SEARCH FOR A NEW DFNB LOCUS IN PAKISTANI POPULATION

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By

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(2009)
DECLARATION

This thesis which is being submitted for the degree of Ph.D in the University of the Punjab, does not contain any material which has been submitted for the award of Ph.D degree in any other university and to the best of my knowledge and belief, neither does this thesis contain any material published or written previously by another person, except when due reference is made to the source in the text of the thesis.

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SUMMARY

Deafness is ascribed as partial or complete hearing impairment and is the most prevalent sensory defect in humans the world over. The etiology of childhood deafness is markedly diverse, involving numerous environmental and genetic factors. It can be divided into two groups; syndromic and nonsyndromic, on the basis of any associated phenotype other than deafness. In the past decade tremendous advances have been made in the field of hereditary hearing loss. Considering the complexity of the hearing process, it has been estimated that at least 1% of 30,000 human protein-coding genes are involved in the hearing process (Friedman et al. 2003). To date, 142 loci for nonsyndromic hearing loss have been reserved and 49 genes have been identified. Thus, search for new deafness loci/genes is imperative for a better understanding of genetic and molecular basis of auditory functions. Moreover, the genetic dissection of non-syndromic deafness in humans and mice has identified many genes involved in hearing, and in some cases has provided mechanistic insight as well. Recessively inherited deafness in the Pakistani population (1.6per1000) is higher than the world average (1per1000) due to high consanguinity (Hussain and Bittles. 1998, Jaber et al.1998, Elahi et al.1998). Hence Pakistani population is a valuable genetic resource for studying deafness, moreover large families with multiple affected individuals are easily available.

The major goal of the study on autosomal recessive non-syndromic hearing loss was to localize novel ARNSHL locus. In our efforts to identify a new DFNB locus, the modern “candidate gene approach” as well as the most prevailing technique the “classical approach” was used. For the first part, linkage analysis was performed according to “candidate gene approach”. 11 candidate genes were selected for which corresponding orthologous mice genes were present and were involved in hearing mechanism in mice. STR markers (mostly intronic) were selected for these strong candidate genes. A large number i.e seven hundred consanguineous families segregating autosomal recessive non-syndromic deafness (present in CEMB DNA bank) were screened for these candidate genes. Unfortunately haplotype analysis of these families,
showed no evidence of linkage with all the selected candidate genes. The reason might be that the selected genes are expressed in mice ear but they are not expressed in human ear or due to the probability that these gene mutations might be rare in Pakistani population. Moreover there might be some error during screening or haplotype analysis.

For the second part of the study, linkage analysis was based on the “classical approach”. Fifty families segregating hearing loss were identified from different geographically remote areas of Pakistan. Out of them eleven consanguineous families were enrolled for further molecular studies. All the families had three or more hearing impaired individuals and showed recessive mode of inheritance. Written informed consent, medical and family histories and pure–tone audiograms were collected from a subset of study participants. Genomic DNA was isolated after collecting blood samples from affected individuals, normal siblings, their parents, grandparents if alive, and other related family members. Genotype analysis was pursued on the enrolled families for linkage to all the known recessive deafness loci by typing at least three informative STR markers. As a result, one family PKFD153 was found linked to DFNB7/11, confirming the fact that it is a very common mutation in Pakistani population (Kitajiri et al. 2007), while another family PKDF539 was linked to DFNB32 with causative gene unknown (Masmoudi et al. 2002), narrowing down DFNB32 locus critical region from 16Mb to 13.8Mb and mapping it to chromosome 1p21.1-p22.1. Approximately 79 candidate genes lie within the refined DFNB32 region. The fact that a large number of families were not linked to any of the known loci supports the notion that still a large number of loci/genes remain undiscovered.

Genome wide linkage analysis studies were performed on selected unlinked families which helped in identification of a novel nonsyndromic autosomal recessive deafness locus DFNB75. DFNB75 was mapped to chromosome 5q23.3-q31.1 in a large highly consanguineous family PKDF365, segregating recessively inherited, profound congenital deafness. DNA samples of 200 additional Pakistani families segregating HL were also available from the CEMB DNA repository and were screened for this novel locus (DFNB75) but no additional family was found linked to this locus. Approximately one hundred and forty candidate genes fall in linkage interval of DFNB75 locus (UCSC
Identification of a new deafness locus (DFNB75) and refinement of DFNB32 region are two major advances in isolation of gene mutations leading to deafness, which will in turn facilitate us to better understand the development and functioning of the auditory system.

