	TM1	p.Ile33Thr (missense mutation)	Possibly damaging	R
	rs111033196	c.380G > A	R127H	CL	p.Arg127His (missense mutation)	Benign	R
	rs111033186	c.457G > A	V153I	TM3	p.Va153Ile (missense mutation)	Benign	R
	rs3751385	c.*84T > C	–	–	3′-UTR variant	Benign	R
	rs9237	c.*1067G > T	–	–	3′-UTR variant	Benign	R
	rs7988691	c.*1277T > C	–	–	3′-UTR variant	Benign	R
	rs7623	c.*1152G > A	–	–	3′-UTR variant	Benign	R

Abbreviations: CL, M2-M3 cytoplasmic loop; N, new; R, recorded; TM1, transmembrane domain 1; TM2, transmembrane domain 2; TM3, transmembrane domain 3.
Note: The position of the nucleotides following the 3′ of the translation loop codon is indicated by the c* number. The genotype frequencies are based on the North Karnataka population.

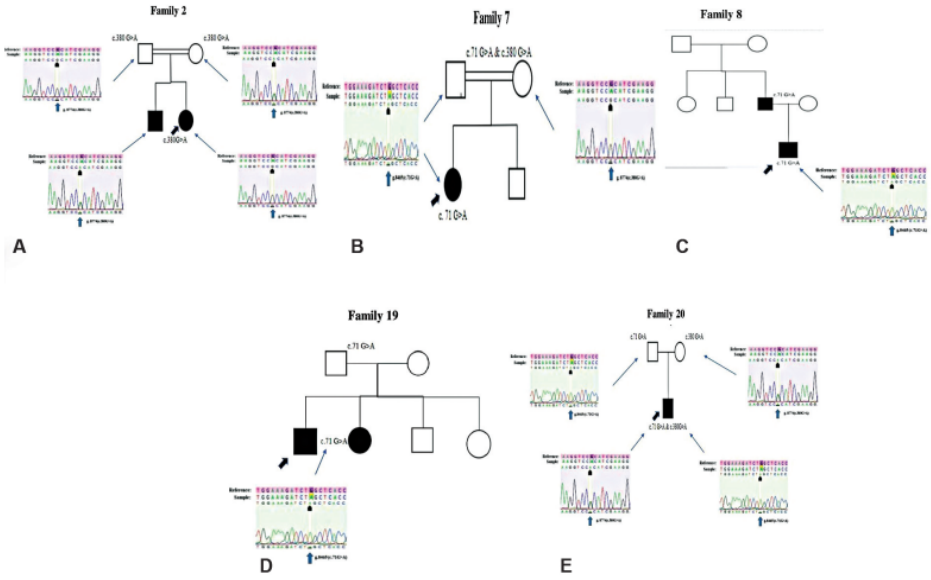

Fig. 1 Five-family pedigree. The graphic depicts *GJB2* gene mutations (**A–E**). (**A**) Pedigree and electropherograms of the missense mutation c.380G > A in Family 2. (**B**) A pedigree demonstrating the inheritance pattern of the nonsense mutation c.71G > A in Family 7. (**C**) A pedigree demonstrating the inheritance pattern of the nonsense mutation c.71G > A in Family 8. (**D**) Pedigree showing the pattern of inheritance of the nonsense mutation c.71G > A (p.Trp24Ter) in Family 19. (**E**) The pedigree of Family 20 demonstrates the inheritance pattern of a compound heterozygote of c.71G > A and c.380G > A, as well as electropherograms. (The pedigree was created with the visual paradigm online diagram tool [https://online.visual-paradigm.com/drive/#diagramlist:proj=0&new=PedigreeChart]).

