Genetic profile among children with congenital nonsyndromic hearing impairment, a study from a tertiary care centre in India

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE RULES AND REGULATIONS FORTHE M.S.(BRANCH IV) OTORHINOLARYNGOLOGY EXAMINATION OF THE TAMIL NADU DR. MGR MEDICAL UNIVERSITY TO BE HELD IN APRIL 2014

CERTIFICATE

This is to certify that the dissertation entitled '**Genetic profile among children with congenital nonsyndromic hearing impairment, a study from a tertiary care centre in India'** is a bonafide original work of Dr. Vijay Singh, submitted in partial fulfilment of the rules and regulations for the **MS Branch IV, Otorhinolaryngology** examination of The Tamil Nadu Dr. M.G.R. Medical University to be held in **April 2014**

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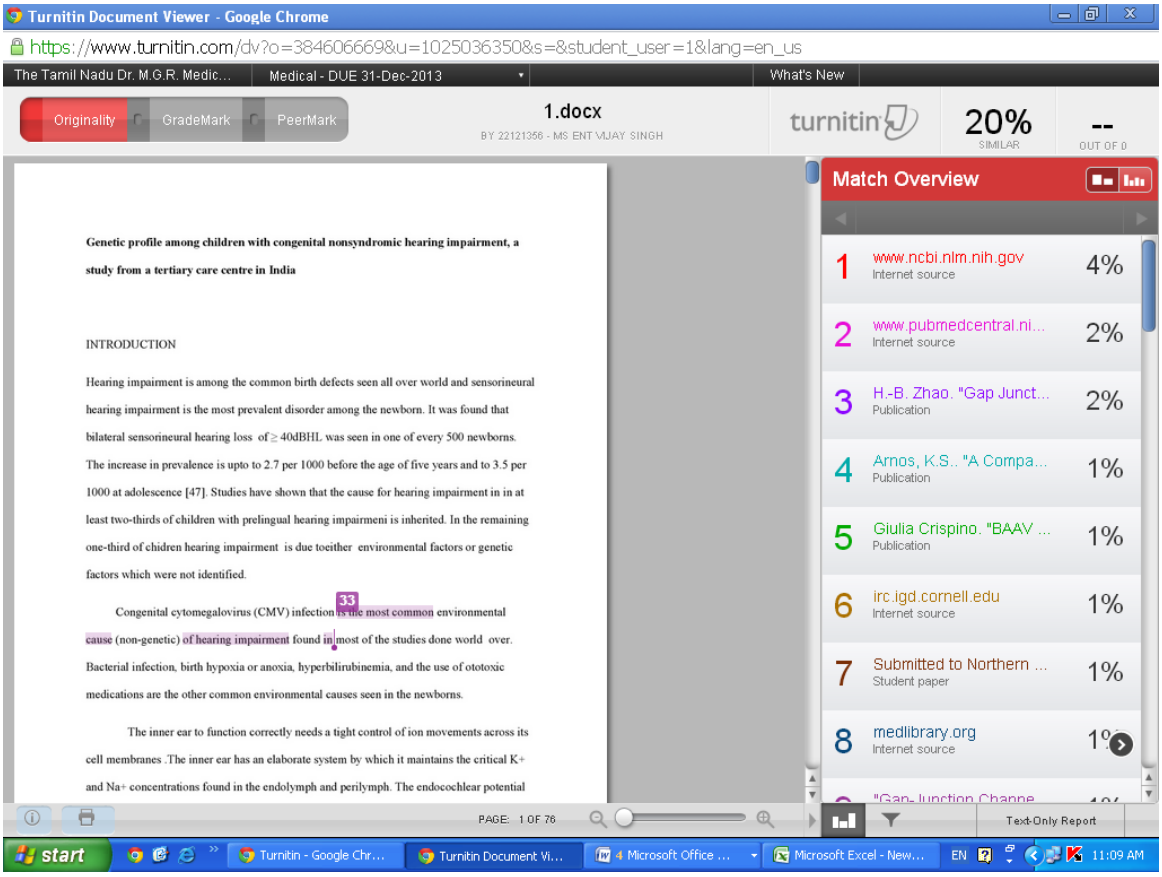
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Genetic profile among children with congenital nonsyndromic hearing impairment, a study from a tertiary care centre in India
INTRODUCTION
Hearing impairment is among the common birth defects seen all over world and sensorineural hearing impairment is the most prevalent disorder among the newborn. It was found that bilateral sensorineural hearing loss of ≥ 40dBHL was seen in one of every 500 newborns. The increase in prevalence is upto to 2.7 per 1000 before the age of five years and to 3.5 per 1000 at adolescence [47]. Studies have shown that the cause for hearing impairment in in at least two-thirds of children with prelingual hearing impairmeni is inherited. In the remaining one-third of chidren hearing impairment is due toeither environmental factors or genetic factors which were not identified.
Congenital cytomegalovirus (CMV) infection is the most common environmental cause (non-genetic) of hearing impairment found in most of the studies done world over. Bacterial infection, birth hypoxia or anoxia, hyperbilirubinemia, and the use of ototoxic medications are the other common environmental causes seen in the newborns.
The inner ear to function correctly needs a tight control of ion movements across its cell membranes .The inner ear has an elaborate system by which it maintains the critical K+ and Na+ concentrations found in the endolymph and perilymph. The endocochlear potential
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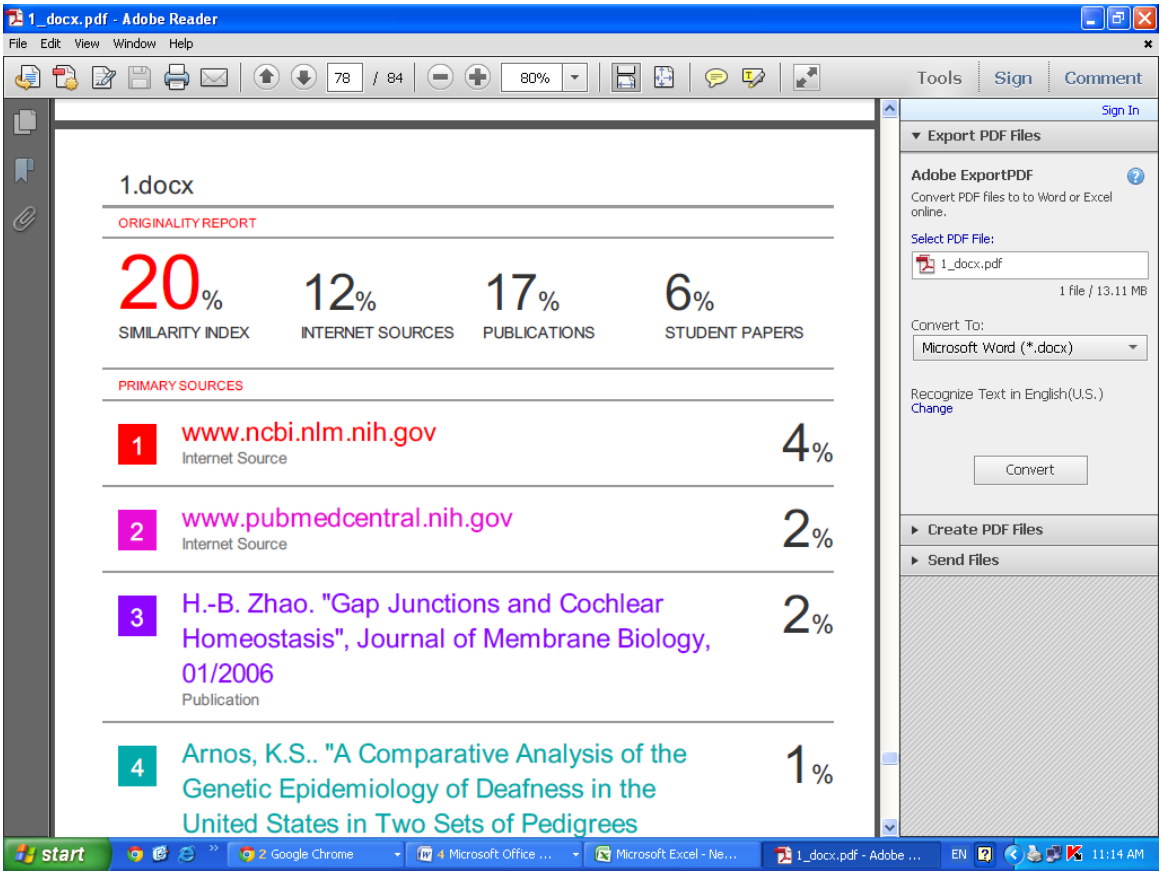
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ABSTRACT

Title of the abstract : Genetic profile among children with congenital nonsyndromic hearing impairment, a study from a tertiary care centre in India

Objective: To detect the percentage of GJB2 gene mutation in congenital non-syndromic hearing impaired children presenting at a tertiary care centre

Methods: This is a pilot study conducted, 96 children were included in the study – 86 being retrospective.

Children presenting with history of nonsyndromicsensorineural hearing impairment in the department of ENT of our hospital were enrolled in the study. . History was obtained based on information obtained by a combination of self-reporting and physician assessment. Clinical evaluation including physical examination, hearing evaluation, blood investigations including genetic test were done. Genetic test were done in Department of Clinical Genetics at CMC.

Results: In this study there were 96 probands, 36 males and 60 females. Among the probands 46 i.e. 47.9% had profound hearing loss. In our study we found Connexin 26 mutation in 13.5% of children with nonsyndromicsensorineural hearing loss. Out of the 36 boys, 19.4% had gjb2 mutation and 10% of the girls had that mutation however. In simple words, there is no evidence of association. We also found in our study all probands with GJB2 mutation had profound hearing loss. In our study we found 47% of GJB2 positive probands tested positive for W24X. 6.3% of the total probands tested positive for W24X mutation. Out of the total probands tested,5.6% of males and 6.7% of females tested positive for W24X. We also found that all probands with W24X had profound hearing loss.In our study among the probands tested 38.5 % were born to consanguinous marriage. There are 37 of the children from

consanguineous marriage of which 29.7% had GJB2 mutation and 3.4% out of non-consanguineous had mutation and this difference was highly significant Among probands born to consanguineous marriage, 62.2% had profound hearing loss as compared to 39% born to nonconsanguinous marriage

This study has shown that Connexin 26 mutation is less common in India than in western population.In our study nearly half of probands with GJB2 mutation were W24X positive and this is the commonest mutation in Indians. Nearly one third of consanguineous marriage with non-syndromicsensorineural hearing loss were GJB2 positive. In this study we also found that all children who were GJB2 positive had profound hearing loss. To conclude, genetic test have many benefits and most impotantly if found positive it helps in early intervention.

Key Words : Hearing Impairment, GJB2 Mutation, W24X gene, RFLP

CONTENTS

INTRODUCTION

Hearing impairment is among the common birth defects seen all over world and sensorineural hearing impairment isthe most prevalent disorder among the newborn. It was found that bilateral sensorineural hearing loss of ≥ 40dBHLwas seen in one of every 500 newborns. The increase in prevalenceis uptoto 2.7 per 1000 before the age of five years and to 3.5 per 1000 at adolescence [1].Studies have shown that the cause for hearing impairment in in at least two-thirds of children withprelingual hearing impairment is inherited.In the remaining one-third of children hearing impairment is due toeither environmental factors or genetic factors which were not identified.

Congenital cytomegalovirus (CMV) infection isthe most common environmental cause (non-genetic)of hearing impairment found in most of the studies done world over. Bacterial infection, birth hypoxia or anoxia, hyperbilirubinemia, and the use of ototoxic medications are the other common environmental causes seen in the newborns.

The inner ear to function correctly needs a tight control of ion movements across its cell membranes .The inner ear has an elaborate system by which it maintains the critical K+ and Na+ concentrations found in the endolymph and perilymph. The endocochlear potential is anequilibrium potential due to K+ ion concentration. The potential is produced by the striavascularis.

Gap junctions are the mainchannels for intercellular communication. These channels are important for passage of ions and metabolites across adjacent cells. Hundreds tothousands of gap-junction channel units are required to form protein structures that get inserted into plasma membrane and form gap junctions. Gap junction channels span two neighbouring

cells. Striavascularis needs the K+ ions to move via gap junctions before they are secreted back into the endolymph..(2) Connexins (Cxs) are proteins, they are the building blocks forming thegap-junction channel (GJCh). Connexins form themain routes for intercellular communication. Many diverse hereditary disorders of humans, like cataracts, oculodentodigital dysplasia, and nonsyndromic or syndromichearing impairment are known to be caused due to mutations involving connexion gene.(3)Among the gap junctions seen in supporting cells of cochlea the most commonly expressed isoforms are Cx26 and Cx30.

GJB2 isthe gene encoding for connexin26 (Cx26). It was the first gene found to be associated with an autosomal recessive form ofdeafness(4).Inherited hearing loss is monogenic in most of the cases. Among the neonates who are not able to pass the newbornscreening for hearing impairment and are found to have genetic hearing loss, 80% of them will have no other positive physical findings. In these neonates the hearing impairment is classified as nonsyndromic. In the rest 20% of neonates, the hearing impairment is associated with other physical findings. These neonates are said to be syndromic [5]. There are more than 400 syndromes recognised which are associated with hearing impairment. Among these syndromes Usher syndrome, Jaervell and Lange-Nielsensyndrome and Pendredsyndrome are the most frequent [6].

There are different ways of inheriting monogenic hearing impairment.Autosomal recessive nonsyndromic hearing loss (ARNSHL) is the cause in about 80% of children with genetic hearing impairment. Most of these children haveprelingual, hearing impairment. Autosomal dominant nonsyndromic hearing loss (ADNSHL) accounts for hearing impairment in the rest 20% of children. In these childrenhearing impairment is most often postlingual. In less than 1% of children, the hearing impairment is due to

inheritance through the X-chromosome or the mitochondria [7] Genetic test are useful as they allow parents to understand the cause of their child's deafness and help on deciding further course of action.

AIMS AND OBJECTIVES

To detect the percentage of GJB2 gene mutation in congenital non-syndromic hearing impaired children presenting at a tertiary care centre

REVIEW OF LITERATURE

Hearing impairment is among the common birth defects seen all over world and sensorineural hearing impairment isthe most prevalent disorder among the newborn. [1]. Hearing impairment is classified in several ways. Conductive hearing loss mainly refers topathology of the outer or middle ear whereas sensorineural hearing loss implies an inner ear defect. Mixed hearing loss is when both conductive and sensorineural hearing impairment occur together. Depending on the age of onset, hearing loss is also classified as prelingual (before development of speech), also referred to as congenital (all prelingualhearing impaired children cannot be classified as congenital) or postlingual (after development of speech).

Hearing impairment is clinically and genetically heterogeneous. When impaired auditory function is the only clinical finding it is called non-syndromicforms of deafness.When hearing impairment is associated with other symptoms or anomalies, it is called syndromic forms of deafness.Mutational changes involvingConnexin 26 is the most common molecular defectseen in nonsyndromicautosomal recessive deafness. Connexin 26 is a gap junction protein seen in cochlea. It is encoded by GJB2 gene.Hearing impairment can be caused by defect in any two of the four alleles from GJB2 and GJB6. There can be either monogenic or digenic inheritance with these genes. There are different ways of inheriting monogenic hearing impairment.Autosomal recessive nonsyndromic hearing loss (ARNSHL) is the cause in about 80% of children with genetic hearing impairment. Most of these children have prelingual, hearing impairment. Autosomal dominant nonsyndromic hearing loss (ADNSHL) accounts for hearing impairment in the rest 20% of children. In these childrenhearing impairment is most often

postlingual. In less than 1% of children, the hearing impairment is due to inheritance through the X-chromosome or the mitochondria [7]

Since the beginning of the 19th Centuryit was known that heredity plays an important role in hearing impairment. In 1857, William Wilde, an Irish otologistconducted a studyfrom data collected in census records by asking questions about deaf individuals. He found that deafness in one or both the parents and consanguinity among parents were important indicators for hereditary aetiology in some of the hearing impaired individuals.(8)

In 1883, a report was published by Alexander Graham Bell "Memoir upon the Formation of a Deaf Variety of the Human Race". This memoir had a retrospective study of records taken from schools for the hearing impaired in the United States.(9) Bell did a study on the frequency of relatives of deaf students who had hearing impairment.. He also analysed the hearing levels of the children born of marriages among those who were congenitally deaf and compared his data with individuals who were adventitiously deaf. After the study, Bell had presented his concern about ''the formation of a deaf variety of the human race in America,''. Bell found that there was arise in the frequency of congenital deafness in the population. Bell argued that the increase in numbers of residential schools for education,the use of sign language, and the formation of societies and conventions for deaf people led to decreased mating choices and fostered intermarriage. This led to increase in frequency of deaf individuals in the population. Geneticists all over world did not approve of Bell's concerns. They argued that due to extreme heterogeneity of genes causing hearing impairment, this was highly unlikely. However, recent evidence have shown that, assorted mating among the hearing impaired population in combination with relaxed selection have led to preferential amplification of the commonest forms of recessive hearing impairment.(10)

ANATOMY

The cochlea is a bony structure which is spiral in shape like a snail and it forms the sensory organ of hearing. The cochlea in humans has a little more than two and a half turns. If it is uncoiled and made straight the cochlea has a length of 3.1–3.3 cm. The cochlea has a height of approximately 0.5 cm in humans . The cochlea (together with the vestibular organ) is totally enclosed in the temporal bone, which is one of the hardest bones in the entire body. The cochlea and the vestibular organs are together referred to as the labyrinth. Bony labyrinth is formed by the bony structures of inner ear and inside it contains membranous labyrinth. The cochlea is made of three canals filled with fluid: the scala vestibule, the scalamedia and the scalatympani . The scala media is located in the middle of the cochlea. Reissner's membrane separates scala media from the scalavestibuli. The basilar membrane separates scala media from the scala tympani. There is similarity in the ionic composition of the fluid of scala media and intracellular fluid. Scala media is rich in potassium and low in sodium. The ionic composition of fluid in scalavestibuli and scala tympani is similar to extracellular fluid such as the cerebrospinal fluid, it is rich in sodium and poor in potassium.