The benefit of this study on one hand is it to provide genetic evidence for the identification of new DFNB locus /gene in Pakistani population, showing the wide genetic heterogeneity that characterizes hearing impairment and the genetic diversity in Middle-Eastern populations on the other hand it provides knowledge and awareness through screening of carrier status and genetic counseling to reduce the socio-economic burden on the affected families and decrease the incidence of deafness in Pakistan.

Mainly this study offers the basic research which will aid in understanding the function of genes controlling the mechanism of hearing, which will further facilitate the development of intervention strategies to prevent and treat hearing loss.
ACKNOWLEDGMENTS

All the praises be for Almighty Allah, the lord and the creator of the universe, compassionate for man-kin, bestowed upon us that we not knew. the bountiful blessings and exaltation of Almighty Allah flourished my thoughts and trived my ambitions in the from of this writer up. All respect to Holy Prophet Hazarat Muhammad , be peace upon him, who enlightened our conscience with the essence of faith in Allah, covering all the kindness upon him.

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May Allah bless us all, and bestow upon us the secret of real knowledge to serve humanity sincerely.

UZMA SHAUKAT

_________________________ DEDICATED TO MY FATHER
# ABREVIATIONS

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<th>Abbreviation</th>
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<tr>
<td>AA</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>ABR</td>
<td>Auditory brain Stem response</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cM</td>
<td>Centimorgan</td>
</tr>
<tr>
<td>dB</td>
<td>decibels</td>
</tr>
<tr>
<td>DFNA</td>
<td>Deafness, Autosomal Dominant</td>
</tr>
<tr>
<td>DFNB</td>
<td>Deafness, Autosomal Recessive</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide phosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminotetraacetic acid, disodium salt</td>
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<td>Hz</td>
<td>Hertz</td>
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<td>Kb</td>
<td>Kilobases</td>
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<td>M</td>
<td>Molar</td>
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<td>MgCl₂</td>
<td>Magnesium Chloride</td>
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<td>Minutes</td>
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<td>mm</td>
<td>Millimeter</td>
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<td>µl</td>
<td>Microlitre</td>
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<td>µM</td>
<td>Micromolar</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>pmole</td>
<td>Pico moles</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl Sulphate</td>
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<tr>
<td>STR</td>
<td>Short Tandem Repeat</td>
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<tr>
<td>TAMRA</td>
<td>Carboxy tetramethyl rhodamine</td>
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<td>USH</td>
<td>Usher</td>
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<td>Male</td>
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<td>Nonconsanguineous marriage</td>
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<td>Deceased</td>
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INTRODUCTION
Hearing loss is the most common sensory deficit in humans, including perturbations due to both environmental (e.g., due to viral or bacterial infection, acoustic trauma, ototoxicity) and genetic causes, with varying times of onset ranging from congenital deafness to presbycusis (hearing loss related to aging). Moreover, one in 1000 children become severe to profound deaf before adulthood (Morton.1991) and 50% of these prelingual deafness cases in developed countries are attributable to genetic factors (Marazita et al. 1993, Rehm.2003). By age 65, one third of the human population has significant hearing impairment with a higher incident in males as compared to female. A severe defect of congenital nature has dramatic effects on speech acquisition and literacy, as a result intensive specialized education is needed for affected children to optimize their potential. Later onset of severe hearing defect seriously compromises the quality of life, as the affected individual becomes increasingly isolated socially.