India.[5,19,21] In this study also, 8 (8/35) probands showed c.71G > A (W24X) mutation. This mutation is present on TM1 of the connexin 26 protein. As a result, the protein was truncated to one-tenth the sequence of the wild-type protein. This is due to the G > A transition at c.71, which results in a stop codon at p.24 (W24X) of connexin 26. The bioinformatics results also support the deleterious effect of CX26 protein by this mutation (shown in ►**Table 4**). This c.71G > A dominance in the Indian population might be due to the founder effect.[5,21] Other variants identified in this study were c.380G > A (R127H) and c.457G > A (V153I), which were classified as "*others*" because protein function

Table 3 Clinical features of probands and family details

Patient code		Sex/age	Clinical feature	Heavy medication history	HL level	HL type	Age onset	Variant finding
Family	Pedigree							
DMF2	Father	M/45	Healthy	No	Normal	BN	NK	c.380G > A
	Mother	F/30	Healthy	Yes (second pregnancy)	Normal	BN	NK	c.380G > A
	Brother	M/13	NSHL	–	Moderate	Unilateral HL (right ear)	6 y	
	Proband	F/10	NSHL	–	Profound	Bilateral sensorineural high-frequency HL	By birth	
DMF7	Father	M/35	Healthy	No	Normal	BN	–	c.71G > A
	Mother	F/30	Diabetic	Yes	Normal	BN	–	c.380G > A
	Brother	M/10	Healthy	–	Normal	BN	–	–
	Proband	F/7	NSHL	–	Profound	Bilateral sensorineural HL	By birth	c.71G > A
DMF8	Grand father	M/70	Healthy	–	Normal	BN	–	–
	Grand mother	F/60	Healthy	–	Normal	BN	–	–
	Father	M/48	NSHL	–	Severe	Bilateral HL	7 y	c.71G > A
	Mother	F/40	Healthy	No	Normal	BN	–	–
	Proband	M/15	NSHL	No	Profound	Bilateral sensorineural HL	By birth	c.71G > A
DMF19	Grand father	M/75	Healthy	–	Normal	BN	–	No DNA available
	Grand mother	F/61	Healthy	–	Normal	BN	–	No DNA available
	Father	M/40	Healthy	No	Mild	Unilateral HL(left ear)	NK	c.71G > A
	Mother	F/36	Healthy	–	Normal	BN	–	–
	Sister 1	F/18	Healthy	–	Normal	BN	–	–
	Brother	M/15	Healthy	No	Mild	Bilatreal HL	NK	No DNA available
	Sister 2	F/10	NSHL	No	Severe	Bilateral sensorineural HL	8 y	c.71G > A
	Proband	M/8	NSHL	No	Profound	Bilateral sensorineural high-frequency HL	By birth	c.71G > A
DMF20	Father	M/50	Healthy	–	Normal	BN	–	c.71G > A
	Mother	F/39	Healthy	–	Normal	BN	–	c.380G > A
	Proband	M/6	NSHL	No	Profound	Bilateral sensorineural high-frequency HL	By birth	c.71G > A & c.380G > A

Abbreviations: BN, bilateral normal; HL, hearing loss; NK, not known; NSHL, nonsyndromic hearing loss.

Table 4 Clinical significance of identified *GJB2* variant by insilico analysis

Variant	dbSNP	PROVEAN	PhD-SNP	x	SNPs&GO	CADD	DVD
I33T	rs575453513	Deleterious Score: −3.722	Disease P: 0.548	Possibly damaging Score: 0.792	Disease P: 0.548	25.2	Pathogenic
V153I	rs111033186	Neutral Score: −0.205	Neutral P: 0.149	Benign Score: 0.003	Neutral P: 0.083	23.4	Benign
R127H	rs111033196	Neutral Score: −0.786	Disease P: 0.658	Benign Score: 0.001	Disease P: 0.589	23.2	Benign

Abbreviations: CADD, combined annotation dependent depletion.
Note: *PROVEAN*: If the prediction score is −2.5, the impact is "destroys." *SNPs&GO*: If the probability is more than 0.5, it is expected to be a disease-causing nsSNP. *PolyPhen-2*: With a score close to 1, the most disease-causing capacity is "probably damaging." With a score of 0.5 to 0.8, "possibly damaging" has less disease-causing capacity. "Benign" means that it has no effect on protein functions and has a score close to 0. *PHD-SNP*: If the likelihood is more than 0.5, the mutation is projected to be "disease," and if the probability is less than 0.5, the mutation is anticipated to be "neutral."