The scala media becomes narrow towards the apex of the cochlea, it ends just short of the apical termination of the bony labyrinth. There is an opening near the apical termination of the bony labyrinth which is called helicotrema, it allows communication between the scalavestibuli and scala tympani. In humans, the area of this aperture of helicotrema is approximately 0.05 mm2. The basilar membrane separates the sounds according to their frequency (spectrum). The organ of Corti is situated along the basilar membrane, it is made of sensory cells (hair cells). These cells transform the vibration of the basilar membrane into a neural code.

Organ of Corti

The organ of Corti is made of many different kinds of cells. The sensory cells are the hair cells and they are so named because of the hair like bundles that are located on their top. The hair cells are arranged in rows along the basilar membrane. The hair cells have bundles of stereocilia on their top . Two main types of hair cells are seen in the organ of Corti, the outer hair cells and the inner hair cells.

The human cochlea has approximately 12,000 outer hair cells which are arranged in 3–5 rows along the basilar membrane, and approximately 3,500 inner hair cells that are arranged in a single row. On each outer hair cell, they have 50–150 stereocilia. The stereocilia are arranged in 3–4 rows that assume a W or V shape whereas the inner hair cells stereocilia are arranged in flattened U-shaped formation. Tunnel of Corti lies between the row of inner hair cells and the rows of outer hair cells. . Tunnel of Corti is bordered by inner and outer pillar cells . The outer hair cells are different from the inner hair cells in several ways. The outer hair cells appear cylindrical in shape while the inner hair cells appear flask shaped or pear shaped . The stereocilia are linked to each other with specific structures called crosslinks. The tallest tips of the outer hair cell stereocilia lie embedded in the tectorial membrane seen overlying the stereocilia,.

The tips of the inner hair cell stereocilia are not embedded in the tectorial membrane. The outer hair cells in the apical region of the cochlea are longer than in the more basal regions. The outer hair cells are approximately 8 μm long in the apical region and less than 2 μm in the base. The diameter of the longest outer hair cell is thus approximately one tenth of the diameter of a human hair. Inner hair cells have similar dimension in the entire cochlea and all have approximately the same number of stereocilia (approximately

60). The stereocilia on inner hair cells that are situated at the base of the cochlea are shorter than stereocilia of hair cells that are located in the apical region of the cochlea. In addition to hair cells, there are other types of cells found in the cochlea. Supporting cells seen in organ of Corti are the Deiter's cells and Henson's cells, inner phalangeal and inner border cells. The striavascularis is located between the perilymphatic and the endolymphatic space along the cochlear wall. The striavascularis has a rich blood supply and its cells are rich in mitochondria, indicating that it is involved in metabolic activity. Many of striavascularis intermediate cells have a high content of melanin. The spiral ligament, to which the basilar membrane is attached, supports the striavascularis.

Basilar Membrane

The basilar membrane is formed by connective tissue and it forms the floor of the scala media. It has a width of approximately 150 μm in the base of the cochlea and it becomes wider at the apex, it is approximately 450 μm wide at the apex.Basilar membrane changes in stiffness from base to apex, it is more stiff in the basal end than at the apex. Due to this gradual change in stiffness, sounds that reach the inner ear create a wave on the basilar membrane which travels from the base towards the apex of the cochlea. Basilar membrane in different regions responds differently to this wave, and the same region on basilar membrane will respond differently to waves of different frequency. This travelling wave motion forms the basis for the frequency separation that the basilar membrane provides before sounds activate the sensory cells that are seen along the basilar membrane. The frequency analysis in the cochlea is complex; it involves interactions between the basilar membrane, the surrounding fluid, and the sensory cells. The outer hair cells interact actively with the motion of the basilar membrane.

Innervations of Hair Cells

There are three different types of nerve fibers that innervate the cochlea: afferent auditory nerve fibers, efferent auditory fibers (olivocochlear bundle) and autonomic (adrenergic) nerve fibers. The afferent auditory nerve fibers are bipolar cells, their cell bodies are located in the spiral ganglion. The spiral ganglion is located in a bony canal, called the Rosenthal's canal.

The auditory nerve fibers innervating the cochlea pass through the habenulaperforata before they continue as radial fibers to the inner hair cells. In humans, the auditory nerve has approximately 30,000 afferent nerve fibers. There are two types of afferent fibersseen in cochlea. Type I afferent fibres are myelinated and have large cell bodies. Type I afferent fibres comprise about 95% of the auditory nerve fibers. Type II afferent fibres are unmyelinated and they have small cell bodies. Type II afferent nerve fibres comprise 5% of the auditory nerve. The auditory nerve fibers connect to the hair cells via synapses. These synaptic connections are different for inner and outer hair cells. Many type I auditory nerve fibers terminate on each inner hair cell while a single type II auditory nerve fiber connects to many outer hair cells. It has been estimated that each inner hair cell receives approximately 20 nerve fibers. The nerve fibers (type II) that make synaptic contact with the outer hair cells cross over the cochlear tunnel to reach the rows of outer hair cells, where each nerve fiber, called an outer spiral fiber, innervates many hair cells and extends apically as much as 0.6 mm along the outer hair cell region.

The inner radial fibers (type I) are thus different from the spiral fibers (type II) as they terminate on single inner hair cell. The hair cells also receive different connections from the descending auditory nervous system, the olivocochlear bundle also known as

Rasmussen's bundle. Outer hair cells receive the largest number of such nerve fibers. The efferent fibers (approximately 500–600 in humans) have their cell bodies in the nuclei of the superior olivary complex (SOC) of the brainstem. These fibers are of two kinds: One kind is the medial olivocochlearfibers that are large myelinatedfibers that originate in the medial superior olivary (MSO) complex and which terminate on outer hair cells. These fibers mostly originate from cells on the opposite side and thus cross the midline. Each outer hair cell receives many efferent fibers and each efferent fiber connects to many outer hair cells (11).

ION HOMEOSTASIS IN THE EAR K+ Na+

The inner ear homeostasis refers to the processes by which the chemical equilibrium of inner fluids and tissues is maintained. The inner ear can function correctly only if there is tight control of ion movements across its cell membranes. The inner ear function includes hair cell function, regulation of extracellular endolymph and perilymph composition, and nerve impulse conduction. The major ions involved are Na + and K+ but a significant role is also assigned to calcium (Ca), chloride (Cl) and others. The hair cells of the inner ear have advantage of operating within a unique ionic microenvironment of two distinct fluids. The apex of the hair cell and stereocilia are immersed in endolymph, whereas the cell body is bathed in perilymph. The reticular lamina separates the two fluid spaces, endolymph from perilymph. The hair cells maintain a negative intracellular potential of -80mV relative to surrounding perilymph. The high K+ concentration of endolymph creates an endocochlear potential of +80mV relative to perilymph. This +80mV endocochlear potential couples with the -80mV hair cell intracellular potential for a differential potential of 160mV. This arrangement of endolymph and perilymph is important for hair cell depolarization when its stereocilia are deflected by travelling wave.

Stereocilia get displaced laterally either by shearing movement of the tectorial membrane or motion of the endolymphatic fluid. Stereocilia displacement allows K+ ions to enter the hair cell through apical transduction channels, which leads to hair cell depolarization and neurotransmitter release. The flow of K+ ions is down an electrochemical gradient that brings K+ into the cell from the endolymph and eventually out the base of the cell body into the perilymph. If the entire hair cell was surrounded by endolymph, the hair cell will not be able to function because the K+ ion concentration inside and outside the cell would be equal. Thus the higher K+ ion concentration seen around the stereocilia provides for a receptor current that does not require an energy dependent pump in the hair cell. When these two fluids are not different, transduction is compromised and hearing loss occurs (2).

Na+ - K+ Transport Mechanism

The sound stimulus cause vibration of ossicles of middle ear. Thesevibrationsare sensed by the transduction machinery contained in the cochlea in the inner ear. The cochlea is made of threeparallel tubular compartments lying adjacent to each other: the scalavestibuli, the scala tympani, and the scalamedia. (3) The epithelial cells, fibrocytes, and hair cells form the principal cellular components of the cochlea. These cells are located in the wall of the cochlea. There arethree compartments forming the cochlea. They are filled with two types of fluids. The perilymph,fills the scala tympani and scala vestibule .The ionic composition of perilymph is similar to that of the extracellular fluid, it is rich in Na+ and poor in K+. The endolymph,fills the scala media. The endolymph has a high concentration of K+ (150 mM).The endolymph has a high positive potential. Studies have recorded endocochlear potential of approximately +80 mV. This endocochlear potential is important for the cochlea to function normally. The

endocochlear potential is due to equilibrium potential of potassium ions. This isproduced by secretion of potassium ions bystriavascularis. The striavascularis is a two-layered epithelium. It forms the lateral wall of the scala media (12). The potassium channelsfound in the plasma membrane of intermediate cells and K+ transporters seen in striavascularis in the basal membrane of marginal cells play a major role in K+ homeostasis. For the activation of hair cellsthe presence of endocochlear potential is essential. The sound produces which is transformed into electrical signals. These electrical signals are transmitted to the brain by the hair cells in the basilar membrane of the scala media. The hair cells are polarized cells. They have ciliated apical membrane which is exposed to the endolymph. Their cell bodies are in contact with the perilymph (or cortilymph). Sound coming into ear causes vibration in the basilar membrane. This vibration induces deflection of hair cell cilia which lead to opening of mechanosensitivepotassium channels (13). Opening of these channels allow endolymphatic K+ to enter into hair cells. This leads to depolarization of hair cells. The large endocochlear potential increases the sensitivity of hair cells to vibration caused by sound.This forms a large driving force of +160 mV for K+ influx. It is the difference between the resting potential of hair cells (+80 mV) lying in the perilymph and the endolymph potential (+80 mV). K+ is released into the perilymph after activation of the hair cells.The K+ than moves laterally via gap junctions. Potassium ions move from the perilymph back to the endolymph through the "cochlear lateral wall".This is also indicated by studies which showed that inhibition of flow of potassium ions from the perilymphatic space leads to inhibition ofendocochlear potential. Studies have also shown that endocochlear potential decreases after perilymphatic perfusion of potassium ion free solution. Different cellular components found in the cochlear wall form this recirculation pathway. K+ is transported along the supporting and epithelial cells in the basilar membrane. Potassium ions are transported to

fibrocytes in the spiral ligament which forms lateral wall connective tissue. K+ ions are eventually transported to epithelial cells of the striavascularis, from where they are pumped into the endolymph. The K+ circulation is in cochlea is by formation of two autonomous syncytia in the lateral wall of cochlea. One is formed by an epithelial gap-junction network (epithelial cellsand supporting cellsseen on the basilar membrane), and the other is formed by connective tissue gap-junction network (epithelial cells of striavascularisand fibrocytes of spiral ligament). These two groups of networks promote the K+ circulation from perilymph to endolymph.The endocochlear potential is created by the striavascularis. The endocochlear potential is reduced significantly following loss of Cx30 in the mouse ear (45) without changes in the potassiumion concentration and amount of endolymphin scala media.There are mutations in Cx26 which do not affect ionic conductance, like V84L, but they selectively affect IP3 permeability. These mutations have been associatedwith hearing impairment.Thefunctionof gap-junction channels in the potassium circulation, and generation of the endocochlear potential is complex and related to transport of other metabolites also.

The inner ear has an elaborate system by which it maintains the critical K+ and Na+ concentrations in the endolymph and perilymph. Following release by the hair cells K+ moves laterally through gap junctions between supporting cells in the organ of Corti. When ions reach the spiral ligament, the gap junctions between fibrocytes move ions up to the striavascularis for secretion back into the endolymph. The striavascularis has a well developed ion transport system of diverse channels and transporters that move K+ into the endolymph and Na+ out of the endolymph and into the perilymph. Other ions, such as Cl, H+ and Ca2+ also are actively transported into or out of the endolymph by the striavascularis.

The major ion channels and transporters which are involved in stria ion movement include Na K adenosine triphosphatase, the Na K 2Cl cotransporter, epithelial Na channel, several different K channels, Cl channels and the K H exchanger.The position of these ion channels and transporters in the striavascularis are determined by the direction of movement of a particular ion. Many of these ion channels and transporters also occur in secretory or absorptive tissues, but their relationship to the luminal or basal sides of epithelial cells varies. It depends on the required direction of K+ and Na+ flow. The +80 to +90mV endocochlear potential is generated by the intermediate cells within the interstrial space. The marginal cells, even though serving as the final transport epithelium to move K+ into the endolymph, they do not appreciably alter the endocochlear potential.

The cellular mechanism for moving K+ to the strial intermediate cells can be thought of as an epithelial complex made up of the fibrocytes in the spiral ligament, the basal and intermediate cells of the stria and the capillary endothelial cells. All of these cells are interconnected by gap junctions, facilitating the transport of K+ between them and into the intrastrial space. The tight junctions of the vascular endothelial cells and basal cells also contribute to the endocochlear potential by limiting intercellular leakage of ions. The exchange of ions through these cells is driven by the Na K ATPase system in the fibrocytes, intermediate cells and marginal cells. Thus any cochlear disorder that compromises these strial cells layers, channels, transporters or tight junctions will reduce the endocochlear potential and cause hearing loss. Also, the ion transport components of the stria system are controlled by multiple genes, making the production of endolymph susceptible to a variety of genetic disorders(2).

COCHLEAR HOMEOSTASIS

The mammalian cochlea has a complex anatomy. It is made of sensory hair cells and different types of supporting cells. The supporting cells seen in cochlea include Hensencells, pillar cells, Deiters cells, and Claudius cells. Gap junction channels couple these supporting cells bothelectrically and metabolically by forming channels for transport of ions and metabolites..Gap junctions in cochlea when compared to other cells in the body, are not found between the sensory cells i.e. inner and outer hair cells. No gap junctions are seen between sensory and non-sensory cells in mammals .

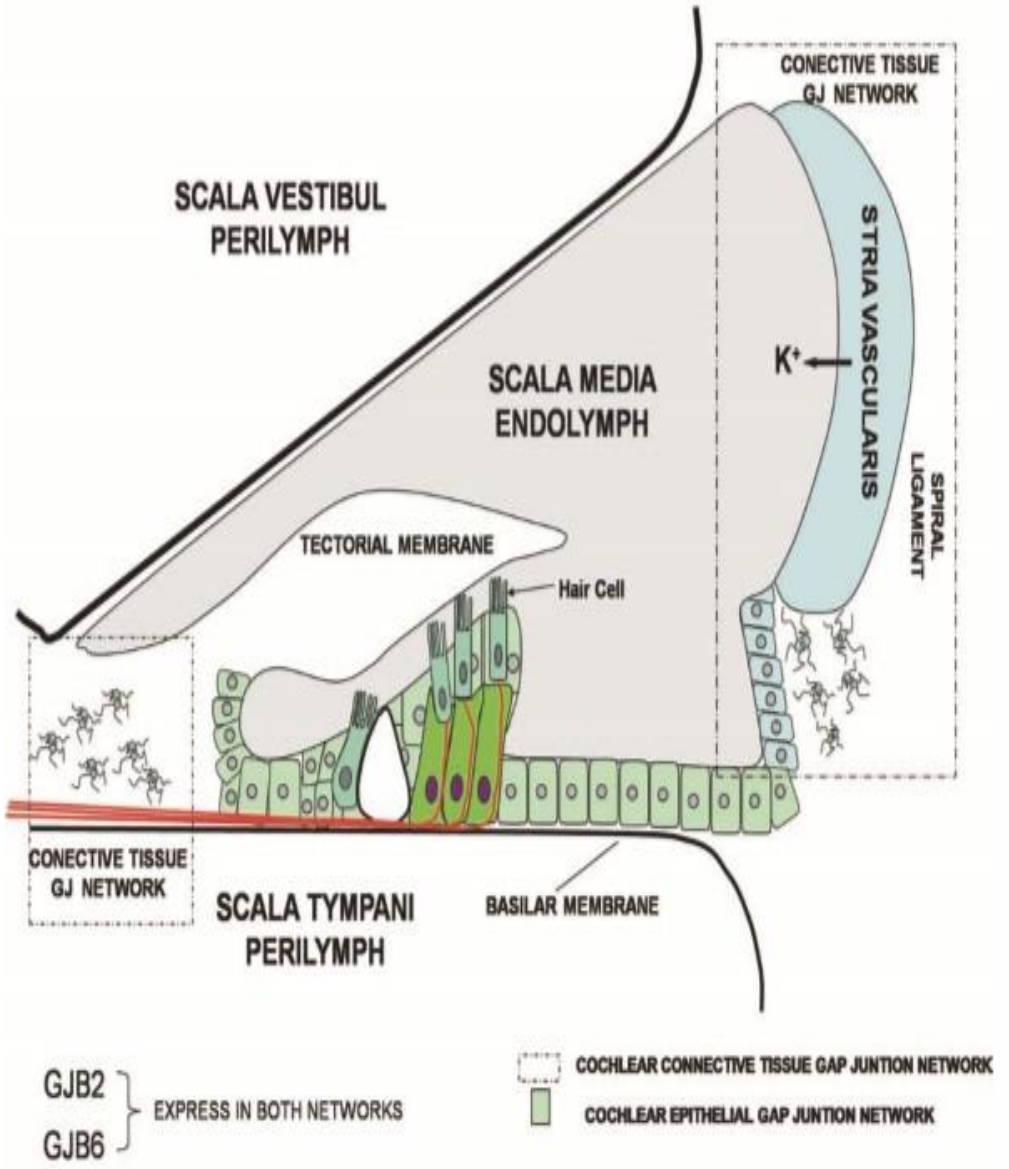


FIG. 1. Diagram of the cochlea cellular systems showing the gap-junction networks. Deiter cells (green) and supporting and epithelial cells (light green) constitute the epithelial gap-junction network. Connective cells (fibrocytes; star morphology) and cells in the stria vascularis (light blue) constitute the connective tissue gap-junction network. Cells in both networks coexpress Cx26 (*GJB2*) and Cx30 (*GJB6*). It has been proposed that K^+ recirculates back to the endolymph through these networks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Other than the gap junctions seen between supporting cells, additional gap junctional coupling is seen in the striavascularis, the spiral limbus, and other cochlear structures .Two distinct types of gap networks are seenin the cochlea. There are epithelial cell gap junction network between the non-sensory epithelial cells. There is also a connective tissue gap junction network seen among the connective tissue cells of the lateral wall of cochlea. These gap junction networks are important for cochlear ionic homeostasis.