Clinical categories of hearing loss are based on conductive (referring to external and/or middle ear defects) versus sensorineural (defects from the inner ear to the cortical auditory centers of the brain) causes as well as other factors such as audiologic profile, environmental influences, severity (mild, moderate, severe, profound), age of onset of inheritance and accompanying symptoms in other organ systems. The environmental factors include birth injury, postnatal trauma, hypoxia, hypoglycaemia of the fetus, maternal diabetes, neonatal jaundice, erythroblastosis fetalis, congenital viral infections such as rubella and cytomegalovirus, infectious diseases like meningitis, advancing age, iodine deficiency, and ototoxic drug (Chen.1988). Approximately one in every 500 newborns receives a diagnosis of congenital hearing loss (Mehl and Thomson.1998, 2002). The phenotypic spectrum of prelingual deafness is broad ranging from simple deafness without other clinically recognizable abnormalities (nonsyndromic-70%) to genetically determined syndrome (30%) of a more pleiotropic nature. Over 400 syndromes associated with hearing loss have been identified with common anomalies of the eye, kidney, muscle, nervous system, and skin etc (Gorlin et al. 1995, Bergstrom et al. 1971). Genetic heterogeneity is seen within some clinically types of hearing loss, indicating that accurate diagnosis and classification of type of deafness can only be possible with elucidation of specific gene involvement and fundamental understanding of molecular mechanisms. Roughly 80% of genetically caused prelingual nonsyndromic hearing loss (NSHL) has an autosomal recessive mode of inheritance, while autosomal dominant cases account for approximately 15%, and
Search for a new DFNB locus in Pakistan Population


Although hearing loss is common worldwide, yet there are problems inherent in the study of communities with single gene hearing disorders, where societies living style and open culture, may confound traditional genetic approaches due to genetic heterogeneity and environmental factors contribution to the phenotype (Petit. 2001, Reardon.1992). Furthermore, inner ear contains many different cell types that are necessary for sound detection (Frolenkov et al. 2004, Forge and Wright. 2002). Because of the small quantity of cells present in the cochlea, classical biochemical and physiological approaches to characterize hearing processes in humans are often not feasible. A genetic approach to identify the molecular players in auditory processes is an alternative strategy and large consanguineous families with inherited hearing impairment have been a key to the mapping and identification of the majority of the mutated genes associated with deafness (Friedman and Griffith 2003). In consanguineous families homozygosity mapping is based on the assumption that a rare mutation is inherited from a common ancestor through both parents, so that affected siblings are homozygous by descent, for polymorphic markers close to the disease locus. Moreover congenital deafness is relatively common in geographical remote areas with high consanguinity, which simplifies genetic linkage analysis using homozygosity. Pakistan has a unique socio-cultural set up and consanguineous marriages are common. Approximately 60% of marriages are consanguineous, of which more than 80% are between first cousins (Hussain and Bittles.1998). According to one estimate, the prevalence of profound bilateral hearing loss is 1.6 per 1000 individuals in Pakistan and 70% of the hearing loss arises in consanguineous families (Elahi et al. 1998, Jaber et al. 1998). To date twenty four non-syndromic deafness loci DFNB16, DFNB20, DFNB 26, DFNB29, DFNB35, DFNB36, DFNB37, DFNB38, DFNB39, DFNB42, DFNB44, DFNB46, DFNB47, DFNB48, DFNB49, DFNB51, DFNB55, DFNB56, DFNB62, DFNB63, DFNB65, DFNB67, and DFNB68, DFNB72 have been mapped in Pakistani population. Thus, the population in Pakistan provides an excellent resource to identify the genes involved in hearing impairment.

Considering the intricacy of the hearing phenomena, it has been estimated that ~300 genes are involved in the hearing process (Friedman and Griffith .2003). To date 142 non-syndromic hearing loci (NSHL) have been genetically mapped, 57 of these loci are inherited
in an autosomal dominant mode in which the onset of hearing loss is most frequently progressive and post-lingual in nature. The remaining 77 non-syndromic loci mapped so far are inherited as autosomal recessive of which only 49 genes have been identified (Hereditary hearing loss homepage, http://dnalab-www.uia.ac.be/dnalab/hhh). Therefore, identification of new deafness loci/genes is fundamental for a better understanding of genetic and molecular basis of auditory functions.