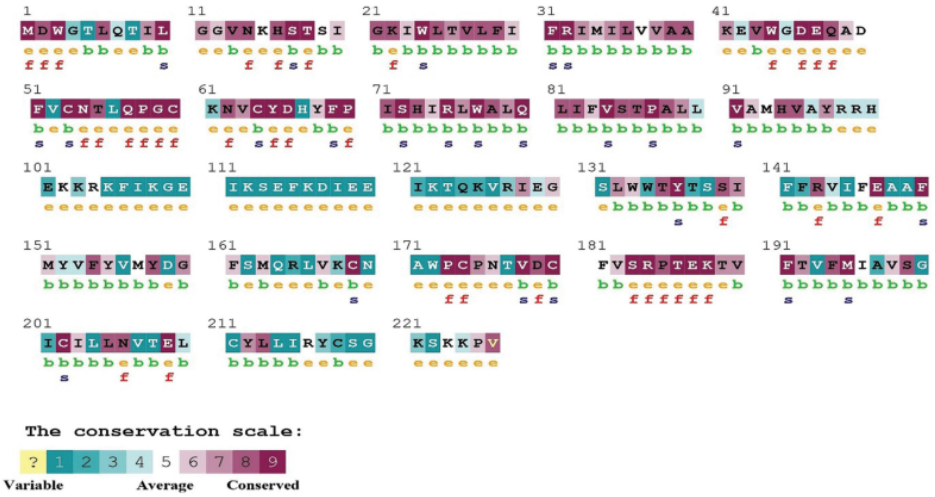

Fig. 2 Protein (connexin 26) conservation of p.W24X and p.R127H (p.W24X is highly conserved and buried, and p.R127H mutation is variable).

prediction tools such as PROVEAN and PolyPhen-2 classified these variations as benign, whereas PhD SNP, SNPs&GO, SNAP2, and PANTHER classified them as pathogenic[22] (shown in ►**Table 4**). c.380G > A (R127H) mutation found on the cytoplasmic domain of CX26, which affects the residue is not highly conserved among the β connexin.[22] This implies the nonpathogenic nature of R127H mutation shown in ►**Fig. 2**. However, functional studies of this variant were nonconsistent.[23] Previous studies conducted on HL in India also recorded c.380G > A (R127H) mutation in high frequency,[5,23–25] like in this study, 5 probands were recorded with c.380G > A variant (5/35). Our pedigree study confirms that the c.380G > A variation is not pathogenic and does not induce HL in the families. Previous research on the Indian population backs up this claim.[5,23,25] In this study, another mutation was recorded which is also involving a premature stop codon resulting in nonsense mutation c.231 G > A (W77X). Only a single proband has been diagnosed with this mutation (1/35), even though the frequency of this mutation was very less in previously reported HL subjects.[5,12,26] The commonly found GJB2 mutation c.35delG in white and c.235delC in Chinese and Japanese were surprisingly not seen in our cohort.[16,17,20] Although the variant c.35delG was reported in the North Indian population,[27] the frequency of the mutation was low compared with the W24X and R127H mutations. We have found four 3′-UTR variants in *GJB2* in our study group, but no functional analysis was conducted on these UTR variants.

The frequency of W24X mutation was found to be very high in our study population, and the absence of c.35delG, c.235delC, and 167delT mutations could be population specific.

Conclusion

The current study's findings show that mutations in the *GJB2* gene are a substantial contributor to NSHL in the North Karnataka community, which varies from the findings of other ethnic groups' studies. Nineteen (54%) deaf-mute patients were detected with the *GJB2* mutations. Out of

that, 13 (37%) patients were homozygous. W24X and R127H mutations were recorded in high prevalence in our study group compared with the other missense variations and 3′-UTR variations. W24X mutation was recorded as pathogenic and R127H mutation was recorded as benign. Thus, W24X mutation in the *GJB2* gene appears to play a major role in familial deafness. Further investigation of the other discovered gene regions, as well as the search for other genetic reasons in the genetic deafness family group, is required. This study shows that investigation of the *GJB2* gene is a preliminary step before going to next-generation sequencing.[9] We also wanted to add that analysis of genes using Sanger sequencing for mutation study may be economical and also be a faster diagnostic technique.

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Conflict of Interest
None declared.

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Epidemiological study on nonsyndromic hearing impairment: North Karnataka, India

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Abstract:

BACKGROUND: In India, hearing loss is a major but often ignored problem. Sixty-three million individuals (6.3%) in India have severe hearing loss.

OBJECTIVE: The present study is the first to examine epidemiological factors that are pertinent to children from North Karnataka, India, who have nonsyndromic hearing loss.