The anatomy of cochlea is organized in a such a manner that it forms separate compartments containing perilymph and endolymph. Cochlea separates the ionic environment in its compartments presented to the sensory cells within this organ. The cochlea consists of three compartments, which are filed with the perilymph and the endolymph . The perilymphatic space is similar to other extracellular fluids, it contains a high Na+ and low K+ solution. In contrast, the apices of the cochlear epithelial cells face the endolymph, which has an opposite cationic composition similar to intracellular fluid of high K+ and low Na+ ions. The endolymph has a positive endocochlear potential of +80 to +100 mV and the perilymph has a negative potential of about -80 mV. Acoustically evoked receptor potentials in response to sound are generated by movement of K+ ions via mechanosensitivecation channels on stereocilia, into the sensory hair cellsfrom the endolymph. These potassium ions are then released into the basolateral extracellular spaces of the perilymph by the cochlear epithelial cells. These K+ ions are then taken up by supporting cells of cochlea and secreted back into the endolymph. The gap junction networks formed by supporting cells are important forrecirculation of cochlear K+ ions. They provide an intercellular route of transport of ions and metabolites.(15)

Connexins (Cxs) are proteins, they are the building blocks forming the gap-junction channel (GJCh). Connexins form important channels for intercellular communication.Gap junctions are important for ionic and metabolic coupling of adjacent cells. Hundreds to thousands of gap-junction channel units are required to form protein structures that get inserted into plasma membrane and form gap junctions. Gap junction channels span two neighboring cell.(3)andhelp in transport of different small molecules, including ions, nucleotides, siRNAs, and inositol phosphates . Under transmission electron micrographs each gap junction is recognized as a uniform narrow gap of about 2 nm between segments of plasma membranes of two adjacent cells .Each gap junction is made of channel particles which are closely aggregated in the membraneas seen in freeze-fracture electron micrographs. These intramembranous channel particles are made of assemblies of sixconnexin proteins. There are different connexin (Cx) subunits like Cx26, Cx30, Cx31 and Cx43 seen in the mammalian inner ear.(15)

Docking of two independent hemichannels, also called as connexions present in the plasma membranes of the two adjacent cells lying apposedto each other will form a gap-junction channel. Six connexin subunits oligomerize in Endoplasmic reticulum and Golgi bodies to constitute a hemichannel. This hemichannelis than transported to the plasma membrane by the secretory pathway. This movement is caused by microtubules attached to the vesicles (16). At sites of cell apposition on plasma membrane, there is docking of twohemichannel to form a gap-junction channel. This gap junctional channel allows formation of an aqueous pore. The aqueous pore connects cytoplasms of two adjacent cells. Theseaqueous pore allow transport of ions and small molecules.

These channels transportmolecules with a size of about 1 kDa includingsecond messengers (cAMP, cGMP, ATP, etc.) and diverse metabolites (sugars, amino acids, glutathione, etc.).(3)

It has been found that hemichannels which lie in plasma membrane undocked with other hemichannels can open and allow transport of ions and metabolites . These hemichannels are seen to work in both physiological and pathological conditions.(16).There is a family of homologous geneswhich encodes for all the different connexins. Human genomic database was screened and20 connexin genes were identified (17). All connexinshave the same basic structure in the plasma membrane. The connexions have their one intracellular loop, amino and carboxyl terminifacing the cytoplasm. Connexins are made of four transmembrane domains with two extracellular loops.

The similarityamong different connexin molecules is high but there are different proteins found in the intracellular loop and the carboxyl terminus. The difference between the proteins is significant and it is here where many molecules likecytoskeletal binding proteins and kinases act to regulate its activity.Heteromeric channels and heterotypic channels add to the variations in different gap junctions.Heteromeric channels are made of more than one type of connexin molecule. Heterotypic channels are constituted by the docking of two hemichannels where each is made by a different connexin molecule. These diverse connexinmolecules will form channels with variable functional and regulatory properties. The importance of gap-junction channelwas understood when many genetic diseases were found to be associated with mutations in variousconnexin genes coding for gap junctions found in different cells of the body.

Many diverse hereditary disorders of humans, like cataracts, oculodentodigital dysplasia, and nonsyndromic or syndromichearing impairment.are known to be caused due to mutations involving connexion gene.Many of the syndromic hearing impairment are found in association with various skin disorders (3). Among the different diseases caused by mutation of connexin in humans, hearing impairment is the most common. Even though inherited hearing impairment is genetically heterogeneous, a large number of patients in every population tested around the world, has been seen to be related with mutations in the gene encoding Cx26 (GJB2). Mutations in four other connexins, Cx30, Cx31, Cx32, and Cx43, are also associated withnonsyndromic or syndromicsensorineural hearing impairment (5, 6). Hearing impairment in mutations associated with Cx43 is controversial, as they were found to be located in the Cx43 pseudo gene on fifth chromosome. But in recent studies in Taiwanese hearing impaired patients,a new mutation was found in functional Cx43 gene.(3)

Among the gap junctions seen in cochlear supporting cells the most commonly expressed isoforms are Cx26 and Cx30. Both Cx26and Cx30 are seen in both the epithelial cell and the connective tissue cell gap junction system, of the cochlea. There are other types of connexions molecules present in the cochlea .

Different connexin molecules contribute different types of connexin proteins to the gap junction(heteromeric and heterotypic gap-junctional channels). This in turn increases the functional diversity of gating and permeability. Gating of different gap junction channels depends on differentfactors like (a) transjunctionalvoltage (b) membrane potential , (c) pH,(d)calcium, or (e) membrane tension . Different connexin channels have different sensitivity to voltage-gating. Hybrid channel configurations of gap junction add to the diversity of these channels. A homotypic channel has an even structure which will have

symmetric voltage-gating properties on both sides. A heterotypic channel with each hemichannel having its own exclusive gating can have disproportionately rectified voltage-gating properties. Asymmetrically rectified voltage gating is seen in both Cx26 and Cx30 channels when both form heterotypic channels in vitro. Further structural diversity is seen when Cx26 and Cx30 coassemble to form heteromerichemichannels in the liposomes. The existingmatch of different expressed connexin subunits can profoundly change the type of substances that can be transported across the gap junction.

Both syndromic and nonsyndromic forms of hearing impairmentcan be caused by mutations in human Cx26, Cx30, or Cx31 (GJB2, GJB6, and GJB3). Hearing impairment by mutation of Cx26 or Cx30 has also been produced by genetically engineered in mice. These observations have proved that normal functioning of the inner ear is dependent on normal functioning of gap junctions. But these observation do not explain why other connexin isoforms are not able to compensate in case of genetic mutation leading to loss of a single gap junction subunit within the cochlea. Further studies have shown that mutations in a single gap junction will lead to changes in permeability, gating properties and change the type of molecules being transportedbetween the plasma membrane of coupled cells.

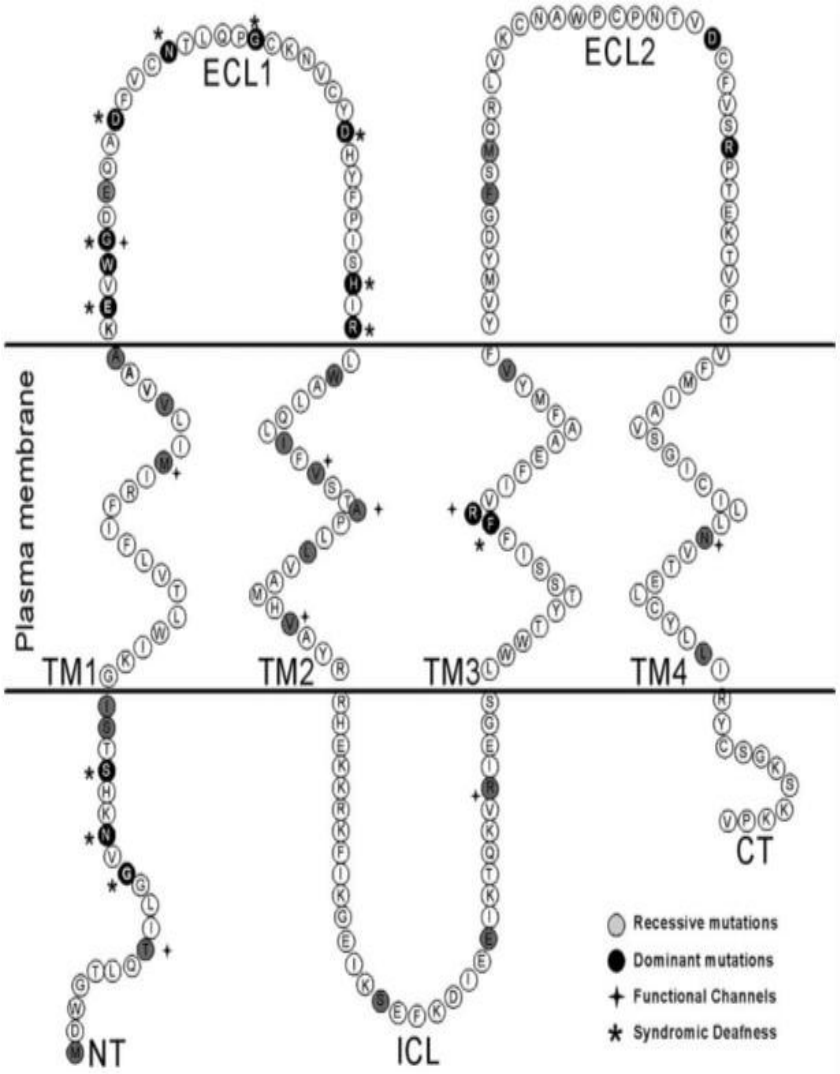


FIG. 2. Schematic topology of Cx26 relative to plasma membrana, showing the distribution of deafness-related mutations of Cx26. The amino acid position mutated (missense point substitutions) are labeled.

CONNEXINS

Connexins are tetraspantransmembrane proteins extending across cell membranes in contact with each other. Connexions arehexameric assemblies of connexin seen in the plasma membrane. These connexions dock with other connexions in plasma membrane of neighbouring cells clustering into a plaque and forming gap junction channels.There is a small extracellular gap of 2–3 nm between the two adjacent plasma membranes with connexions forming the plaque [18]. GJB2 is the gene coding for connexin26 (Cx26).

It was the first gene found to be associated to an autosomal recessive form of hearing impairment, DFNB1.(4) It was also associated with a dominant type of rare hearing impairment , DFNA3 [19]. In the studies done on connexin mutations, 90 recessive mutations have been discovered forGJB2, further studies are still going on. These mutations can be diverse and include (a) nonsense, (b) missense, (c) splicing, (d) frame–shift mutations and (e)inframe deletions [21]. These GJB2 mutations are responsible for about 50% of sensorineuralnonsyndromic hearing loss in many populations as seen in studies done around the world. There is a carrier frequency of 1 in 33 for GJB2 mutation among the Mediterranean population [20].

Two large deletions take placein initial part of GJB2 in GJB6 are associated with DFNB1–linked inherited cases with absentmutation in GJB2. On humanchromosome 13 is the gene coding for connexin30 (Cx30) lying 30 kb telomeric to GJB2 [21]. Only one mutation has been reported for connexin 30 where at position 5 there is a threonine–to–methionine substitution [22]. Detailed structure of wild connexin 26 is described by the recent 3.5–A˚ crystal structure [23]. Cx26 and Cx30 have 77% of similarity in their amino

acid. Both the connexions are commonly expressed in nonsensory cells of the inner ear [24,25] They form two discrete intercellular gap junction networks [26].

The epithelial gap junction network in the murine cochlea, starts development in embryo around sixteenth day.The organ of Corti is formed by supporting cells located in the sensory epithelium . These cells are connected by gap junction network.In comparison the connective tissue gap junction network starts development around birth [27] [28]. Mouse models confirmed the importance of Cx26 and Cx30 for normal auditory role [29]. Mouse models have helped to understand the connection between connexin expression, inherited deafness, endolymphatic K+ concentration and endocochlear potential [30,31,32]. Mouse models also helped to understand movement of nutrients among the cells of sensory epithelium of the inner ear [33]. They also helped in understanding cellular degeneration associated with connexin mutation occurring in cochlea.[34]

Organization of Gap Junction in the Mammalian Cochlea

Kikuchi et al. In 1995 described the gap junction networks in cochlea. They found that there exist two differentgap junction networks, the epithelial cell system and the connective tissue cell system. They found that both Cx26 and Cx30 appear very frequently throughout these systems . The epithelial cell gap junction system includesdifferent types of cells like interdental cells found in the spiral limbus, cochlear supporting cells, and root cells seen in lower part of the spiral ligament. Gap junctions are absent between the sensory hair cells and the surrounding supporting cells. The connective tissue cell gap junction system is made of different types of cells. It includes (a) fibrocytespresent in the spiral ligament and suprastrial zone, (b) basal and intermediate cells seen in the striavascularis, mesenchymal cells seen lining the

perilymphatic space of the scalavestibuli, (c) the supralimbalmesenchymal dark cells and (d) the fibrocytes in the spiral limbus. The cells forming the epithelial cell gap junction system are lined by a basement membrane. The epithelial cell gap junction have no direct contact with the underlying connective tissue cells.

Recycling Mechanism for Potassium ions via Gap Junctions in the Cochlea

Endocochlear potential formed by the different ionic composition of endolymph and perilymph is important for the normal auditory function of cochlea.Acoustic stimuli of cochlear sensory cells leads toentry of potassium ions into the hair cellsfrom the endolymph. Thepotassium ions are then transported basolaterallyout of the hair cells to the extracellular spaces. Cochlear supporting cells move potassium ions to the lower part of the spiral ligament laterally via the epithelial cell gap junction system. The potassium ions move further laterally to extracellular space within the spiral ligament. The spiral ligament is the outermost element of the epithelial cell gap junction system. Potassium ions are taken up by the Na, K-ATPase and Na-K-Clcotransporter (NKCC1) present in the type II fibrocytesplasma membrane. Potassium ions move into the connective tissue gap junction system after the uptake by type II fibrocytes. At the basal cell layer of the striavascularispotassium ions pass through the tight junctional barrier. Potassium ions are then transported to the striavascularis. The marginal cells seen in the striavascularissecrete the potassium ions into the endolymphatic space, to be reused formechanosensory transduction, after taking up the ions from extracellular space by Na, K-ATPase and Na-K-Clcotransporter (NKCC1). There is an anothermechanism for potassium cycling. It involvescalcium waves induced by inositol triphosphate.

Voltage Gating of Cx26 and Cx30 in Cochlear Cell Pairs

Irregular current flow between cells in the cochlea are produced due to asymmetry in transjunctional voltage gating due to diverse coupling of different connexions and also due to dependence of coupling on membrane potential.

Voltage gating properties of Cx26 and Cx30 in vitro have been documented in various studies. Both Cx26 and Cx30 are mainly invoved in forming gap junctions between cochlear supporting cells. Many studies have shown that heterotypic or heteromeric gap junction channels are seen amongsupporting cells. These gap junctions will have variable stoichiometry and posttranslational modifications leading toirregular voltage gating seen in cochlear supporting cells. Thisinduces a directional transjunctional current amid coupled supporting cells .After acoustic stimulation this directional currents can lead to a directed funnelling of ions like potassium ions away from the sensory cells. Studies have shown that variable permeability is seen inheteromeric Cx26 and Cx30 channels when compared with homomeric gap junctions. This increasesthe functional diversity of gap junction channels in the cochlea. It was also found that Cx26 and Cx30 heteromeric channels had faster Ca2+ intercellular signalling which may prove to have critical functional consequences..(3)

In addition to the gap junction channels which promote intercellular communication, hemichannels are also seen on plasma membrane in both physiologic and pathologic conditions. (35) These hemichannels lead to increased plasma-membrane permeability. The mature organ of Cortihas functional hemichannelswhich under certain conditionspermit uptake of anionic molecules with a large size, as well as the release of ATP. Studies have shown that mutation of connexin leading to hearing impairment can be

due to hybrid Cx26 with Cx30 channels. Mutation of either connexion of a hybrid Cx26/Cx30 channel can change the channel function even when the other docking connexion is normal. This mutation can disrupt cochlear homeostasis and hinder K+ recycling. Presence of hybrid channels can explain why homotypic Cx26 or Cx30 cannot compensate for the mutations in the other gene.

Inner Ear Gap Junction Permeability

All connexins have 1 to 1.5 nm diameter of the gap junction channel lumen.Itcorresponds with the diameter of molecules that can pass through the gap junctions. Despite the common size of pore diameter, different connexin isoforms forms different channels that behave differently. Gap junction channels have preferences for charge and discrimination for metabolites with diameters below the upper limit of the pore size. It has been shown in studies that in transfected cells, both cationic and anionic dyes can pass through Cx26 whereas Cx30 channels can pass cationic dyes but do not allow passage of Lucifer yellow and Alexa Fluor 488 which are anionic dyes. A similar charge selectivity is also seen in gap junctional coupling in cochlear sensory epithelium. It has been found in experiment done on permeability thatCx26 preferably allows passage of anions as it allows passage of negatively charged dyes. Important molecules for cell signalling likeATP, cAMP, cGMP, glutamines and IP3 are all anions. So it is believed that Cx26 channels are important in intercellular signalling and passage of nutrients across plasma membrane in cochlea.Mutations causing deafness on functional analysis showed similar findings.

Pharmacological Modulation of Gap Junction Coupling in Cochlear Supporting Cells

Studies have shown that drugs causing ototoxicity influence supporting cell gap junctional coupling leading tocochlear pathology. Isolated Hensen cellshad decreased permeability of its gap junctions on exposure to gentamicin. It was also found that Cisplatin exposure led to uncoupling of fibroblast gap junctions.Further experiments showed that gentamicin ototoxicity was reversed by catalase and Hydrogen peroxide application promoted similar ototoxicity.These experiments proved that gentamicin initially stimulates production of Hydrogen peroxide which can inhibit gap junction coupling.Further studies are needed to find out the effect of free radicals and whether free radicals can influence permeability in gap junctions.

Another pharmacological modulator studied is Nitric oxide (NO). Experiment showed that on applying sodium nitroprusside (SNP), which is a NO donor,on to Dieter cells led to inhibition of gap junction coupling.