The most prevailing technique to identify new deafness loci is the “classical approach” which encompasses analysis of polymorphic markers to exclude linkage to known DFNB loci and after exclusion genome-wide screening and fine mapping is done. However another interesting phenomenon has been observed, that structural and functional homologies extend across distantly related species. Human and mouse genome are reported to be 80% identical. The differences are a few hundred of ~35,000 genes in both organisms. Therefore the “modern candidate gene approach” was introduced. According to it once a gene or its localization is known in mice or other mammals it is possible to predict the likely location of its orthologous gene in humans. Hearing mechanism in mice is genetically similar to humans therefore mouse models represent powerful tools for advancing the understanding of hearing loss and to identify novel genes or novel functions of known genes that underlie deafness and to develop innovative treatment strategies for deafness in humans using mouse genetic models. Human non-syndromic deafness genes like OTOA (DFNB22), TMIE (DFNB6), TMC1 (DFN B7/11), MYO6 (DFNA22), POU3F4 (DFN3), POU4F3 (DFNA15) and EYA4 (DFNA10) were identified using corresponding deaf mice models. Mouse deafness models have also given an insight in understanding and confirming human deafness genes and their functions, likely in case of genes DIAPH1, COL11A2, TECTA, GJB2, GJB3, SLC26A4, MYO7A, MYO15A, OTOF, CLDN14, and TFCP2L3 (Freidman and Griffith. 2006, Avraham.2003).

The study ascertained here was designed to identify new locus causing deafness in Pakistani population. In first half of the study, in order to isolate a novel locus, linkage analysis was performed through modern “candidate gene approach”.  11 candidate genes were selected for which corresponding orthologous mice genes were present and were involved in hearing mechanism in mice. STR markers (mostly intronic) were selected for these strong
Search for a new DFNB locus in Pakistan Population

candidate genes, and were screened on 700 deaf families present in CEMB DNA bank. Unfortunately no family was found linked to these candidate gene markers.

To identify a novel locus or gene, in second half of the study linkage analysis was done via “classical approach”. A total of 50 families were identified through the deaf children schools present in Punjab, Sindh, and Balochistan provinces while 11 families were enrolled. Blood samples were collected and processed for DNA extraction after written informed consents. All the enrolled families were studied for linkage to known loci, one family was found linked to DFNB7/11/TMC1, while another family PKDF539 was linked to DFNB32 (it’s causative gene is unknown), narrowing down DFNB32 locus critical region from 16Mb to 13.8Mb. PKDF539 refined DFNB32 locus region to chromosome 1p21.1-p22.1.~79 candidate genes lie within the refined DFNB32 region. Some of the unlinked families were separated for a genome wide linkage analysis studies. Consequently, a novel locus DFNB75 was mapped on one of the families. Novel locus DFNB75 was mapped to chromosome 5q23.3-q31.1 in a large highly consanguineous family PKDF365, segregating recessively inherited, profound congenital deafness. DNA samples of 200 additional Pakistani families segregating HL were also available from the CEMB DNA repository and were screened for this novel locus (DFNB75) but no additional family was found linked to this locus. Approximately one hundred and forty candidate genes fall in this region.

The benefit of this study on one side is to provide knowledge and awareness through screening of carrier status and genetic counseling to reduce the socio-economic burden on the affected families and decrease the incidence of deafness in Pakistan. On the other hand identification of a new deafness loci (DFNB75) and refinement of DFNB32 are two major advances in isolation of gene mutations leading to deafness, that were not identified so far in Pakistani population, which in turn will help us to better understand the development and functioning of the auditory system.
CHAPTER-1
REVIEW OF LITERATURE
SECTION-I

SOUND TRANSDUCTION MECHANISM

ANATOMY OF AUDITORY SYSTEM
The human auditory system is one of the most intricate, miraculous, and an ingenious creation designed to transfer sound waves from environment to brain in a most efficient and precise manner. The ear can be described as both an analytic microphone and a microcomputer, sending sound impulses to the brain. Ear is capable of turning the tiniest disturbances to a form that brain can understand and doing so instantaneously, over an enormous range of pitch and loudness. Being extremely complicated organ, it performs dual function of balancing and perceiving sound.