MATERIALS AND METHODS: This study was conducted by attending government and private ENT hospitals and special schools for deaf children in North Karnataka. A total of 638 children with hearing loss of age ranging from 1 to 18 years were screened. Two hundred and seventy out of 638 were excluded from the study because of other anomalies and some were not ready to participate in the study. A semistructured interview was followed for data collection. All the required personal demographic details and medical details of ear examination of inspection history, otoscopy, tympanometry, and pure-tone audiometry were noted. Age, gender, hearing loss type severity, family history, and consanguinity were considered epidemiological factors in the study.

RESULTS: Statistical analysis showed that there was no significant association of age difference between the two genders, but the majority of male children were deaf compared to female children (63.9% vs. 36.1%). Prelingual hearing loss was observed in 94.6% and postlingual hearing loss was in 5.4%. 93.5% of children had bilateral deafness and only 6.5% had unilateral deafness. In the present study population, 3.5% of children had mild, 7.3% had moderate, 2.7% had severe, and 86.4% had profound hearing loss. 83.2% of the children had a family history of deafness. There was no significant association of consanguinity to deafness in our study group ($P = 0.07$).

CONCLUSION: The prevalence of hearing loss was high in the North Karnataka population. We observed the following in our research group: gender, poor education or lack of awareness of pregnancy, and lack of knowledge of consanguinity. so, People need to Know more about these disorders and this is only possible by the Research or education.

Keywords:

Deafness, hearing impairment, nonsyndromic, North Karnataka

Hearing loss is a serious problem in India, although it is frequently overlooked. India's severe hearing loss affects 63 million people (6.3%).[1] Hearing loss can occur by different reasons like Presbycusis, middle ear infections such as chronic suppurative otitis media etc. and genetic and congenital reasons [Table 1]. In 1981, the National Sample Survey Organization (NSSO) carried out the first extensive national survey of people with physical disabilities. Following that, a comprehensive census of India's population with disabilities was carried out in 2002 as part of the 58th round of the NSSO. For every 100,000 persons, there are 291 people with severe to profound hearing loss, according to the 58th round of the NSSO. A substantial portion of the 291 persons

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Table 1: The etiologic factor of acquired hearing loss

Disease Type	Affected by
Prenatal infectious disease	Rubella, measles, mumps, poliomyelitis, pneumonia caused by a virus, herpes zoster, toxoplasmosis, hepatitis, other severe virus infections
Noninfectious disease	Alcohol, drugs, aminoglycosides, diabetes mellitus, X-rays, thalidomide, hypoxic events nephropathy
Perinatal	Asphyxia, rhesus-incompatibility, congenital heart failure, herpes, premature birth, birth trauma with intracerebral hemorrhage hyperbilirubinemia
Postnatal	Meningitis, sepsis, measles, herpes zoster, ototoxic drugs, sound exposure

group is made up of children between the ages of 0 and 14.[1]

Hearing loss is a big global issue. In Southeast Asia, deafness affects 4.6% to 8.8% of the population. Low-income or developing countries (15%–26% of the global population) had the highest prevalence.[2] The prevalence rate of deafness has been determined from a population-based research undertaken by several countries.[3] Explanation of deafness continues to be explored in developing countries such as India. Understanding the cause and frequency of hearing loss is critical for providing a good health-care system.[3,4]

Nonsyndromic hearing loss (NSHL) and prelingual deafness (before language has been established) are the main subjects of this research work. Congenital hearing loss has a genetic component in more than 50% of cases. Over 400 illnesses can include hearing loss or decreased hearing abilities. In more than 70% of cases, however, nonsyndromic deafness is present. Familial or spontaneous occurrences of NSHL are common, and transmission is mostly autosomal recessive. Several genes responsible for deafness have been discovered using linkage studies: more than 50 loci associated with NSHL have been identified (dominant and recessive autosomal, and X-linked transmission types) http://dnalab-www.uia.ac.be/dnalab/hhh/.[5]

The recessive DFNB1 locus on chromosome 13q12 is by far the most prevalent genetic variation despite the high genetic diversity. The Gap junction genes GJB2 and GJB6, which, respectively, encode connexin 26 (CX26) and connexin 30 (CX30), are located in this area (CX30). These proteins form homo- and hetero-connexons in hexamers.[6,7] To create a functional channel that facilitates the flow of potassium ions, which are required for typical sensory hair cell excitation, two connexons from adjacent cells dock.[6]

It is critical to diagnose hearing loss early to comprehend auditory stimulations. The modification of the auditory path occurs throughout the first 18 months of life.[5] Hearing aids can be used to prevent mutism during this period.