Experiment have been done on other modulators of gap junction coupling like membrane tension, temperature and pH but their mechanism of action is still doubtful.

Loss of Function of Cochlear Connexin studied on Mouse Models

Studies were done on mice after mutating or deleting Cx26 and Cx30 genes. These studies have shown importance of gap junctional coupling forcochlear homeostasis.In experiments done on mice, they were not able to survive after completely knocking out of Cx26. So targeted ablation of Cx26 was done in cochlea to study the effects. It was found that sound stimulation led to increased postnatal cell death in cochlea after normal

development of cochlea for fourteen days. This led to significant hearing impairment.These cell death were seen to occur near inner hair cells along with a decrease in endolymphatic potential. Endolymphatic potassium concenteration was found to be low. These experiments showed that loss of Cx 26 function led to reduced recycling of potassium in cochlea after potassium entered hair cells following sound stimulus.This potassium was secreted basolaterally by hair cells leading to increased potassium concenteration in extracellular perilymph. This blocked uptake of glutamateandled to cell death in cochlea.

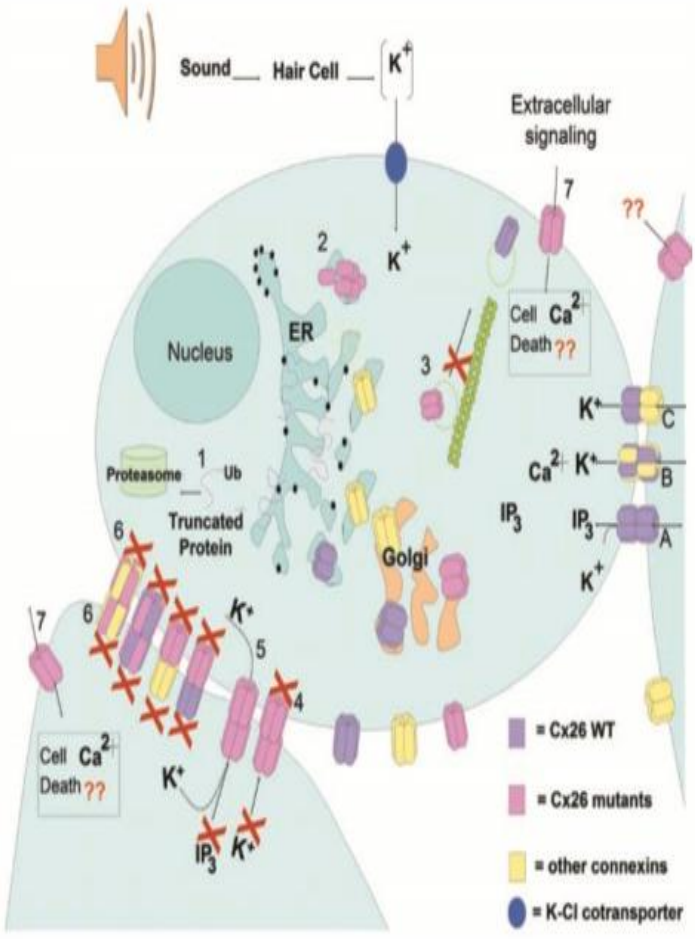


FIG. 3. Pathogenic mechanism of deafness-associated Cx26 mutations. Wild-type connexins oligomerize in the ER/Golgi. Hemichannels traffic to plasma membrane through the secretory pathway by a cytoskeletal-dependent mechanism. Epithelial and supporting cells in the cochlea express both Cx26 and Cx30. (**A**) Cx26 homomeric GJCh are permeable to ions, like K^+, and bigger molecules, like IP_3. Cx30 homomeric GJCh have high permeability to K^+ but lower permeability to IP_3. (**B**) Heteromeric Cx26–Cx30 GJCh. (**C**) Heterotypic channels. Deafness-associated Cx26 mutations may produce **1.** Truncated protein connexin subunits; **2.** Oligomerization defects impeding the assembly of hemichannels; **3.** Defective trafficking of the hemichannels, impeding targeting to the plasma membrana; **4.** Nonfunctional channels; normal trafficking and assembly into the plasma membrane and gap-junction plaque formation, but the GJCh are closed or their pore structure severely affected, impeding the diffusion of ions and small metabolites; **5.** Functional channels permeable to ions but with reduced permeability to bigger molecules like IP_3, affecting propagation of calcium waves or other metabolites; **6.** Mutant Cx26 that can act as dominant negative of co-expressed wild-type connexins. Mutant Cx26 can oligomerize with wild-type connexins, producing nonfunctional heteromeric channels. Heterotypic combination between mutant Cx26 hemichannel and wild-type hemichannels can also lead to nonfunctional channels; **7.** Aberrant functionality of free hemichannels in the plasma membrane, allowing an increase in plasma-membrane permeability that may lead to cell death due to either loss of important intracellular metabolites (like ATP or NAD), or increase intracellular calcium concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

These experiments proved the important role of epithelial gap junction network in cochlear function. These experiments could not elaborate all the functions of Cx26 as Cx30 was not able to compensate for the loss of function of Cx26.

Another study was done using a different mouse model by transgenically expressing R75W in inner ear which is the dominant mutant negative for Cx26 to study the pathology of dominant mutations .R75W mutation caused syndromic hearing impairment along with skin manifestations.These mice developed profound hearing impairment. These mice also developed histological changes leading to degeneration of hair cells and tunnel of corti in mice cochlea. These experiments showed that R75W affected potassium ion circulation in organ of corti and not extracellular perilymph, as was seen in other experiments. These experiments demonstrate that different pathologies can occur depending on type of connexin 26 mutation and the type of inheritance.

Another animal study was done by knocking out Cx30.These mice had normal cochlear development but they were unable to maintain endocochlear potential. On exposure to sound stimulus apoptosis of cells in cochlear sensory epithelium was seen. This experiment again demonstrated that inspite of normal Cx26 function, no compensation for loss of Cx30 function was seen. These experiments suggest that gap junctions have other roles and they allow passage of other metabolites also.

Functional Properties of Mutant Channels

Most of the studies have shown that mutions of Cx 26 are the most common reason for both autosomal recessive and autosomaldominant hearing impairment. These mutations are also seen in sporadic congenital deafness . These mutations produce different effects leading to nonsense mutations or insertion and deletion. These mutations cause

premature termination of protein translation. This leads tochange in a single amino acid by missense mutation. The most commonly found mutation involving Cx26 is 35delG. In this mutation there is termination of protein translation by deletion of one base resulting in a frame shift. This introduces a premature stop codon and results in cessation of protein translation.

Studies have shown that since potassium is high intracellularly, the mutant channels may retain their permeability to potassium . It is believed that hearing impairment due to mutation is not fully due to problems in potassium recirculation but can be due to involvement of other molecules. It was found in certain studies that certain mutations led to decreased permeability of cell signalling molecule inositol 1,4,5-trisphosphate (IP3). Studies have shown that inositol 1,4,5-trisphosphate is important for calcium wave propagation for normal functioning of cochlea. This study showed that Cx26 mutations leading to hearing impairment where the gap junctions retain their ionic coupling is due to reduced permeability to other metabolites.(3).

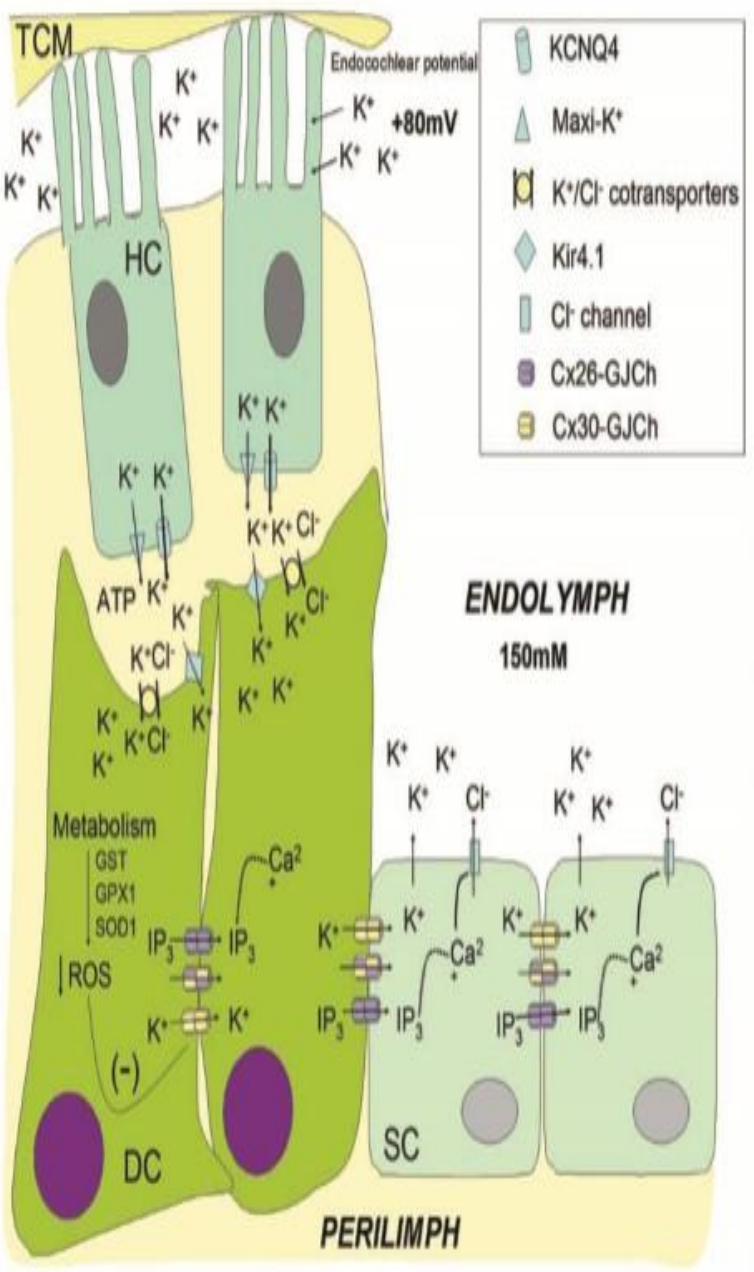


FIG. 4. K^+ recirculation in the cochlea may be affected by the level and properties of gap-junction intercellular communication. Activation of hair cells (HCs) by sound-induced movement of the tectorial membrane (TM) produces an increase in extracellular K^+ at the basal surface by activation of KCNQ4 and maxi-K^+ potassium channels in the basal membrane of HC. Thereafter, K^+ is buffered by supporting and epithelial cells. K^+ enters Deiter cells (DCs) through K^+/Cl^- cotransporters and K^+ channels like Kir4.1. Then K^+ is redistributed to supporting cells (SCs) and epithelial cell by means of GJCh connecting these cells (epithelial GJ network). In addition, some extracellular signals, like ATP (activating purinergic receptors), induce IP_3 production and Ca^{2+} release from the ER compartment. Diffusion of IP_3 through Cx26-GJCh allows calcium wave propagation, a signal for many cellular functions. It has been proposed that Ca^{2+} may activate Cl^- channels of supporting cells, allowing the efflux of Cl^- to the extracellular milieu that favors K^+ circulation to the endolymph. High metabolism of cochlear cells favors generation of reactive oxygen species (ROS) that are under the control of antioxidant enzymatic systems (GTS, GTX1, SOD1). ROS are negative regulators of GJCh, reducing intercellular coupling in many systems. Aging-induced hearing loss may be associated with inhibition of GJCh by increasing ROS production and reduction in the function of antioxidant enzymatic systems in the cochlea. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Cx26 mutation leading to hearing impairment can be categorised into four types:

a) mutations that can reducehemichanneltransport to the plasma membrane or gap junctional channel assembly;
b) mutationswhere the channels do not function but the gap junctions are being formed
c) mutations that createGJChwhich are functioning but have atypical gating or permeability properties, like decreased IP3 permeability; and
d) mutations that produce hemichannelslocated at the plasma membrane which will open under physiologic conditions, they may influencea ionic balance or the homeostasis of vital molecules that reduce cellular viability

Etiology of Congenital and Early-Onset Hearing Impairment

A lot of studies have been done to look at possible genetic and environmental causes of congenital and early onset hearing impairment.Tools of genetic epidemiology, including linkage analysis and molecular testing have helped to determine the causes of congenital hearing impairment. Studies have shown that prevalence and reasons of hearing impairment vary in different populations studied at different times. It was found in studies that autosomal recessive, autosomal dominant, and X-linked genes were accountable for 76%, 23%, and 1% of the inherited cases (36). Studies done in United States by analysing data collected from sample of deaf school children showed that hearing impairment in 36% of the children was sporadic (nongenetic), hearing impairment in 64% was caused dueto genetic causes (autosomal- recessive inheritance seen in 74%and autosomal-dominant inheritance seen in 26%) (37). By combining the

data from different sources it wasfound that in the United States, substantial hearing impairment is present in around 1.9 per 1000 infants at birth.(1)

There are a large number of genes causing hearing impairment. Mutations at a single locus, DFNB1 led to hearing impairment in a large number (30%–40%) was not expected.DFNB1 includes GJB2 and GJB6 genes. These genes code for the Connexin 26 (Cx26) and Connexin 30 (Cx30) subunits of gap-junction proteins.Inherited hearing impairment is commonly transmitted as a monogenic trait. Studies have shown digenic transmission also.(52)

It was observedin many studies that individuals who were digenic compound heterozygotes had more severe hearing impairment when compared to GJB2 35 del G homozygotes. . Mutations at a single locus, DFNB1 leading to hearing impairment in a large number was believed to be due tointense assortative mating seen in hearing impaired populations and the relaxed genetic selection against hearing impairment due to introduction of sign language. Both these factors were significant, and were more pronounced after sign language was introduced 400 years ago.It was found using computer simulation, that these factors could have twice the frequency of DFNB1 associated hearing impairmentover the past 200 years in the United States.

Effect of Consanguinity in the Population

Both assortative mating and consanguinity form important attributes for a population. They both can affect the incidence of hearing impairment in a population. In a population whenever a new recessive mutation first appears, due to stochastic processes there is a significant chance that it will be lost in further generations. Consanguinity in a population leads to phenotypic expression of some recessive mutations. These mutations may or may

not be naturally selected.Assortative mating comes into play once these mutations are expressed in the population in response to relaxed selection.Consanguinity, in a population, will influence all recessive mutations whether or not associated with hearing loss.AssortativematinG comparatively will influence recessive mutations associated with hearing impairment.In a given population recessive mutation associated hearing impairment will be increased byassortative mating .Genetic inheritanceenhanced by both consanguinity and assortative mating can increase the survival, expression, and spread of genes for hearing impairment. Sign language has improve the ‘‘genetic fitness’’ of the hearing impaired population, more so whensign language is used by both hearing impaired and normal family members.This resulted in acceptance of deaf individuals into the community. This promoted assorted deaf to deaf marriages and further led to increase in hearing impairment in the population as there was usually only one type of genetic deafness prevalent in the population. It is well known that gene drift and consanguinity play important roles in the persistence and initial phenotypic expression of genes in these populations.It was also noted that relaxed selection and assortative mating further promoted the increases recorded in both gene and phenotype frequencies. This points to a founder effect in recessive mutation leading to hearing impairment. (38)

LOCALIZATION AND ISOLATION OF GENE

Loci fornonsyndromic hearing loss (NSHL) were discovered only after 1992 and mapped on the human genome. It is significant that research into genetic causes for hearing impairment lagged behind research into other sensory handicaps like blindness.Cloning of genes which were responsible for human hearing impairmentby genetic tests has been delayed for a long time because the important proteins seen in normal hearing and hearing impairment have been identified only in the last ten years. Positional cloning of genes

associated with hearing impairment was also delayed than that of other disease genes. It was believed that mapping of genes responsible for hearing impairment was not possible by using linkage analysis. This was thought so because all the studies done by genetic community concenterated on prelingual hearing loss in Western countries. Prelingual hearing loss is congenital and severe leading to deaf-mutism. This led to social isolation of hearing impaired and affected theirgenetic fitness.Most pedigrees seen in West contained only a restricted number of prelingual deaf individuals in affected families as this has an autosomal recessive manner of inheritance. It was thought that due to large genetic heterogeneity of nonsyndromic hearing loss the linkage data seen in different families cannot be shared as the number of genes involvedis large.Due to high heterogeneity in genes causing hearing impairment, localization of genes by conventional linkage analysis in such one-generation pedigrees was too trivial to localizeas the linkage power was too small. Because of this no recessive gene were mapped in Western pedigrees in initial studies. Another reason wasassortative mating introducedin a single family, different mutant genesin the deaf community, this reduces the linkage analysis. Reservoir of consanguineous multiplex families were found in different ethnic communities in developing countries. In these families autosomal recessive hearing loss was seen in many family members due toconsanguinity.The benefit of studying these ethnic isolates was absence of assortative mating. The other benefit was that these families had limited living area and extended pedigrees were living together. This helped in accessibility to a large number of family members with hearing impairment. This has led to mapping of most autosomal recessive forms of hearing impairment in these families from developing countries.

LOCI FOR NONSYNDROMIC HEARING LOSS

There is a standard classification for mutation loci. DFN (DeaFNess) represents deafness. It is followed chronological order of identification of the locus indicated by a number. Autosomal dominant loci is denoted as DFNA, autosomal recessive loci is denoted as DFNB, X-linked loci is denoted as DFN.