The auditory system is highly complex and composed of three anatomical compartments, the external, middle and inner ear, which function as an entity. The boundary between the external ear and the middle ear is the tympanic membrane. The middle ear contains the auditory ossicles (malleus, incus, and stapes). The boundary between the middle ear and the inner ear is the oval window (Fig 1:1). The inner ear has sensory receptors, which utilize the hair cell for sensory transduction.

THE OUTER EAR

The external ear consists of three parts: the outer ear (auricle or pinna), the external auditory canal (auditory meatus) and the eardrum (tympanic membrane).

THE PINNA

Pinna directs sound to auditory canal and is composed of cartilaginous framework of elastic connective tissues which are attached to skull by ligaments and muscles (Fig 1:1).

THE AUDITORY CANAL (AUDITORY MEATUS)

Auditory meatus is a short canal (~ 1"), extending from the pinna to tympanic membrane, carrying ceruminous glands in it. Cerumen (ear wax) excreted from ceruminous glands keeps the tympanum soft, waterproof and prevents entry of foreign objects, collectively with the hairs (Fig 1:1).

TYMPANIC MEMBRANE (EAR DRUM)

Eardrum is a thin, double-layered, epithelial partition (~1 cm in diameter) between the auditory canal and the middle ear. Tympanic membrane seals the delicate organs of the inner parts of the auditory system to protect it from bacterial infections and foreign matter which could clog the system (Fig 1:1). Furthermore, it is designed for efficient transmission of sound.

THE MIDDLE EAR (TYMPANIC CAVITY)
The middle ear is a narrow air-filled cavity located in the temporal bones of the skull and connects the outer ear to the inner ear. It is separated from the auditory meatus by the tympanic membrane while is separated from the inner ear by a bony partition, which contains two windows i.e. the oval window and the round window (Fig.1:1)

**AUDITORY OSSICLES**

Chain of three tiny, linked movable bones, the auditory ossicles i.e. the malleus (hammer), the anvil (incus) and the stapes (stirrup) are connected at one end by ligaments to the tympanic membrane, and then ends with the oval window of the cochlea (Fig 1:1). The intricate function of the auditory ossicles is to transmit and amplify sound waves across the tympanic cavity, from the tympanic membrane into the mechanical movements of oval window. Geometrical organization of ossicles and surface area difference between tympanic and oval window give 20 fold amplification of sound waves. Any limitation of motion (impedance) will not transmit the original sound resulting in hearing loss.

**EUSTACHIAN TUBE**

Eustachian tube is a small tube connecting middle ear to nasopharynx of the throat and it equalizes air pressure on both sides of the tympanic membrane. It allows fresh air to be filled in the middle ear space periodically. Otitis media, an infection of middle ear which occur if the eustachian tube is blocked due to any reason (Fig 1:1).

**OVAL AND ROUND WINDOWS**

These windows separate air filled tympanic cavity from fluid filled membranous labyrinth. Oval window (Fenestra vestibuli) displacement occurs through movement of tympanic membrane via ossicles, and causing fluid displacement in inner ear. Round window (Fenestra cochlea) displacement is opposite to that of oval window because of incompressible nature of inner ear fluid (Fig 1:2).
The human ear consists of three sections: the outer ear, the middle ear, and the inner ear. The outer ear includes the auricle (pinna), the visible part of the ear that is attached to the side of the head, and the waxy, dirt-trapping auditory canal. The tympanic membrane (eardrum) separates the external ear from the middle ear, an air-filled cavity. Bridging this cavity are three small bones—the malleus (hammer), the incus (anvil), and the stapes (stirrup). The cochlea and semicircular canals make up the inner ear.

Fig 1.2 Structure of Inner Ear
Search for a new DFNB locus in Pakistan Population

THE INNER EAR

Inner ear regulates two sensory systems simultaneously i.e. the vestibular system for spatial orientation and equilibrium and the cochlear/auditory system for hearing. The inner labyrinth is exceptionally an intricate series of structures, and consists of two parts; the osseous (or bony) labyrinth located within the temporal bone, and the membranous labyrinth within the bony labyrinth with interconnected sacs and tubes.

The osseous labyrinth is lined with the periosteum and is filled with perilymph, a fluid secreted by the cells lining the bony canals (Fig 1:2). Perilymph resembles cerebral spinal fluid (CSF) and normal extracellular fluids in chemical composition i.e. low $K^+$ and high $Na^+$ concentration. Since its osmolarity is similar to plasma; hence in osmotic equilibrium with the blood.