Hearing loss is one of man's most frequent afflictions, with approximately 700 million people suffering from pure-tone hearing loss of higher than 55 decibels globally. According to prelingual hearing loss epidemiological statistics, one out of every 1000 newborns is born with severe-to-profound hearing loss, with 50% of the loss being hereditary. Syndromic and NSHL are the two types of hearing loss. About 70% of congenital cases with hereditary components are classed as nonsyndromic deafness and the remaining 30% as syndromic deafness based on clinical findings.

In their inquiry to determine the origin of bilateral sensorineural hearing loss, In 2000, study found that the age of the initial diagnosis varied from 4 months to 11 years, with a mean of 42 months.[5] It was found that the sex ratio between men and women was about 1:1. In 38% of cases, the cause of hearing loss was found to be acquired, while 18% of cases included genetic inheritance. However, in 44% of cases, the source of the hearing loss could not be determined. Different studies showed parental education was found to be a major demographic component in the cause of deafness.[5,8] In research looking for a risk factor for childhood sensorineural hearing loss, Sutton and Rowe[8] discovered that families with poor socioeconomic status had a considerably higher number of instances of hearing loss. In addition, they found that a considerable percentage of cases (24%) had craniofacial anomalies, such as nonaural abnormalities and dysmorphic characteristics. The current study was carried out to better understand the various epidemiological aspects linked to NSHL in children from North Karnataka due to the severely low epidemiological data for NSHL in children from North Karnataka.[9] Mutations in the *GJB2* gene have been linked to inherited nonsyndromic deafness in a variety of groups. There are several sequence variations in the *GJB2* gene, and the pathogenic consequences of these differences are not always evident. Because the prevalence of certain mutations varies by group, community-specific testing should be a requirement when molecular diagnostics is given. To determine the impact of *GJB2* mutations in the development of deafness, population studies are also necessary.[6] Because there is a scarcity of epidemiological data for NSHL in the North Karnataka region, the current study was undertaken to better understand epidemiological factors such as age, gender, family history, type of hearing loss, severity of hearing loss, and consanguinity related to NSHL in children from the 4 districts of North Karnataka.[9] This study is about sensorineural deafness that is not accompanied by any abnormalities.

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Materials and Methods

This research was carried out in North Karnataka by visiting government offices, ENT centers, and special schools for the deaf. A total of 638 children with hearing loss, ranging in age from 1 to 18 years, were tested; however, only 368 met the inclusion criteria for NSHL and were chosen for this study. The sample size was calculated using the country's prevalence rate (6.3%) (World Health Organization), with a 95% confidence level and a 5% absolute error. The result of a simple random sample was 185 from 7 districts of North Karnataka. However, out of 638 youngsters that were screened, 368 were chosen. Nonsyndromic deafness was found in 368 of the youngsters. Two hundred and seventy of the 638 participants were excluded from the study due to other irregularities, and some were not willing to participate. During the initial assessment, a detailed clinical history and physical examination were conducted. A standard questionnaire was used to collect information on the child's age, gender, religion, parents' occupation, and literacy, as well as the child's birth history, developmental, and family history. In addition to physical and clinical examinations, audiological studies (pure-tone audiometry) were used to establish the type and severity of hearing loss. Wherever it was required, psychologists and ophthalmologists were contacted. Children with nonsyndromic deafness were given a higher priority. The obstetrical report, pediatric data, and neurological findings were all examined to determine the cause of the hearing loss. Parents were questioned about their memories of pregnancy, early childhood, and genetic hearing problems in their immediate family. The cause was divided into three categories: unknown, inherited, and acquired hearing loss, with acquired hearing loss occurring in three stages: unborn, perinatal, and postnatal. These data were placed into a Microsoft® Excel 97 chart, and SPSS® was used for statistical analysis. For the optimal stimulation of residual hearing, binaural amplification was advised for any child who was diagnosed with sensorineural hearing loss.