GENES FOR NONSYNDROMIC HEARING LOSS

In the studies done so far 90 mutations involving Cx26 gene leading to nonsyndromic hearing impairment have been discovered. These mutations constitute nearly half of congenital cases of hearing impairments.(3)

Inspite of the large heterogeneity seen in genetic hearing loss, 50% of non syndromicsenosrineural hearing impairment has been attributed to variants of GJB2 gene mutation inmost of the studies done so far. Studies in different populations have shown different alleles as being more common.Studies done on Ashkenazi Jewish population have shown the 167delT mutation to be more common in theirpopulation (39). Another study done on populations in Caucasians and in Mediterranean regions showed 35delG as more common.(40) In studies done on Japanese population, 235delC mutations are seen commonly.(41)In studies done in Ghana, R143W was found to be more common occurring GJB2 variant (42).Some studies have described the presence of W24X mutation in GJB2 gene (40),only in Indian population is this mutation seen in significant numbers(43).Studies done in the Kerala population on probands with hearing impairment using molecular screening showed that 36% were associated with mutations in the connexin26 gene. These values were similar to those reported in other studies.[44,18].W24X was the most frequent mutation seen in these probands with

hearing impairment.Out of 86 probands 28 were found to be homozygous for W24X mutation.The most common mutation reported from studies done abroad is 35delG, but this mutation was not detected in this study. In another study done recently in a North Indian population, 35delG mutations were found along with W24X mutation (45). These studies have shown that different mutations are seen in different populations. It also implies that certain mutations are specific for same population.(46)

Restriction fragment length polymorphism

RFLP, restriction fragment length polymorphism, is a technique that uses differences in homologous DNA sequences. It refers to homologous DNA molecules with difference among samples that are present at differentpositions of restriction enzyme sites. It also refers to a related laboratory technique by which these segments can be illustrated. In RFLP analysis,restriction fragments are separated according to their lengths by gel electrophoresis after the DNA sample is broken into fragments(digested) by restriction enzymes.

Analysis technique

The basic technique for detecting RFLPs involves a process known as a restriction digest. It is done by fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs. Then agarose gel electrophoresis is used to separate the resulting DNA fragments. An RFLP occurs when among the individuals the length of a detected fragment differs. Each fragment length is accepted as an allele, and can be used in genetic analysis.

Applications

In genome mapping and genetic disease analysis,study of RFLP variation is a vital tool. To find out the chromosomal location of a particular disease gene, the DNA of members of a family afflicted by the disease is examined. Then RFLP alleles showing a similar pattern of inheritance as that of the disease are searched. Once a disease gene was localized, other family members were checked by RFLP analysis to look for who was at risk for the disease, or who was likely to be a carrier of the mutant genes.

GENETIC TESTING

There are very few studies done on the effect of genetic counselling and genetic test for newborn hearing as a screening procedure. This is attributed to the difficulties associated in genetic testing with technical difficulties, time taken for the results and financial implications, also associated are social reasons. This makes genetic counselling for hearing impairment very complex. The other difficulties associated with genetic tests is the underlying etiological heterogeneity seen in mutations associated with hearing impairment. The other problem associated with genetic testing is the limited ways of interpreting the test results, as only positive results are easy to interpret, inconclusive results due to a variant of mutation or a genetic variant of uncertain significance is difficult to interpret in terms of result.These results increase the complexity in terms of understanding inheritance of deafness, genetic information, and chances of recurrence in future generations. It is difficult to explain to the parents when theaetiology for their child's deafness remains unknown. In the case of GJB2/GJB6 testing, a positive result reveals that a child's deafness is genetic in origin, whereas, negative or inconclusive results do not provide definitive information about why a child is deaf. Importantly,

negative or inconclusive GJB2/GJB6 results do not rule out a genetic form of deafness because there are many untested genes that can cause deafness. Although the number of studies evaluating knowledge and understanding of genetic information is small, the most common finding is misinterpretation of the meaning of a lack of explanation for why a child is deaf, either as an outcome of evaluation of clinical and family history information or as a result of a negative genetic test result, where individuals tend to equate a negative result with 'not genetic' and a zero percent recurrence chance.

Parents can understand concepts of heterogeneity and inheritance in abstract terms after genetic counselling. Studies have shown that the way test results are interpreted by parents varies according to the result of the genetic test, whether positive, negative or inconclusive. Most of the parents were able to understand when the test results are positive. Studies have shown that parents are able to interpret that their child has connexion related deafness, it is inherited, and there is a 25% chance of recurrence in further pregnancies. All this information can be conveyed to parents by genetic counselling.Studies have shown that when parents received inconclusive test results, they could understand that the child may still have an inherited deafness and that the aetiology of their child's deafness was undetermined. These parents have less understanding as regards tochances of recurrence. This difficulty in understanding and interpreting of results by the parents can be overcome by pre-test and post-test genetic counselling given to the parents. More studies are needed to be done to identify the ways of genetic counselling and the amount of information to be given to the parents.

Various studies have demonstrated the diversity of the attitudes and beliefs of hearing parents, deaf parents, and deaf adults regarding genetic testing for deafness, the social complexities associated and the difficulties in dealing with these.Genetic tests are useful

in various ways, they help in finding out the cause of hearing impairment of the child, knowing any chance of recurrence in future pregnancies, and desciding on future family planning. Although there are different reasons to do the genetic tests, studies have shown that factors like cultural, religious and ethnic heritage determine the acceptability and other use of this genetic information.One common concern expressed by most is that this genetic information will be used to decide on future reproductive decisions. Another concern is that prenatal diagnosis and termination of foetuses with genotypes associated with hearing impairment may be practised.

The hearing screening process for the newborn starts at bedside with audiometric screening of all newborns before they are discharged. Any newborn who fails the screening test, further diagnostic audiologic evaluation is done in outpatient department. During this process of screening parents of the child are in contact with a number of different health care professionals including obstetricians, paediatricians, hearing screeners, audiologists, otolaryngologists, and early intervention teachers. At present there is no guidelines or formal protocol on who among these health care professional gives genetic counselling and advices parents regarding the genetic tests. Among the health care professional paediatricians and audiologists are in repeated contact with the parents,they should be the one to inform parents regarding the need and availability of genetic testing. Many studies have shown that most parents feel that genetic testing and evaluation if needed should be initiated early.

Many studies have shown that parents do not believe that genetic testing of their hearing impaired children is harmful. Studies have shown that most parents who receive positive results in genetic testing feel benefited by the study. They feel that they can understand the cause of their child's hearing impairment better. This helps them in deciding upon

future treatment options and also with future family planning. Parents who receive inconclusive or negative results feel less benefit from genetic tests but they do feel some benefits in terms of future family planning. In contrast, parents who feel genetic test are harmful and will lead to stigma, their views are unlikely to change when they learn their child's test result. It is important to do pre- and post-test genetic counselling as it can improve understanding of parents of negative test results.(47)

Clinical Utility of Genetic Testing for Hearing Loss

There are several advantages of genetic tests when compared to conventional hearing loss evaluation without genetic testing. (48,49,50) The benefits anticipated from genetic testing include:

1. Reduction of additional time consuming, invasive, and expensive testing;
2. Choice of early interventions such as hearing aids, cochlear implants, or sign language that significantly improve language ability and quality of life outcomes;
3. Information on the progression of the condition;
4. Ability to monitor associated clinical manifestations and complications, particularly for certain syndromic forms of hearing loss;
5. Information on the chance of recurrence in the family that can inform reproductive decisions; and
6. Information pertinent to risks and health care decisions (e.g., avoiding aminoglycoside antibiotics among those with MTRNR1 mutations). (51)

It was found in many studies that when compared to traditional evaluation genetic testing is more sensitive and specific. A study done at Cincinnati Children's Hospital on children with hearing impairment found that after the regular evaluation, 80% of hearing impaired

chidren remained undiagnosed.(52) Studies have shown that the age at which the hearing loss was identified ranged from 12–60 months among children with hearing impairment. This delay in diagnosing hearing loss affects language learning and reduces further management options. A study about cochlear implants reports, “There seems to be a substantial benefit for both speech and vocabulary outcomes when children receive their implant before the age of 2.5 years”(21)

GENE THERAPY

Studies were done for gene therapy by targeted deletion of Cx26 in the inner ear of a mouse. The mouse had a GJB2 mutation, 35delG, produced by targeted deletion of a single base. This mutation led topremature termination of the Cx26 protein by causing a frame shift at the 12th amino acid. Cx26 gene was delivered using bovine adeno-associated viral (BAAV) vectors. Bovine adeno-associated viral (BAAV) vectors are efficient in gene transfer and have minimal toxicity.Cochlear non–sensory cells were maintained in organotypiccultures.andCx26 gene was delivered using bovine adeno-associated viral (BAAV) vectors to restore gap junction coupling. This study showed that transduction of cochlear organotypic cultures of inner ear of mouse with targeted deletion with bovine adeno-associated viral (BAAV) vectors at P5 restored Cx26 to the plasma membrane of cochlear nonsensory cells. It was also shown that these new molecules of connexin 26 also induced formation of functional intercellular channels by docking with other molecules at points of contact between adjacent cells of cochlea.(53).

Gene therapy is a veru useful way of modulating gene expression in the inner ear. Gene therapy can help in treating cochlear disorders. Recombinant bovine adeno-associated viral (BAAV) vectors have several advantages which make them very useful as means of

gene transfer in the inner ear. Recombinant bovine adeno-associated viral (BAAV) vectors havedemonstrated efficient gene transfer both in vivo and in vitro. BAAV have a unique serological identity and have low toxicity. Bovine associated adeno virus (BAAV) show tropism for the inner ear cells. They are also well tolerated by the cochlea [54,55]. Studies have reportedtransduction of most of the non-sensory cells by BAAV and they are well tolerated. Studies have also shown that in vivo gene therapy it is possible to have apical transduction of cochleain utero which would be preferred [48]. Studies have shown that sialic acid containing gangliosides are important for BAAV entry and transduction.Mammalian cochlea expresses multiple gangliosideson its cell surface[50]. Bovine adeno-associated virus (BAAV) exhibits lead tosporadic transduction of sensory inner and outer hair cells with increased tropism for cochlear non– sensory cells [48,49]. These results in this study showed that suggest that normal connexin levels by gene delivery via recombinant AAV can be restored. Thus gene therapy can be used to restore hearing function in DFNB1 mouse models. This in future can lead to application in therapeutic interventions designed for humans, mainly for children with congenital nonsyndromic hearing impairment. (56)

PATIENTS AND METHODS

This is a pilot study conducted in Department of ENT and Medical Genetics, at CMC, VELLORE. 96 children were included in the study – 86 being retrospective.

Children presenting with history of nonsyndromicsensorineural hearing impairment in the department of ENT of our hospital were enrolled in the study. . History was obtained based on information obtained by a combination of self-reporting and physician assessment. Clinical evaluation including physical examination, hearing evaluation, blood investigations including genetic test were done. Genetic test were done in Department of Clinical Genetics at CMC.

Methodology of genetic test : Extracted DNA was amplified using specific primers for GJB2 gene by PCR. This was checked in agarose gel & the product were digested using restriction enzyme Alu1 to look for W24X mutation. Those negative for above mutation, in them PCR was repeated followed by sequencing

The test were done in the following steps

1. DNA EXTRACTION – MINI (QIAGIN KIT METHOD)

AIM: To isolate DNA from the blood sample using QIAGINI KIT method

MATERIALS REQUIRED:

1. Blood sample
2. Water bath
3. Eppendorf tube
4. Vortex
5. Absolute alcohol
6. Centrifuge
7. Qiagini kit

PROCEDURE:

1. Samples must be at room temperature
2. Water bath set at 56^0 C
3. Label the eppendorf tube with patient's name and number
4. Add 20 µl protease K to eppendorf
5. Add 200 µl EDTA blood to eppendorf
6. Add 200 µl of AL buffer. Do not shake
7. Vortex spin 30 seconds X 3 times

8. Ordinary spin 30 seconds

9. Water bath set at 56^0 C for 5 minutes

10. Vortex spin 30 seconds X 3 times

11. Ordinary spin 30 seconds

12. Water bath set at 56^0 C for 5 minutes

13. Vortex spin 30 seconds X 3 times

14. Ordinary spin 30 seconds

15. Add 200 µl of absolute alcohol

16. Vortex spin 30 seconds X 3 times

17. Ordinary spin 30 seconds

18. Label columns and pour mixture

19. Centrifuge at 14000rpm for 3 minutes

20. Discard the well containing the filtrate, remove upper column and fix in new wells

21. Add 500 µl of AW1 buffer

22. Centrifuge at 14000rpm for 3 minutes

23. Discard the well containing the filtrate, remove upper column and fix in new wells

24. Add 500 µl of AW2 buffer

25. Centrifuge at 14000rpm for 4 minutes

26. Discard the filtrate and place the spin column in the 1.5ml eppendorf tube and add 200 µl of AE buffer.

27. Incubate at room temperature for 20 minutes

28. Centrifuge at 14000rpm for 2 minutes

29. Incubate at room temperature for overnight and store the extracted DNA

2. POLYMERASE CHAIN REACTION

AIM: To amplify the desired region using PCR

PRINCIPLE:

Polymerase chain reaction (PCR) is a primer mediated enzymatic amplification of specifically cloned or genomic DNA sequences. PCR process was invented by Kary Mullis and it has been automated for routine use in laboratories worldwide. The main purpose of the PCR process is to amplify template DNA using thermostable DNA polymerase enzyme which catalyzes the buffered reaction in which an excess of an oligonucleotide primer pair and four deoxynucleoside triphosphates (dNTPs) are used to make millions of copies of the target sequence.

There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation at 94°C:

During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop.

Annealing at 54°C:

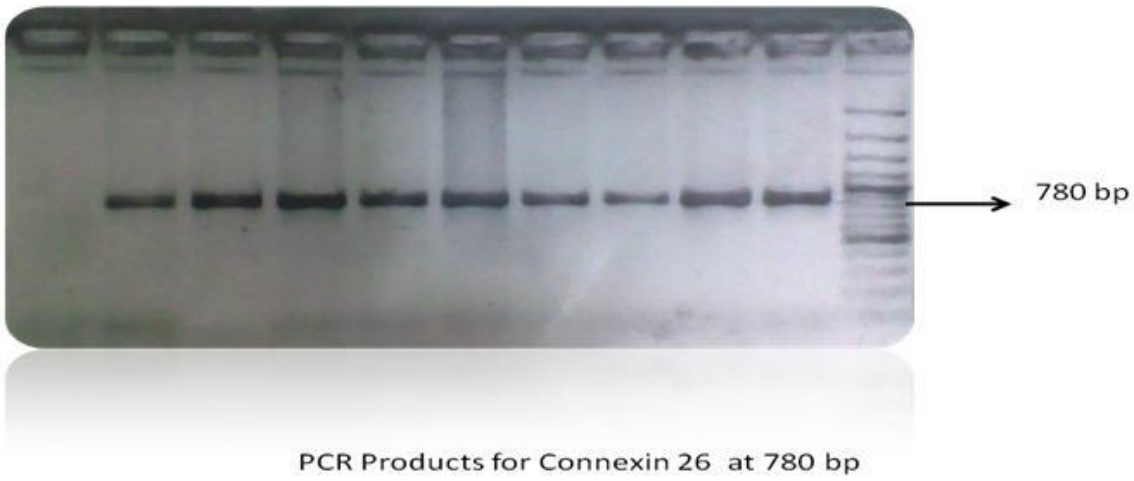
The primers are jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

Extension at 72°C:

This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match get loose again (because of the higher temperature) and do not give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template)

3. Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. The total number of DNA copies after n number of cycles is 2^n.

Amplification of the GJB2 gene by PCR



PCR Products for Connexin 26 at 780 bp

COMPONENTS:

1. **Taq DNA polymerase**

The DNA polymerase, known as 'Taq polymerase', is named after the hot-spring bacterium Thermusaquaticus from which it was originally isolated. The enzyme can withstand the high temperatures needed for DNA-strand separation, and can be left in the reaction tube. The cycle of heating and cooling is repeated over and over, stimulating the primers to bind to the original sequences and to newly synthesised sequences. The enzyme will again extend primer sequences. This cycling of temperatures results in copying and then copying of copies, and so on, leading to an exponential increase in the number of copies of specific sequences. Because the amount of DNA placed in the tube at the beginning is very small, almost all the DNA at the end of the reaction cycles is

copied sequences. Higher Taq DNA polymerase concentrations than needed may cause synthesis of non-specific products.

2. Template DNA

Nearly any standard method is suitable for template DNA purification. An adequate amount of template DNA is between 0.1 and 1 µg for genomic DNA for a total reaction mixture of 100 µl. Larger template DNA amounts usually increase the yield of non-specific PCR products.

3. Primers (forward and reverse)

1. PCR primers should be 10-24 nucleotides in length.

2. The GC content should be 40%-60%.

3. The primer should not be self-complementary or complementary to any other primer in the reaction mixture, to prevent primer-dimer and hairpin formation.

4. Melting temperatures of primer pairs should not differ by more than 5°C, so that the GC content and length must be chosen accordingly.

5. The melting and annealing temperatures of a primer are estimated as follows: if the primer is shorter than 25 nucleotides, the approximate melting temperature is calculated with the formula: $Tm = 4 (G + C) + 2 (A + T)$.

6. The annealing temperature should be about 5°C lower than the melting temperature.

4. $MgCl_2$ Concentration

Because Mg2+ ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of $MgCl_2$ has to be selected for each experiment. Too few Mg2+ ions result in a low yield of PCR product, and too many will increase the yield of non-specific products. The recommended range of $MgCl_2$ concentration is 1 to 3 mM, under the standard reaction conditions specified.

5. dNTP's

The concentration of each dNTP (dATP, dCTP, dGTP, dTTP) in the reaction mixture is usually 200 µM. These concentrations must be checked as being equal, because inaccuracies will increase the degree of misincorporation.