The tubular chambers of the membranous labyrinth (Fig1:2) are filled with a second fluid, known as the endolymph, having an unusual composition than perilymph i.e. high $K^+$ concentration (~140 mM) and a very low $Na^+$. In the cochlea, but not the vestibular system, endolymph has a high positive electrical potential (~+80 mV) depending on an active secretion of $K^+$, which involves fibroblasts, different support cells and the stria vascularis (Graham et al 2000). These fluids provide the media for vibrations involved in hearing and the maintenance of equilibrium and are essential for the functioning of the sensory cells of the inner ear (Hudspeth et al 1989). An important feature of the endolymphatic space is that it is completely bounded by tissues and there are no ducts or open connections between perilymph and endolymph. The border between the two fluids lies at the level of the junctions between the epithelial cells surrounding the endolymphatic spaces. Maintenance of this permeability barrier is essential for function of the inner ear

OSSEOUS LABYRINTH

The osseous labyrinth consists of three structural and functional divisions, vestibule, semicircular canals, and cochlea.

VESTIBULE

The vestibule is the central part of the bony labyrinth. The lateral wall of vestibule contains the oval window shown in (Fig 1:2) as the bean shaped white blotch between the utricle and saccule.
SEMICIRCULAR CANALS
The three bony semicircular canals (superior, posterior, and lateral) are oriented at right angles to each other and are positioned posteriorly (dorsally) to the vestibule. At one end of each is a dilatation called ampulla which connects to the vestibule (Fig 1:2).

COCHLEA
The cochlea is a sense organ for hearing. Its purpose is to take the vibrations from middle ear and transform them to nerve impulses, detected by the brain (Fig 1:2).

MEMBRANOUS LABYRINTH:
A second series of tubes made out of delicate cellular structures called the membranous labyrinth lies within the bony labyrinth. Structures of the membranous labyrinth include: Utricle and saccule (within the vestibule), three semicircular ducts and their ampulla (within semicircular canals), and Cochlear duct (within the cochlea).

Three types of epithelium surround the membranous labyrinth endolymphatic compartment, sensory epithelia, ion transporting epithelia and relatively unspecialized epithelia.

NEUROSENSORY EPITHELIA (MECHANOTRANSDUCERS)
The neurosensory epithelial sheets responsible for sense of position and sound are located in specific areas within the respective structures called as:
For Vestibular system
  Maculae of the utricle and saccule
  Three Crista ampullaris in the ampulla of each of the three semicircular ducts
For Cochlear system
  Organ of Corti within the cochlear duct

Sensory epithelia are composed of sensory hair cells and accessory supporting cells. Hair cells are surrounded by supporting cells so that no two hair cells contact each other. They are called hair cells due to characteristic cuticular plate and tuft of stereocilia bathing in the surrounding endolymph. The cell body itself is localized in the perilymph compartment. There are 50-100 stereocilia/cell in vestibular system and inner hair cells of cochlear system and 100-300 stereocilia/cell in outer hair cells of the cochlear system. The sensory epithelium is covered by an acellular extracellular matrix structure: the tectorial membrane in the cochlea, the otolithic membranes of macular organs, and the cupulae of the cristae. Hair bundles deflection either caused by sound waves or changes in head position modulates the opening/closing ion channels, depending on direction of movement of stereocilia. Opening
of ion channel result in flow of K+ ions from endolymph through the hair cells, altering the hair cell’s resting electrical potentials and exciting their cell activity. Hair cells are thus mechanotransducers converting a mechanical stimulus (movement) into an electrical signal.

**ION TRANSPORTING EPITHELIA**

The ion transporting epithelia, the stria vascularis of the cochlea and the dark cell regions of the vestibular system, are involved in active (energy consuming) ion transport necessary to maintain the unusual endolymph composition (Fig 1:3A).