To gather data, a semistructured interview was used. In addition, medical information on the ear examination of inspection history, otoscopy, tympanometry, and pure-tone audiometry was noted. All necessary personal demographic information, such as age, sex, height, and education details, were also provided. From all the 368 children, written informed consent was taken before taking the data. Inclusion criteria: Nonsyndromic deaf children who were ready to participate in the study and they should be below the age of 18 years. Exclusion criteria: Children should not have syndromic deafness and also should be above the age of 18 years. Moreover, those who are not ready to participate were excluded. The Shri B M Patil Medical College Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura's Institutional Ethical Committee granted their clearance for the study (Ref no. BLDE (DU)/IEC/335/201819).

Statistical analysis

The details of each quality were provided. For continuous variables, the summary statistics of mean and standard deviation were used. The numbers and percentages were used in the data summaries and diagrammatic display of categorical data. The Chi-square[2] test was used to determine whether two category variables were related. The means of the study variables were compared between two distinct groups using an unpaired *t*-test. To determine whether there was a difference in the means of the analysis variables between more than two independent groups, analysis of variance and the *f*-test of assessing equality of variance were utilized. The results were considered statistically significant if the $P < 0.05$; otherwise, they were considered nonsignificant. To analyze the data, SPSS software version 23.0 was employed.

Results

Even with amplification, deafness is defined as the inability to use the ears as a major route for hearing speech. As a result of the impairment in speech and language development, hearing loss impacts several facets of a child's overall development. In addition, it could impede and damage the child's financial and social relationships. Hearing impairment is only considered when both ears are completely deaf, or when the person has a hearing loss in the better ear of 70 decibels or more. A person with hearing levels of 61–70 dB was immediately excluded from the hearing disability category, according to the Rehabilitation Council of India Act, 1992.[10]

A simple random sample of 638 hearing-impaired children was taken from several districts in the North Karnataka area of Karnataka, India. The study included 368 children with NSHL (57.7%), whereas the remaining 270 children with syndromic hearing loss (42.3%) were eliminated. In the current study, 235 (63.5%) of the 368 children were male and 133 (or 36%) were female. Patients in the research ranged in age from 5 to 19 years. The majority of patients were between the ages of 10 and 19 years, accounting for 80.7% of the total. Others were between the ages of 10 and 19 years, accounting for 19.3% of the total [Table 2]. The average age was 11.5 years old.

In Karnataka majority of the population is Hindu (84%, Census of India, 2011).[11,12] Hence, Hindus contributed more, whereas Muslims were less (12.92%, Census of India, 2011) and the rest other religions were not seen in our study population.

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In our study, there were 316 children (85.9%) with a low degree of hearing tone, followed by moderate (32; 8.7%) and high (20; 5.4%) degrees of hearing tone. Surprisingly, bilateral deafness affected the greatest number of children (344; 93.5%) compared to unilateral deafness (24; 6.5%) [Table 2]. Consanguineously married parents accounted for 155 (42%) of the deaf children in the current study. In the current study sample, however, there was no significant relationship between hearing loss and consanguinity ($P = 0.07$) [Table 3]. However, 139 (89.7%) of children with substantial hearing loss have parents who are married consanguineously. According to a family history research, 62 hearing loss youngsters (16.8%) had a family history of deafness.

Discussion

In the present study, out of 368 studied children, 235 children (63.5%) were male and 133 children (36%) were female. Similar male predominance was observed in several previous studies performed in the Indian population.[11,13-17] Only few studies recorded female predominance;[18] this may be due to male and female ratio in respective studies. Maximum number of children belonged to the 10–19 years of age followed by <10 years of age (80.7% v/s. 19.3%, respectively). A 1997 study which sampled 1200 children between the ages of 4-17 found high prevalence among those aged 10 and under (8.2%) compared to those over 20 (2.8%).[19] Age-wise prevalence is similar to our results that we recorded high prevalence of <10 years age group. Maximum number of children had prelingual deafness (94.6%) [Table 2].