MATERIALS REQUIRED:

1. DNA sample
2. Master mix
3. Forward Primer
4. Reverse Primer
5. Double distilled water
6. Thermo cycler (PCR machine)
7. PCR tubes (0.2ml)
8. Micro pipettes

PROCEDURE:

1**.** Label the PCR tubes

2. Add the following to each of the PCR tubes

Components	Tube
PCR master mix	11.5 µl
Primer (F)	1 µl
Primer (R)	1 µl
Water	9.5 µl
DNA sample	1.5 µl
Total	24.5 µl

3. Spin the PCR tube with components added using low speed small micro centrifuge machine

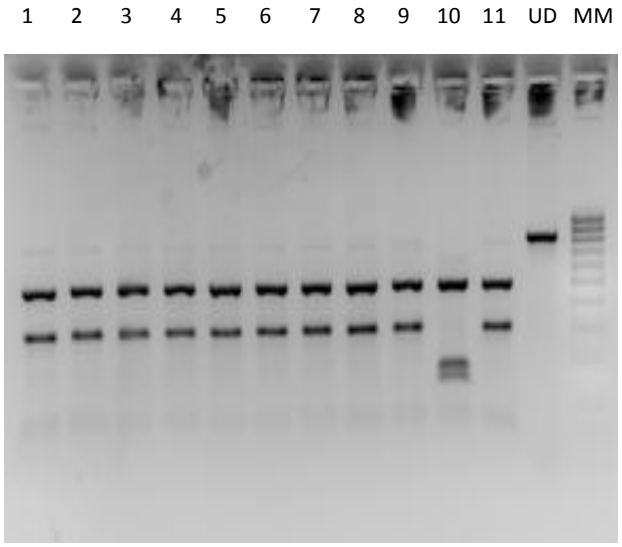
4. Place the tubes in the Thermo cycler and set the programme as follows,

Temperature	Time	Condition	Cycles
95^0 C	2 min	Denaturation	1
94^0 C	30 sec	Denaturation	35
58^0 C	30 sec	Annealing	35
72^0 C	30 sec	Extension	35
72^0 C	10 min	Final extension	1

5. Run the programme.

6. PCR Product checked on 2 % agarose gel.

Gel picture showing the products after digestion with Restriction enzyme *AluI* 10th patient is having W24X mutation



4. RESTRICTON DIGESTION

AIM: To digest the DNA using Apa 1

PRINCIPLE:

Restriction endonucleases are enzymes which recognize and cleave double stranded DNA at specific sequences, and are named for the cellular strain from which they are isolated. The natural function of the enzymes is to destroy foreign DNA, while the cell's own DNA is protected from cleavage because it has been specifically modified by cellular methylases. Typically, the recognition sites consist of 4-6 (or more) bases arranged in a palindromic sequence. Type II restriction enzymes are mostly used in cloning than the other types.

Alu1:

Digest the PCR product with Alu1 enzyme.

PROCEDURE:

1. Label the tubes

2. Add the following to 10µl PCR product

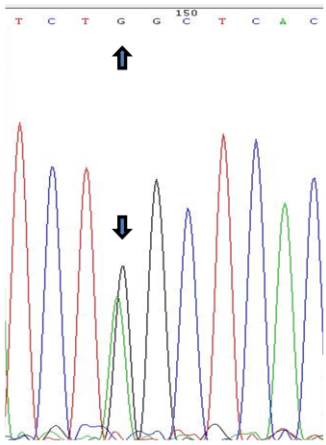
Buffer	2µl
Alu I enzyme	1µl
Sterile water	7µl
Total	10µl

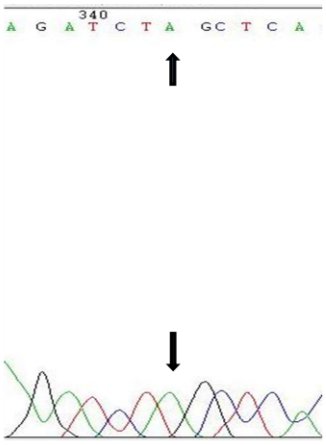
3. Mix well

4. Keep in water bath for 12 hours.

5. Check the products on 1.5% Agarose gel. To interpret W24X mutation.

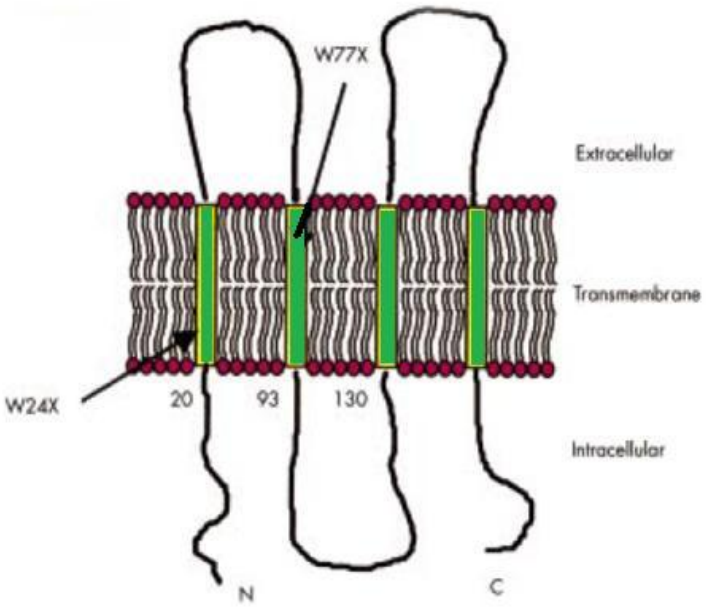
Sequencing done in ABIPrism 3100 sequencer showing heterozygote and homozygote





Heterozygous and homozygous W24X mutation confirmed by sequencing in our patients

Diagram showing the W24X mutation from internet



Inclusion criteria: Children less than 16 years old presenting with history of non-syndromicsensorineural hearing impairment in the department of ENT of our hospital were enrolled in the study. Hearing level criteria included permanent childhood hearing impairment of 40dB or more in better hearing ear averaged across 500Hz to 4KHz.

Exclusion criteria: Children with non-genetic causes of hearing loss (maternal infections, prematurity, low birth weight, birth injuries, ototoxins usage, Rh factor complications, maternal diabetes, toxaemia during pregnancy &anoxia) were excluded from the study.

RESULT

TABLE 1 : Distribution of hearing loss

HEARING LOSS	FREQUENCY	PERCENTAGE
MODERATE	21	21.9%
MODERATELY SEVERE	5	5.2%
SEVERE	24	25%
PROFOUND	46	47.9%
TOTAL	96	100%

Among the probands with nonsyndromicsensorineural hearing loss nearly 22% had moderate, 5% moderately severe, 25% severe and 48% had profound hearing loss.

Distribution of hearing loss among the probands

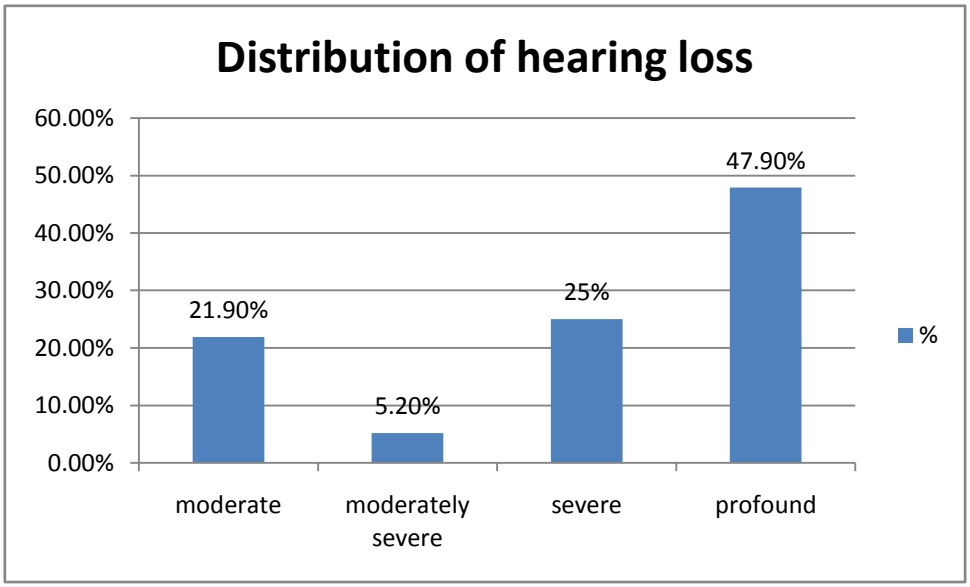


TABLE 2 : Gender distribution of the probands

GENDER DISTRIBUTION	FREQUENCY	PERCENTAGE
MALE	36	37.5
FEMALE	60	62.5
TOTAL	96	100

37.5 % of probands were males and 62.5% females

Gender distribution in the probands

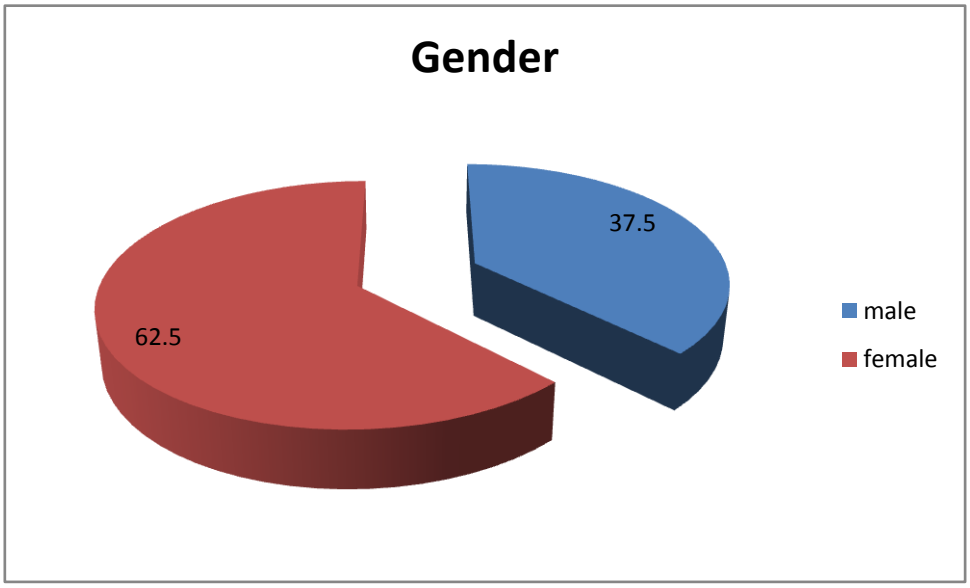


TABLE 3 : Gender distribution across hearing loss

GENDER DISTRIBUTION OF HEARING LOSS	MODERATE	MODERATELY SEVERE	SEVERE	PROFOUND	TOTAL
MALE	8	0	10	18	36
PERCENTAGE WITHIN GENDER	22.2%	0%	27.8%	50%	37.5%
FEMALE	13	5	14	28	60
PERCENTAGE WITHIN GENDER	21.7%	8.3%	23.3%	46.7%	62.5%
TOTAL	21	5	21	46	96
TOTAL PERCENTAGE	21.9%	5.2%	25%	47.9%	

47.9% of probands had profound hearing loss, 50% of males and 46.7% of females probands had profound hearing loss

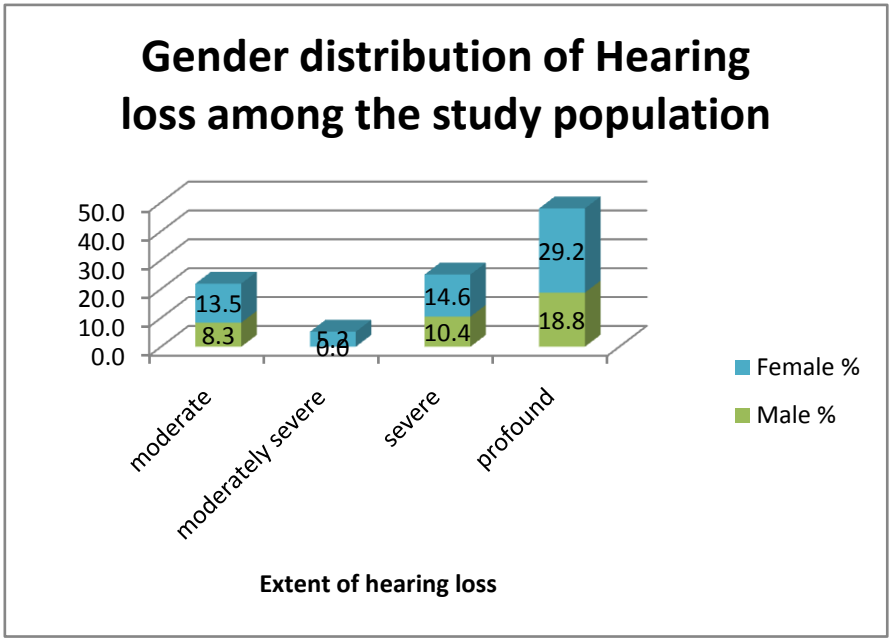


TABLE 4 : Distribution across the type of marriage

MARRIAGE	FREQUENCY	PERCENTAGE
CONSANGUINOUS	37	38.5
NONCONSANGUINOUS	59	61.5
TOTAL	96	100

Among the probands studied 38.5 % were born to consanguinous marriage and 61.5% born to nonconsanguinous marriage

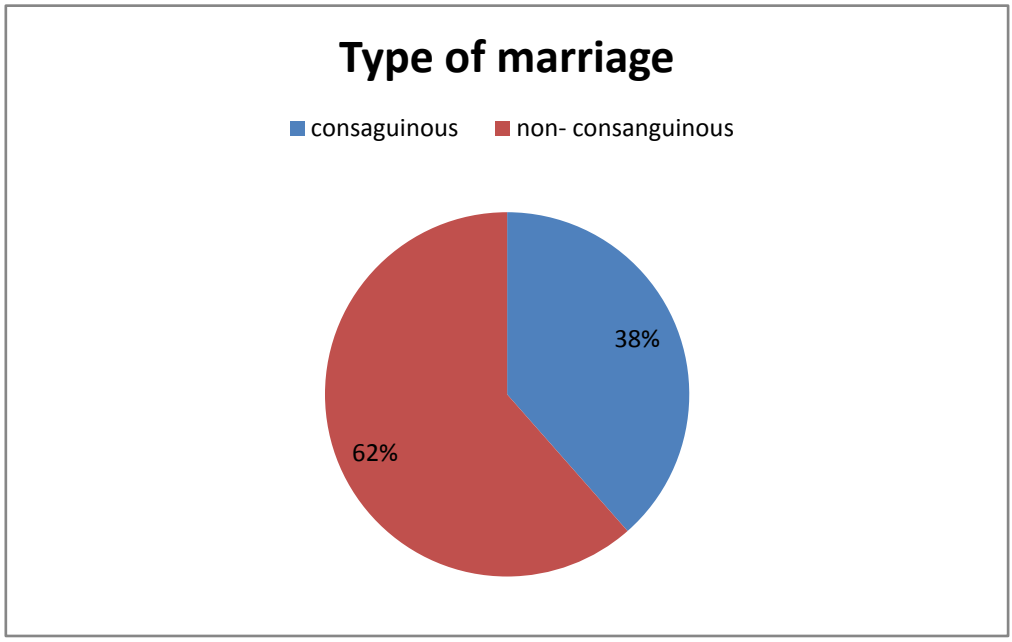


TABLE 5 : Degree of hearing loss in probands born to consanguineous marriages

HEARING LOSS IN CONSANGUINOUS MARRIAGE	MODERATE	MODERATELY SEVERE	SEVERE	PROFOUND	TOTAL	PERCENTAGE
CONSANGUINOUS	5	2	7	23	37	38.5
PERCENTAGE IN CONSANGUINOUS MARRIAGE	13.5%	5.4%	18.9%	62.2%		
NONCONSANGUINOUS	16	3	17	23	59	61.5
PERCENTAGE IN ONCONSANGUINOUS MARRIAGE	27.1%	5.1%	28.8%	39%		
TOTAL	21	5	24	46	96	

Among probands born to consanguineous marriage, 62.2% had profound hearing loss as compared to 39% born to nonconsanguinous marriage, 18.9% had severe hearing loss compared to 28.8%, 5.4% had moderately severe hearing loss compared to 5.1% and 13.5% had moderate hearing loss compared to 27.1%

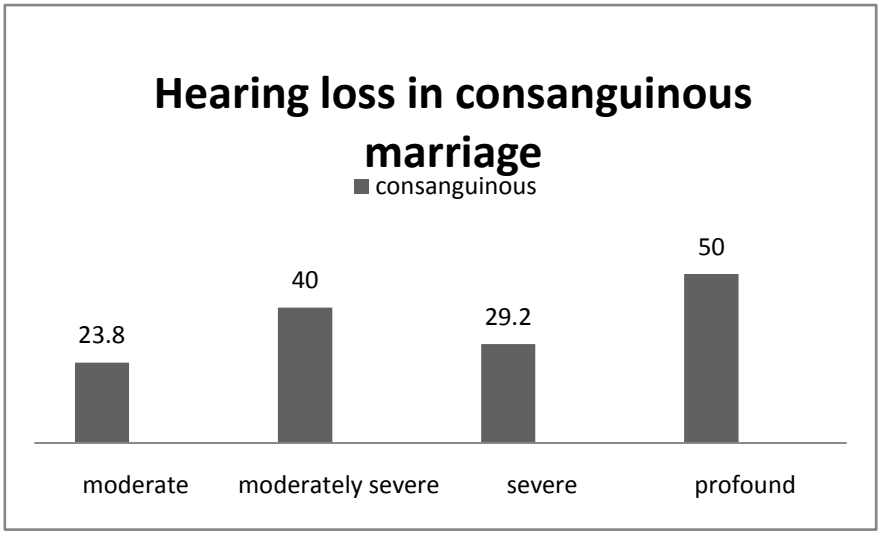


TABLE 6 : Percentage of GJB2 mutation positive in probands

GJB2 MUTATION	FREQUENCY	PERCENTAGE
POSITIVE	13	13.5
NEGATIVE	83	86.5
TOTAL	96	100

13.5% of probands were positive for GJB2 mutation and 86.5% were negative

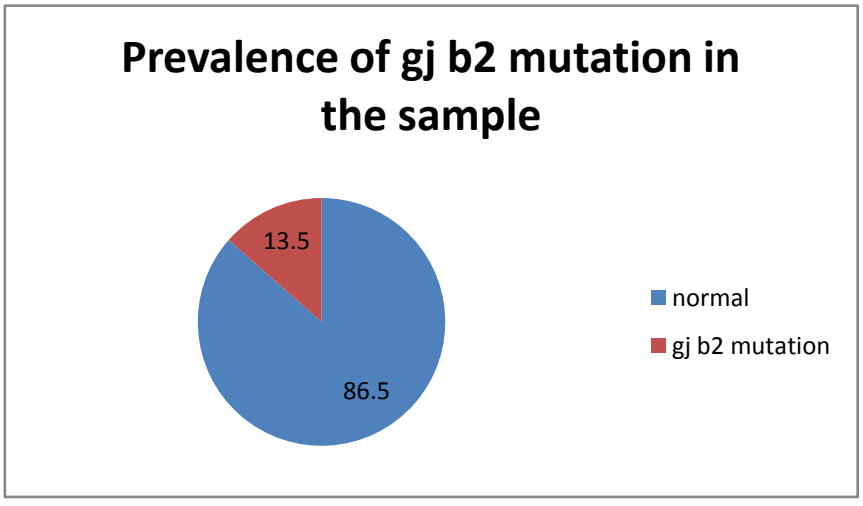


TABLE 7: Gender distribution of GJB2 mutation

GENDER DISTRIBUTION OF GJB2	FREQUENCY	TOTAL	PERCENTAGE
MALE	7	36	19.4%
FEMALE	6	60	10%
TOTAL	13	96	13.5%

There are 36 boys and 60 girls in the study. Out of the 36 boys, 19.4% had gjb2 mutation and 10% of the girls had that mutation however, this difference was not statistically significant. In simple words, there is no evidence of association