**LESS SPECIALIZED EPITHELIA**

The less specialized epithelia, Reissner’s membrane in the cochlea and the epithelium of the roof of the saccule, utricle, ampullae of the semicircular canals form permeability barriers separating the fluid spaces. It is expected that Rupturing of these membranes would result in fluid mixing and physiological dysfunction (Fig 1:3A).

**Vestibular system**

There are five sensory receptor regions associated with the vestibular system, two in the macula of utricle and saccule which contain receptors sensitive to gravity and linear movements of the head and one in each of the three semicircular ducts, cristae ampullaris of semicircular ducts which are sensitive to angular acceleration and deceleration of the head as in rotational movement.

**Cochlear system**

Cochlea is the core element of the inner ear responsible for hearing. Its name come form its spiral structure mimicking a marine snail. The bony spiral makes roughly 2.5 revolutions around a central pillar of bone called the modiolus and is about 35 mm long (range 28–40mm) in humans (Wright *et al.* 1987). Uncoiled, the cochlea is divided along its length into three fluid-filled compartments

- upper, scala vestibule, filled with perilymph
- middle triangular, scala media (cochlear duct), filled with endolymph
- lower, scala tympani, filled with perilymph

The cochlear duct is triangular in shape. Reissner's membrane (vestibular membrane) divides the scala vestibuli from the scala media (cochlear duct) and the basilar membrane divides the scala media from the scala tympani (Fig 1:3A). The oval window is at the base of the cochlea in scala vestibuli while round window is at the base of the cochlea in scala tympani. Perilymph bath both the scala tympani and vestibuli which are continuous till the apex (or tip
of the spiral) through the helicotrema while the cochlear duct is filled with endolymph and terminates at the helicotrema. The cochlear duct contains the sensory organ of hearing the Organ of Corti. Movement of perilymph via oval window displacement causes movement of endolymph in cochlear duct which is sensed by the organ of Corti.

**ORGAN OF CORTI**

The extraordinary ability of the mammalian cochlea to detect and distinguish sounds over a wide range of frequencies depends on the precise organization of its highly specialized neurosensory epithelium, known as the organ of Corti. It is seated on the basilar membrane, covered by proteinaceous tectorial membrane in the middle compartment, scala media of the cochlea. (Fig 1:3). It is composed of the sensory cells, called hair cells, the neurons, and several types of support cells.

On the morphological and physiological basis, there are two kinds of hair cells in Organ of Corti; the inner hair cells (IHCs) and the outer hair cells (OHCs). Schematically, both types of cells, IHCs and OHCs, differ by their shape and the pattern of their stereocilia. In the human cochlea, there are about 12,000 OHCs and 3,500 IHCs. This number is extremely low as compared to millions of photo-receptors in retina and chemo-receptors in the nose. Moreover, hair cells share with neurons an inability to proliferate, they can only be differentiated which means that the final number of hair cells is reached very early in development (around 10 weeks of fetal gestation); from this stage on our cochlea can only lose hair cells.

**OUTER HAIR CELLS: (OHCs)**

12,000 OHCs are regularly arranged in most mammals within three or sometimes four rows. They are shaped cylindrically, like a cane, and have 100 stereocilia at the top of the cell, and a nucleus at the bottom (Fig 1:3B). Their hair bundles form a characteristic ‘W’-shape and contact the underside of the overlying tectorial membrane in which impressions of the longest stereocilia from the OHC can be seen. Although they are much greater in number than the IHCs, they receive only about 5% of the innervations of the afferent nerve fibers from the acoustic portion of the VIII nerve and 80% of the efferent nerve innervations (Andrew *et al.* 2002)
INNER HAIR CELLS: (IHCs)

There is only one row of approximately 3,500 IHCs, having ~ 40 stereocilia (Fig 1:3B). IHC are flask shaped and their hair bundles are in an approximately straight line or wide ‘U’-shape. The hair bundles of the IHC do not appear to contact the overlying tectorial membrane. These cells receive about 95% of the afferent innervations from the nerve fibers from the acoustic portion of the VIII nerve and 20% of the efferent nerve innervations. These cells have primary responsibility for producing our sensation of hearing. When lost or damaged, a severe to profound hearing loss usually occurs.