Our study recorded 316 children (85.9%) with low degree of hearing tone, followed by middle (32; 8.7%) and high (20; 5.4%) degree of hearing tone. Interestingly, maximum number of children had bilateral deafness (344; 93.5%) compared to unilateral deafness (24; 6.5%) [Table 2]. Our results showed deviations to the findings of Bijan Bask, in their study they recorded maximum cases of unilateral hearing loss cases. The study also recorded that 86.4% of the children had profound hearing loss, followed by moderate (7.3%), mild (3.5%), and severe (2.7%) hearing loss [Table 2]. Study conducted in 2001 and 2019, also recorded the high frequency of profound hearing loss, followed by severe, moderate and mild cases.[20,21] 2017 study on north Indian population and records maximum number of individuals with mild hearing loss followed by moderate, severe and profound.[22] This is quite opposite to our results. A significant association of degree of hearing loss with respect to age group was observed in the study cohort ($P = 0.01$) [Table 4]. No significant association was observed for gender and degree of hearing loss ($P = 0.2$) [Table 5].

Table 2: Clinicopathological features of study cohort

Clinical pathological condition	*n* (%)
Age	
<10	71 (19.3)
10-19	297 (80.7)
Gender	
Male	235 (63.9)
Female	133 (36.1)
Nature of deafness	
Prelingual	348 (94.6)
Postlingual	20 (5.4)
Pattern of deafness	
Unilateral	24 (6.5)
Bilateral	244 (93.5)
Degree of hearing loss	
Mild	13 (3.5)
Moderate	27 (7.3)
Severe	10 (2.7)
Profound	318 (86.4)
Family history of deafness	
Yes	62 (16.8)
No	306 (83.2)
Consanguine marriage	
Yes	155 (42.1)
No	213 (57.9)

Table 3: Degree of hearing loss according to consanguineous marriage

Degree of hearing loss	With consanguineous marriage, *n* (%)	Without consanguineous marriage, *n* (%)	*P*
Mild	7 (4.5)	6 (2.8)	0.071
Moderate	8 (5.2)	19 (8.9)	
Severe	1 (0.6)	9 (4.2)	
Profound	139 (89.7)	179 (84.0)	
Total	155 (100.0)	213 (100.0)	

Table 4: Degree of hearing loss according to age

Degree of hearing loss	Age <10 (years), *n* (%)	Age 10-19 (years), *n* (%)	*P*
Mild	8 (11.3)	5 (1.7)	0.001*
Moderate	3 (4.2)	24 (8.1)	
Severe	2 (2.8)	8 (2.7)	
Profound	58 (81.7)	260 (87.5)	
Total	71 (100.0)	297 (100.0)	

*Significant at 5% level of significance ($P<0.05$)

Table 5: Degree of hearing loss according to gender

Degree of hearing loss	Male, *n* (%)	Female, *n* (%)	*P*
Mild	9 (3.8)	4 (3.0)	0.200
Moderate	14 (6.0)	13 (9.8)	
Severe	4 (1.7)	6 (4.5)	
Profound	208 (88.5)	110 (82.7)	
Total	235 (100.0)	133 (100.0)	

One hundred and fifty-five (42%) children of the present studied deaf children has consanguineously married

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parents. However, there was no significant association of hearing loss and consanguinity in the present studied population ($P = 0.07$) [Table 3]. However, interestingly, 139 (89.7%) children with profound hearing loss had parents with consanguineous marriage. Children with consanguineous marriages accounted for 5% of children according to 2019 study;[20] this result is low compared to our study findings.

A family history analysis showed that 62 hearing loss children (16.8%) had a family history of deafness. 2019 study records 4% of children belongs to 0-3 years showed family history of deafness.[20] And also, according to Clifton NA and Swartz's research in 1988,[23] one-tenth of kids with a family history of deafness had hearing impairment. In that, maximum number of deaf children had 2nd and 3rd degree relatives with hearing loss. Overall, 24 hearing loss children had either deaf brother or deaf sister. Statistical analysis showed no significant association of hearing loss and degree of relationship ($P = 0.76$) [Table 6] and also no significant association for family history of the deafness and deafness in the studied children.

The results of the overall research showed that the etiological factors of hearing loss severity, family history, and consanguinity always play a part in the onset of deafness. Hearing loss prevention is still a difficult task, but it is both possible and necessary. The burden of hearing loss must be closely monitored to project future burdens and the resources needed to prevent and control it.