GENDER DISTRIBUTION OF GJB2 MUTATION

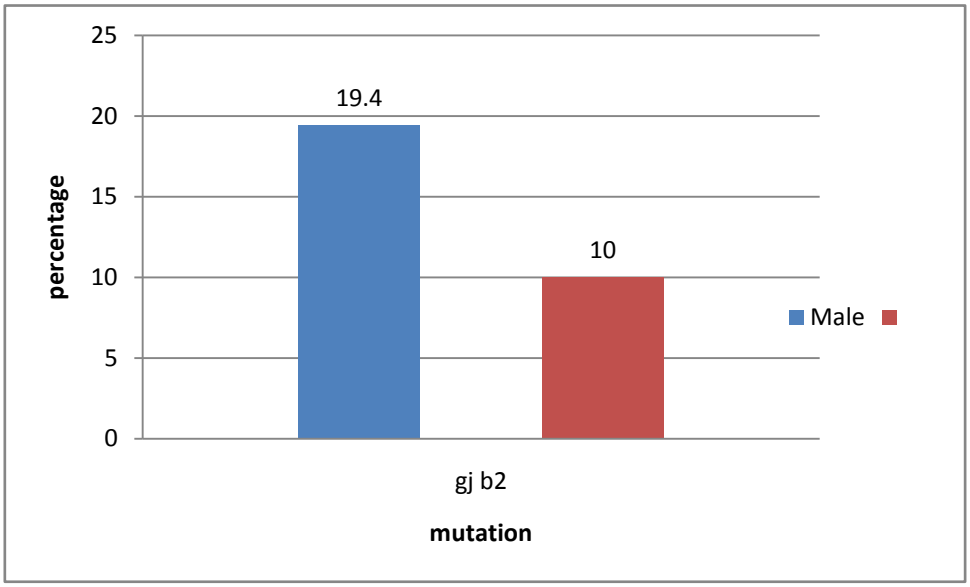


TABLE 8 : Degree of hearing loss across probands with GJB2 mutation

HEARING LOSS ASSOCIATED WITH GJB2	MODERATE	MODERATELY SEVERE	SEVERE	PROFOUND	TOTAL
POSITIVE	0	0	0	13	13
NEGATIVE	21	5	24	33	83
TOTAL	21	5	24	46	96
PERCENTAGE	0%	0%	0%	28.3%	13.5%

All probands with GJB2 mutation had profound hearing loss. 28.3% of profound hearing loss probands were positive for GJB2 mutation

DEGREE OF HEARING LOSS ACROSS PROBANDS WITH HEARING LOSS

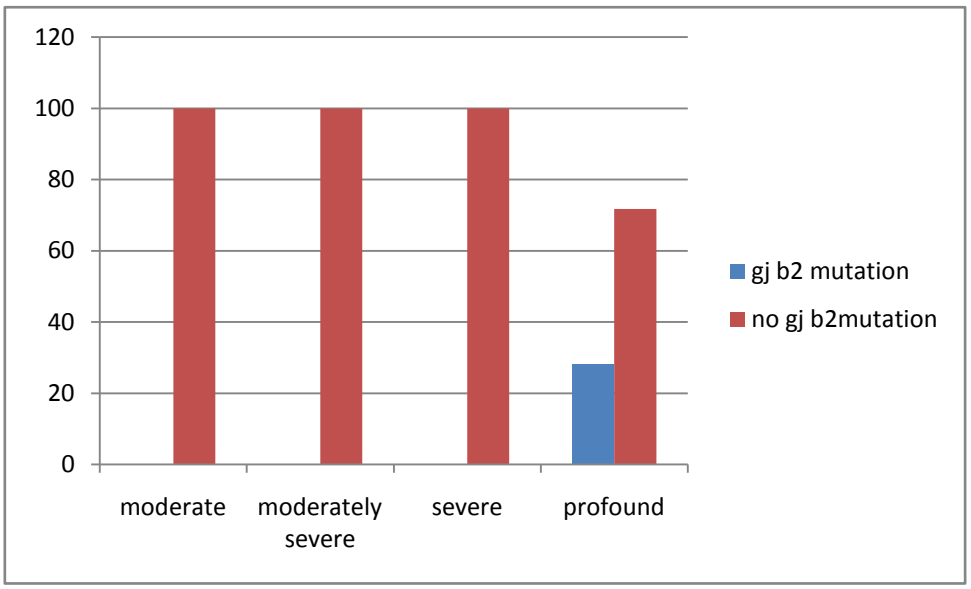


TABLE 9 : Percentage of w24x positive among GJB2 positive

W24 X AMONG GJB2 POSITIVE	FREQUENCY	PERCENTAGE
POSITIVE	6	47%
NEGATIVE	7	53%
TOTAL	13	100%

47% of GJB2 positive probands tested positive for W24X and 53% tested negative

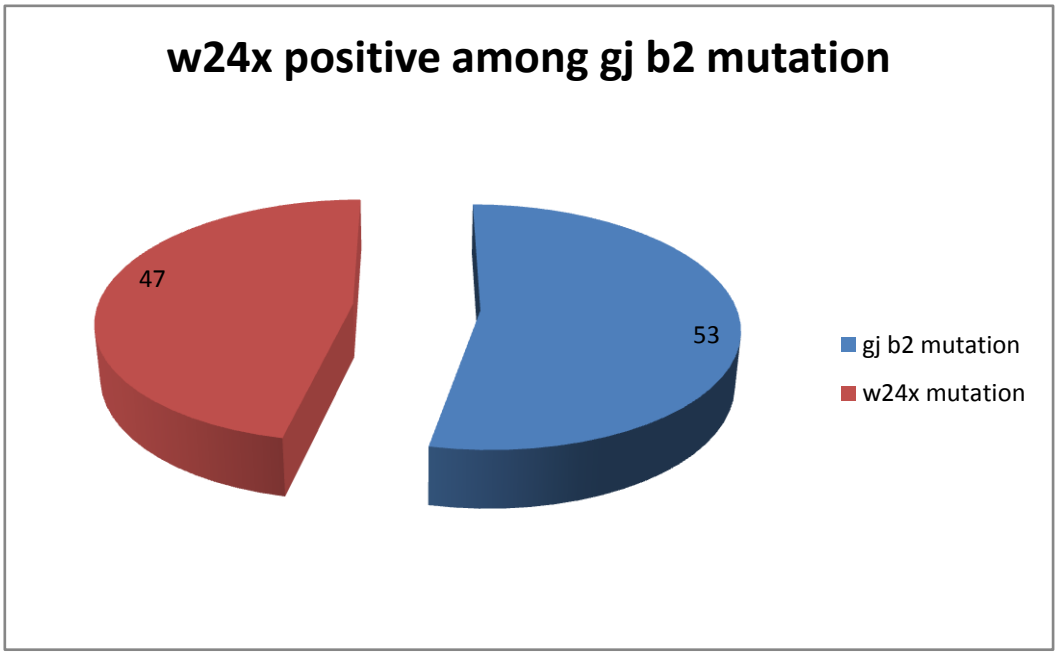


TABLE 10 : Percentage of w24x positive among probands

W24X AMONG NSSNHL	FREQUENCY	PERCENTAGE
POSITIVE	6	6.3%
NEGATIVE	90	93.8%
TOTAL	96	100%

6.3% of probands tested positive for W24X mutation and 93.8% tested negative

W24X POSITIVE AMONG PROBANDS

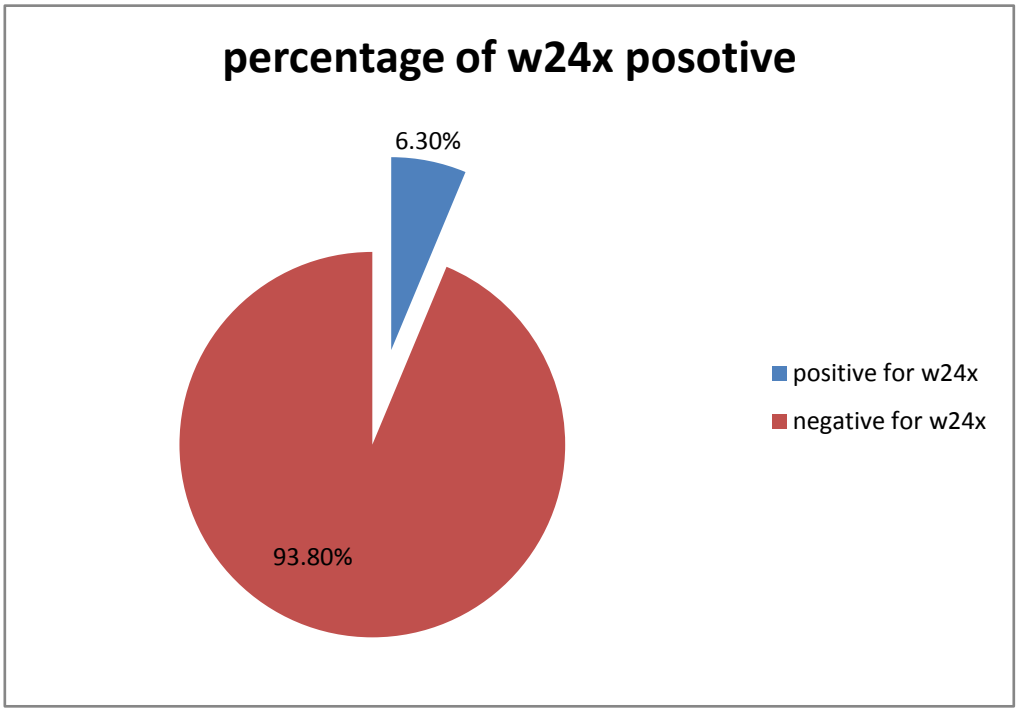


TABLE 11 : Gender distribution of W24X

GENDER DISTRIBUTION OF W24X	FREQUENCY	TOTAL	PERCENTAGE
MALE	2	36	5.6%
FEMALE	4	60	6.7%
TOTAL	6	96	6.3%

5.6% of males and 6.7% of females tested positive for W24X, 6.3% of total probands tested positive for W24X mutation

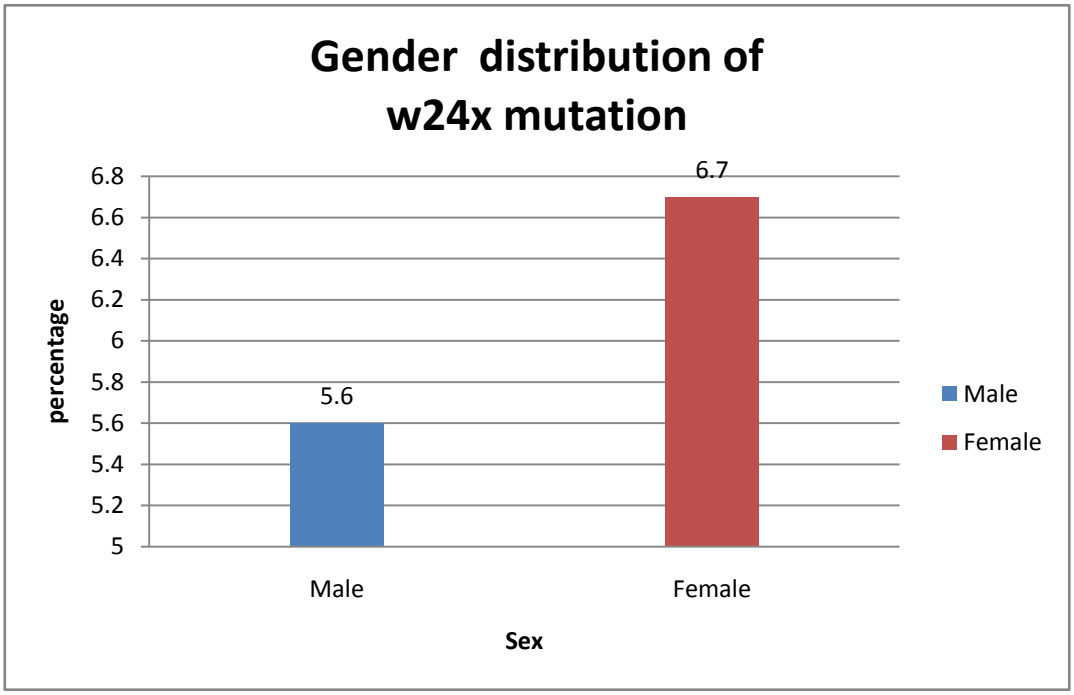


TABLE 12 : Hearing loss across W24X positive probands

HEARING LOSS ASSOCIATED WITH W24X	MODERATE	MODERATELY SEVERE	SEVERE	PROFOUND	TOTAL
POSITIVE	0	0	0	6	6
NEGATIVE	21	5	24	40	90
TOTAL	21	5	24	46	96
PERCENTAGE	0%	0%	0%	13%	6.3%

All probands with W24X had profound hearing loss, 13% of probands with profound hearing loss had W24X mutation.

HEARING LOSS ACROSS W24X POSITIVE PROBANDS

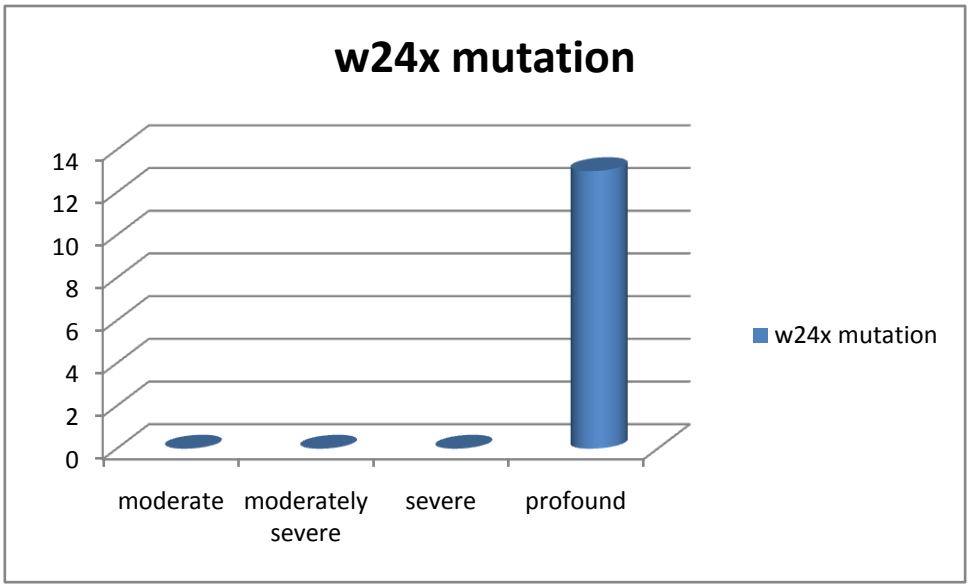
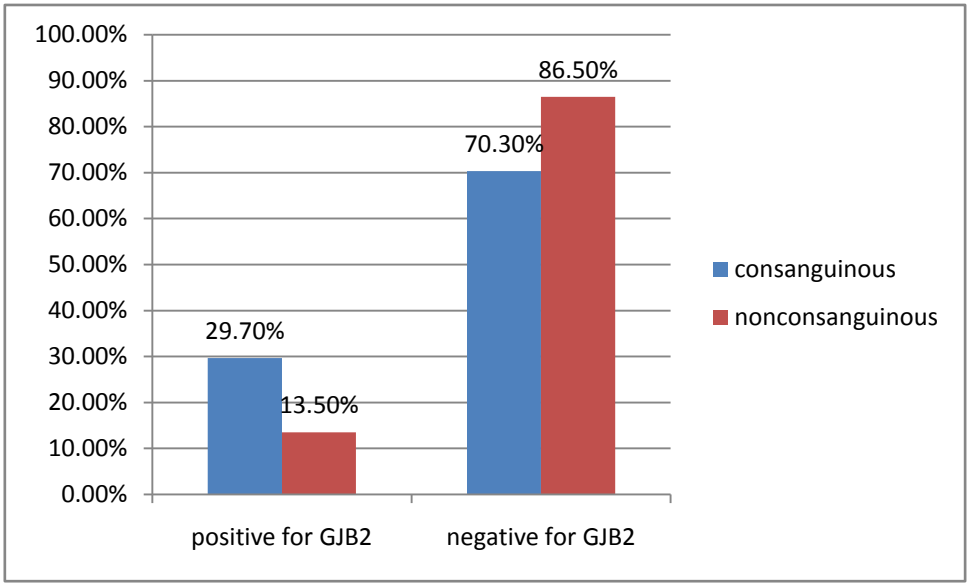


TABLE 13 : Association of consanguinity with GJB2 mutation

MARRIAGE	POSITIVE	NEGATIVE	TOTAL
CONSANGUINOUS	11	26	37
PERCENTAGE OF GJB2 IN CONSANGUINOUS	29.7%	70.3%	
NONCONSANGUINOUS	2	57	59
PERCENTAGE OF GJB2 IN NONCONSANGUINOUS	13.5%	86.5%	
TOTAL	13	83	96

There are 37 of the children from consanguineous marriage of which 29.7% had GJB2 mutation and 3.4% out of non-consanguineous had mutation and this difference was highly significant.

ASSOCIATION OF CONSANGUINITY WITH GJB2 MUTATION



DISCUSSION

This pilot study was done in a tertiary training care centre in India to look at the genetic profile of children with nonsyndromicsensorineural hearing loss who had attended our OPD.

In this study there were 96 probands, 36 males and 60 females. Among the probands 46 i.e. 47.9% had profound hearing loss.