Conclusion

With lesser levels of hearing loss, speech does develop, but it is flawed. The period from birth to age three is crucial for the development of speech and language, therefore early detection and assessment of hearing loss in new-borns and toddlers is imperative.[6] In India, hearing loss is an important but mostly ignored problem. Due to reduced production, greater unemployment, and lower earnings for the hearing impaired, the country suffers a significant economic burden. The actual problem in India is the deplorable lack of deaf facilities of any kind. The North Karnataka region of Karnataka has high rates of consanguineous marriages not only within the state but also across the country.[4,17] Consanguinity is extensively practiced in Indian families. The practices of arranged marriages within families, as well as the public's ignorance of the severe genetic effects of such practices, were also highlighted in this study. The entire analysis exposed the epidemiological factors of age, family history, and consanguinity that play a part in the onset of deafness in the population of north Karnataka. Additionally, the residents of this area are undoubtedly impacted by poverty, a lack of information about deafness, and other reasons. In order to offer high-risk couples with the necessary genetic counseling and help them from having such children, extensive researches on the epidemiology of deafness and the identification of genetically inherited disorders are required.

The small sample size, confined strategy, data collection from limited facilities, lack of additional testing, and imaging all hindered this study.

Future research should focus on a bigger population, with data gathered from all of the region's assessable centers. A full family history, comorbid conditions, medication history, and substance usage history should also be included in the documentation. Impedance audiometry and imaging might also be beneficial in assisting with patient diagnosis.

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Table 6: Degree of hearing loss according to degree of relation

Degree of hearing loss	Degree of relation							*P*
	3rd degree, *n* (%)	4th degree, *n* (%)	6th degree, *n* (%)	First cousin, *n* (%)	Second cousin, *n* (%)	Undetermined, *n* (%)	Nonrelatives, *n* (%)	
Mild	4 (6.8)	0	0	1 (6.7)	0	2 (7.1)	6 (2.8)	0.761
Moderate	4 (6.8)	1 (2.5)	1 (11.1)	1 (6.7)	0	1 (3.6)	19 (8.8)	
Severe	0	1 (2.5)	0	0	0	0	9 (4.2)	
Profound	51 (86.4)	38 (95.0)	8 (88.9)	13 (86.7)	2 (100.0)	25 (89.3)	181 (84.2)	
Total	59 (100.0)	40 (100.0)	9 (100.0)	15 (100.0)	2 (100.0)	28 (100.0)	215 (100.0)	

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study (Department of Higher Education, ED 15 UKV 2018, Bengaluru, Date December 13, 2018).

Conflicts of interest

There are no conflicts of interest.

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PAPER PRESENTATION CERTIFICATE ORAL PRESENTATION:

Paper entitled "In Silico Analysis of Single Nucleotide Polymorphisms (nsSNPs) of *GJB2* Gene in Hearing Impaired children" in 41 annual conference on Recent Trends in Biomedical Research organised by the Indian association of biomedical scientists from 12-14th march 2021.

41 Annual Conference of Indian Association of Biomedical Scientists
International Virtual Conference on
RECENT TRENDS IN BIOMEDICAL RESEARCH
12- 14th March, 2021

CERTIFICATE

This is to certify that Smita Hegde Department of Biochemistry, Sree Balaji Medical College and Hospital, Chennai has participated as and presented Oral Presentation entitled In Silico Analysis of Single Nucleotide Polymorphisms (nsSNPs) of GJB2 Gene in Hearing Impaired children in the "International Virtual Conference on "RECENT TRENDS IN BIOMEDICAL RESEARCH" Organized by the Indian Association of Biomedical Scientists held during 12-14 March 2021.

Dr. G. K. Pal
President
IABMS

Dr. E. Padmini
General Secretary, IABMS
Organizing Secretary

Participated and presented a paper in UNESCO/UNITWIN Network Web Seminar 2020 hosted by BLDE (Deemed to be University). Abstract is published in '*BLDE University journal of Health Sciences'*.

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This is to certify that, **Smita Hegde** of Department of Anatomy, Shri B M Patil Medical College, Hospital & Research Center, Vijayapura, Karnataka, India has participated and presented a paper entitled *"Screening of 35delG, W24X, W77X and Q124X Variants in connexin 26 gene associated with Non Syndromic Hearing Impairment"* during UNESCO/UNITWIN Network Web Seminar held on August 6 & 7, 2020.

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