These children came from different geographic areas of India, and from different cultural background. Most of these children came from Tamil Nadu, Andhra Pradesh, West Bengal, Bihar and the North eastern states. They came from different cultural background with some favoring consanguinity in the population. This study we did not look at any pockets or clusters of deaf population, or any deaf schools. So the results in this study are different from other studies where they have concentrated on families and populations with hearing impairment and also looked at populations where consanguinity is commonly practised.

Deafness associated with mutation at DFNB1 locus on chromosome13q11 is the most prevalent type of nonsyndromicsensorineural hearing loss in most parts of the world. Connexin 26 (Cx26, genesymbol*GJB2*) and connexin30 (Cx30, *GJB6*) are the two genes localised to this chromosomal region and both have been implicated in causing the hearing loss (43). Connexin 26 (GJB2) and connexin 30 (GJB6) have been reported to be the most common cause of nonsyndromicsensorineural hearing loss, accounting for 30% to 40% in different populations (46). Studies in different populations have shown different alleles as being more common.Studies done on Ashkenazi Jewish population have shown the 167delT mutation to be more common in theirpopulation (39). Another

study done on populations in Caucasians and in Mediterranean regions showed 35delG as more common.(40). In studies done on Japanese population, 235delC mutations are seen commonly.(41)In studies done in Ghana, R143W was found to be more common occurring GJB2 variant (42).Studies done in the Kerala population on probands with hearing impairment using molecular screening showed that 36% were associated with mutations in the connexin26 gene. (46). Similar values were reported in other studies done elsewhere in the world .

In our study we found Connexin 26 mutation in 13.5% of children with nonsyndromicsensorineural hearing loss. There are 36 boys and 60 girls in the study. Out of the 36 boys, 19.4% had gjb2 mutation and 10% of the girls had that mutation however. In simple words, there is no evidence of association. We also found in our study all probands with GJB2 mutation had profound hearing loss.

The most common GJB2 mutation found in the other studies done in India in was W24X (46). Interestingly, 35delG, which is one of the most common reported mutations in GJB2 gene in studies done abroad was not detected in most studies done on Indian population. . In another study done recently in a North Indian population, 35delG mutations were found along with W24X mutation (45).These studies have shown that different mutations are seen in different populations. It also implies that certain mutations are specific for same population.(46).

In our study we found 47% of GJB2 positive probands tested positive for W24X. 6.3% of the total probands tested positive for W24X mutation. Out of the total probands tested,5.6% of males and 6.7% of females tested positive for W24X. We also found that all probands with W24X had profound hearing loss.

Studies have shown that high carrier rates of ressive genes is seen in populations with high rates of consanguinity.Previous studies have shown a very high rate of consanguinity and inbreeding coefficient of South Indian population (56). In our study among the probandstested 38.5 % were born to consanguinous marriage. There are 37 of the children from consanguineous marriage of which 29.7% had GJB2 mutation and 3.4% out of non-consanguineous had mutation and this difference was highly significant Among probands born to consanguineous marriage, 62.2% had profound hearing loss as compared to 39% born to nonconsanguinous marriage

The most important benefit of identifying the molecular genetics behind deafness lies in the further management. In our study among the probands with nonsyndromicsensorineural hearing loss 47.9% had profound hearing loss

It has been reported that cochlear implant done in patients with GJB2 mutation related deafness have better speech performance. Identifying the molecular genetics of hearing impairment has benefited the most in deciding on further treatment. Studies have reported that cochlear implant patients with GJB2 related deafness have good speech performance (57). Hence, identifying GJB2 mutations in hearing impaired children will help in providing early intervention and better counselling

LIMITATIONS

Genetic test have high cost and the results take long time. This study needs to be done in larger number of children with non-syndromicsensorineural hearing loss. Specific populations with deafness and consanguinity need to be addressed.

CONCLUSION

This study has shown that Connexin 26 mutationis less common in India than in western population.In our study nearly half of probands with GJB2 mutation were W24X positive and this is the commonest mutation in Indians. Nearly one third of consanguineous marriage with non-syndromicsensorineural hearing loss were GJB2 positive. In this study we also found that all children who were GJB2 positive had profound hearing loss. To conclude, genetic test have many benefits and most impotantly if found positive it helps in early intervention.

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APPENDICES

CLINICAL GENETICS UNIT, CMC, VELLORE
Consensus for DNA banking and consent to be taken for DNA banking

Background

The objective of DNA bank is to store human DNA to preserve for future analysis for the purpose of research and the diagnosis of the genetic diseases.The potential beneficiaries are persons seeking counseling at the later date.

DNA banking is needed for the future requirement of the individuals with a genetic disorder and those requiring DNA analysis for confirmation of the diagnosis at the molecular level, and also in case of presymtomatic diagnosis, carrier state detection, prenatal diagnosis, and resource for research into the molecular pathology of genetic disorder.

DNA banking is indicated for condition where gene is mapped but molecular analysis not readily available and for those where the gene is not mapped.

There is need for formal written consent prior to blood samples for DNA analysis or banking, and hence those willing for are sampled.

Methodology

Transport of blood sample

Blood at ambient temperature for a few days during transport gives adequate DNA yields .Blood sample should not be freezed prior to transporting it, because freezing and thawing will bring down the integrity and yield of DNA by way of its degradation.

Type of samples

Venous blood

Venous blood in EDTA tube is the choice and convenient sample from the pointof view of DNA extraction. It is difficult to extract DNA from clotted sample and also the yield will be low.About 10 ml(split into two 10 ml aliquots one aliquot for near analysis and other aliquot for long term analysis) is adequate for routine use, this provides about 800 micrograms of DNA.Glass container should be avoided as they are unsuitable for freezing.

Till date these are the guidelines and they are subject to change.

Pathological sample(autopsied)

Liver is a rich source of DNA when it is fresh, but it undergoes rapid autolysis, therefore beyond 24 hours spleen is a good choice.

Storage of wholeblood and DNA

Storage of blood

The water shortens the shelf life of DNA. In wet environment DNA undergoes hydrolysis, for long term storage blood should be stored at -80 degree centigrade.

The whole blood can be stored for good DNA yield if samples stored at -20 degree centigrade for up to two years, storage at -70 degree centigrade is preferred as in event of freezer failure it takes longer time to warm up.

Long term storage of blood is not advisable because:

(1) Yield of DNA from whole blood sample is variable,it can be low as zero.

(2)Blood is transported in different sized container which hinders efficient storage

(3)Blood is more vulnerable to freezer failure which may cause degradation of DNA.

(4) Concern that yield may be reduced if blood stored is stored for long time.

Storage of DNA

DNA will be stored in a solution in 2 aliquots -70 degree centigrade,Sample required to be sent for outside testing be kept at 4degree centigrade for a maximum of two weeks.

Samples for long term storage be banked at -70degree centigrade for a maximum of 10 years

Stability of purified DNA can be preserved upto two years when stored at 4 degree centigrade,upto seven years at -18 degree centigrade and longer than seven years when stored at -80 degree centigrade.

Reporting

When the sample is received and no DNA analysis is carried out then a document stating that DNA has been successfully extracted and stored will be given to the patient and another in . patient's record.

Documentation with each specimen-sample should be submitted with a request form.For each family ,the DNA bank should have –Pedigree number, family pedigree, surname,firstname,date of birth,diagnosis,summary of diagnostic criteria.

Our contact address:

Clinical Genetics Unit
OT, Block, 5th Floor
ChristianMedicalCollege& Hospital
Vellore – 632 004
Tamil Nadu.India.
Phone : 0416-3073161
Fax: 0416-2211196
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Reference:

1. Yates John RW et al Journal of Medical genetics 1989 26, 245-250
2. Recommendations of the European Society of Human Genetics – European Journal of Human Genetics (2003) 11, Suppl 2, S8-S10.

Consent for DNA banking

1. I have been explained in my own words that the purpose of DNA BANKING is solely for research purpose.
2. I understand that storing blood for DNA extraction does not guarantee that a test will be done and the result will be available.
3. I hereby consent to store my blood and bloods of individuals of my family for DNA extraction and carrying out research as and when required.
4. I have the right to withdraw from research and I have been explained that DNA banking will not hamper ongoing treatment.
5. I have no objection for discussion and publication of the results in scientific meetings and literatures.
6. I am aware that the DNA will be banked for a maximum of 10 years from the date of collection but there is no guarantee on the DNA quality.
7. A part of this sample not used for diagnostic testing may be stored and used for medical research or education with full confidentiality being maintained. In some cases, it may be possible for the institute to reanalyze the left over DNA samples in the future using new and improved methods.

Proband Name: CMC Hospital No. DNA No.:

Mobile No.:

Land line:

Email id:

Pedigree: (if needed)

Patient's Data

ProbandName	
CMCH No.	
Date of birth	
Date of sample	
ProbandDNA number	Pedigree no. (if applicable)

Family members:

Sl.No	Name	Relation-ship	DNA No.	Date of birth	Signature	CMC No. (if exists)

Name and address of the submitting person	
Diagnostic criteria	1.
	2.
	3.
	4.
	5.
Diagnosis	
Purpose of investigation	1.Diagnostic
	2.Research

Signature of patient/relatives Doctor's name and signature

DNA consent form version 01.11.12
FORM/MGEN/002/P/02/072011
Study on Congenital Nonsyndromicsensorineural Hearing Impairment

Dept. of ENT & Medical Genetics, CMC, VELLORE

Appendix 1

CLINICAL DATA FORM

Proband code / Diagnosis

Proband DNA sample taken yes/no

Family DNA sample taken yes/no

Birthplace / Birth date

Paternal history

Birthplace / birth date

Noise exposure / mutagens exposure yes/no

Deafness / hypoacusis yes/no

Audiometry performed / audiological findings

Other known diseases

Maternal history

Birthplace / birth date

Noise exposure / mutagens exposure yes/no

Deafness / hypoacusis yes/no

Audiometry performed / audiological findings

Other known diseases

Proband gestation

Duration weeks weight gain fetal activity

Infections in pregnancy yes/no

TORCH serology available yes/no

Alcohol yes/no

Drugs in pregnancy

Other problems in pregnancy

Proband birth

Mode birth weight

Jaundice anoxia trauma APGAR score

Other problems at birth

General health

Previous serious illness

Meningoencephalitis

Hospitalization

Exposure to ototoxic agents (a) drugs

(b) infection

(c) noise

Growth

<u>Developmental progress</u>

Normal psychomotor development yes/no

Holding head age Sitting age Standing age walking age

Speech present yes/no lallation age single words age words in sentences age

Behaviour

Age of onset of deafness

Rate of progression of deafness

Suspected causative agents

<u>Pedigree</u>

Proband phenotypic evaluation

TFT

URINE

ECG

PTA

OAE

ABR

MASTER CHART

Sno	Age	Sex	Hearing Loss	Marriage	GJB2 Mutation	W24x gene
1	10 yrs	2	4	2	2	2
2	7 yrs	1	3	2	2	2
3	4 yrs	2	3	2	2	2
4	12 yrs	1	4	1	2	2
5	7 yrs	1	3	2	2	2
6	12 yrs	1	4	1	2	2
7	5 yrs	2	2	2	2	2
8	14 yrs	2	4	2	2	2
9	7 yrs	1	4	2	2	2
10	12 yrs	2	3	2	2	2
11	4 yrs	2	4	2	2	2
12	8 yrs	1	4	2	2	2
13	14 yrs	2	3	2	2	2
14	8 yrs	2	3	2	2	2
15	13 yrs	2	4	1	2	2
16	1 year	1	4	2	2	2
17	6 yrs	2	4	2	2	2
18	4 months	2	3	1	2	2
19	2 months	2	2	1	2	2
20	3 yrs	2	4	1	2	2
21	1 year	2	4	1	2	2
22	3 yrs	2	1	2	2	2
23	4 months	1	3	1	2	2
24	4 months	2	1	2	2	2
25	1 year	2	1	2	2	2
26	3 yrs	1	3	2	2	2
27	3 yrs	1	1	1	2	2
28	8 months	2	3	2	2	2
29	1 year	2	1	1	2	2
30	1 year	2	1	2	2	2
31	6 months	1	1	2	2	2
32	1 year	2	1	1	2	2
33	2 yrs	2	4	1	1	2
34	1 year	1	4	1	1	2
35	2 yrs	2	3	2	2	2
36	6 months	2	1	2	2	2
37	2 months	2	3	2	2	2
38	4 months	1	1	2	2	2
39	6 months	2	2	2	2	2
40	3 yrs	1	4	1	1	2
41	3 months	1	4	2	1	2

42	4 months	2	4	1	1	1
43	9 months	1	1	2	2	2
44	2 yrs	1	3	1	2	2
45	3 yrs	1	4	1	2	2
46	3 yrs	2	3	2	2	2
47	2 yrs	2	4	1	1	2
48	2 yrs	1	1	1	2	2
49	2 yrs	2	2	1	2	2
50	7 months	1	3	1	2	2
51	2 yrs	2	1	2	2	2
52	7 months	2	3	1	2	2
53	6 months	2	1	1	2	2
54	5 months	1	4	2	2	2
55	4 yrs	1	4	1	2	2
56	9 months	2	3	2	2	2
57	2 yrs	2	4	1	1	1
58	3 yrs	1	4	1	1	1
59	3 yrs	2	1	2	2	2
60	8 yrs	1	4	2	1	2
61	1 yr	2	4	2	2	2
62	2 yrs	2	4	2	2	2
63	7 yrs	2	4	2	2	2
64	5, yrs	1	1	2	2	2
65	5, yrs	1	1	2	2	2
66	5, yrs	2	2	2	2	2
67	4 yrs	2	1	2	2	2
68	12 yrs	2	3	2	2	2
69	8 yrs	1	3	2	2	2
70	2 yrs	1	4	1	2	2
71	1 year	1	1	2	2	2
72	4 yrs	2	4	1	2	2
73	2 yrs	2	4	2	2	2
74	2 yrs	2	4	2	2	2
75	1 year	2	3	1	2	2
76	6 yrs	2	4	2	2	2
77	6 yrs	2	4	2	2	2
78	5 yrs	1	4	1	1	1
79	3 yrs	1	3	2	2	2
80	4 yrs	2	1	2	2	2
81	5 yrs	2	4	2	2	2
82	6 yrs	2	4	2	2	2
83	9 yrs	2	4	1	2	2
84	7 yrs	2	3	2	2	2
85	2 yrs	2	1	2	2	2

86	4 yrs	1	4	2	2	2
87	6 yrs	2	4	1	1	1
88	9 yrs	1	3	2	2	2
89	8 yrs	2	4	2	2	2
90	9 yrs	2	4	1	2	2
91	12 yrs	2	4	1	1	1
92	9 yrs	1	4	1	1	2
93	1 year	2	4	1	2	2
94	4 years	1	4	2	2	2
95	7 yrs	1	3	1	2	2
96	1 year	2	4	2	2	2

INSTITUTIONAL REVIEW BOARD (IRB)
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Chairperson, Research Committee & Principal

Dr. Nihal Thomas
MD,MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

May 13, 2013

Dr. Vijay Singh
PG Registrar
Department of ENT
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL:**
Genetic profile among children with congenital nonsyndromic hearing impairment, a study from a tertiary care centre in India.
Dr. Vijay Singh, ENT, Dr. John Mathew, ENT, Dr. Sumita Danda, Medical Genetics, Dr. Achamma Balraj, Dr. Syed Kamran Asif, Dr L. Paul Emerson, ENT.

Ref: IRB Min. No. 8165 dated 09.01.2013

Dear Dr. Vijay Singh,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Genetic profile among children with congenital nonsyndromic hearing impairment, a study from a tertiary care centre in India." on January 09, 2013.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Cvs of Drs. Sandeep Dawre, Shashank Lamba, Ashish Kumar Gupta,
3. Proforma
4. Information Sheet and Informed Consent Form (English, Tamil and Hindi)
5. A CD containing documents 1- 4

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Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

The following Institutional Review Board (Research & Ethics Committee) members were present at the meeting held on January 9, 2013 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliations
Dr. Susanne Abraham	MBBS, MD	Professor, Dermatology, Venerlogy & Leprosy, CMC.	Internal, Clinician
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Surgery (Colorectal), CMC.	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS MCh	Professor, Neurological Sciences, CMC	Internal, Clinician
Dr. P. Prasanna Samuel	B.Sc, M.Sc, PhD	Professor Dept. of Biostatistics, CMC	Internal, Statistician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Dept. of Pulmonary Medicine, CMC.	Internal, Clinician
Dr. Simon Rajaratnam	MBBS, MD, DNB (Endo), MNAMS (Endo), PhD (Endo), FRACP	Professor, Endocrinology, CMC	Internal, Clinician
Dr. Anup Ramachandran	PhD	The Wellcome Trust Research Laboratory Gastrointestinal Sciences	Internal
Dr. Chandrasingh	MS, MCH, DMB	Urology, CMC	Internal, Clinician
Dr. Paul Ravindran	PhD, Dip RP, FCCPM	Professor, Radiotherapy, CMC	Internal
Dr. Anand Zachariah	MBBS, MD, DNB	Professor, Dept. of Medicine, CMC	Internal, Clinician

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Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Mr. Sampath	BSc, BL	Advocate	External, Legal Expert
Mr. Harikrishnan	BL	Lawyer, Vellore	External, Legal Expert
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M.Phil, BL	Legal Advisor, CMC.	Internal, Legal Expert
Mr. Joseph Devaraj	BSc, BD	Chaplain, CMC	Internal, Social Scientist
Dr. B. J. Prashantham (Chairperson), IRB Blue Internal	MA (Counseling), MA (Theology), Dr Min(Clinical	Chairperson(IRB)& Director, Christian Counselling Centre	External, Scientist
Dr. Jayaprakash Muliyil	BSC, MBBS, MD, MPH, DrPH(Epid), DMHC	Retired Professor, Vellore	External, Scientist
Dr. Nihal Thomas	MD MNAMS DNB(Endo) FRACP(Endo) FRCP(Edin)	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Endocrinology & Addl. Vice Principal (Research), CMC.	Internal, Clinician

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

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VELLORE 632 002, INDIA

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Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

A sum of Rs. 38,180/- (Rupees Thirty Eight Thousand One Hundred and Eighty only) will be granted for 1 year.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. John Mathew, Department of ENT, CMC

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788, 2284481 E-mail : research@cmcvellore.ac.in