ARRANGEMENT OF HAIR CELL STEREOCILIA AND LINKS

Hair cells are highly specialized mechanoreceptors having hair-like projections on their apical surfaces that help to translate the mechanical stimuli (sound vibration) into electrical signals, interpreted by the brain. These projections, known as stereocilia, have mechanosensitive ion channels (Corey and Hudspeth.1979, Ohmori.1985) and constitute the hair bundle which is formed of rows of stereocilia that increase in height in one particular direction across the bundle. Stereocilia are generally arranged in three rows of graded lengths and a single kinocilium located behind the row of longest stereocilia. In the hair cells of the organ of Corti, the kinocilium is present only during development, but as the cochlea matures it is reduced to remain as a basal body on one side of the stereociliary bundle. The tallest stereocilia of outer hair cells directly contact the tectorial membrane. The tip of each stereocilium is linked to the shaft of its neighbor by thin tip links which are involved in the mechano-transduction process, stereocilia are also attached by transverse (lateral) links, both in the same row and from row to row. There are thought to be at least three different types of lateral links between stereocilia. Ankle links which are absent from the hair cells of the organ of Corti, but present in the hair bundles of mammalian vestibular organs connect stereocilia at their proximal ends. Shaft connectors are present along the mid-region of the stereociliary shaft. Top-connectors link stereocilia laterally just below the level of the tip-links.
Search for a new DFNB locus in Pakistan Population

Fig 1: A. Membranous Labyrinth: Scala vestibuli with perilymph, Scala tympani with perilymph and Cochlear duct with endolymph and organ of corti B. Organ of Corti: Inner hair cells, Outer hair cells, Tectorial membrane, Basilar membrane, Stereocilia and Supporting cells.
PHYSIOLOGY OF EAR

Hearing involves a complex chain reaction within the ear. Sound creates vibrations in the air somewhat similar to the rippling waves created when a stone is thrown into a pond. Mainly it consists of three parts: Conductive, Sensory, and Neural.

CONDUCTIVE HEARING

Sound waves result from the alternate compression and decompression of air reaches the ear, are directed by the pinna into the external auditory canal. When the waves strike the tympanic membrane it vibrates. The central area of the tympanic membrane is connected to the malleus, which in turn starts vibrating. These vibrations are picked up by the incus, and transmitted to stapes. As the stapes moves back and forth, it pushes the oval window in and out (Fig 1:4).

SENSORY HEARING

The movement of the oval window sets up waves in the perilymph of the scala vestibuli. As the oval window bulges inward, it pushes the perilymph of the scala vestibuli to produce pressure waves. As the pressure moves through the perilymph of the scala vestibuli, it pushes the vestibular membrane inward and increases the pressure of the endolymph inside the cochlear duct. As a result, the basilar membrane moves slightly and bulges into the scala tympani. The pressure in the perilymph of the scala vestibuli is then transmitted through the basilar membrane and eventually to the round window. Following the compression that resulted in the above actions is a decompression that causes the stapes to move toward the tympanic membrane and the above actions are reversed. That is, the fluid moves in the opposite direction along the same pathway, and the basilar membrane bulges into the cochlear duct. A young human ear can hear sounds in the frequency range of 20 to 24,000 Hz, yet can distinguish between sounds that have only a 0.3% difference in frequency. The human ear can detect differences in sound intensity of only 0.1 to 0.5 db. When the basilar membrane vibrates, the hair cells of the Organ of Corti move against the tectorial membrane. This shearing action causes the stereocilia to be deflected and tip links stretch and open the mechanotransduction cationic channels located near the stereocilia tip, which let K+ ions flow into the hair cells from the endolymph (Fig 1:5). K+ ions rush in because the strongly negative potential of the hair cells attracts positive ions. This tends to neutralize some of the negative charge, and brings the potential up towards zero, a process known as depolarization.
NEURAL HEARING

As the depolarization takes place voltage sensitive calcium channels are activated in IHCs and calcium triggers the release of neurotransmitters, which lead to the generation of nerve impulses. The impulses are passed on to the cochlear branch of the vestibulocochlear (VIII) nerve and then to the medulla. Within the medulla, most impulses cross to the opposite side and then travel to the midbrain, to the thalamus, and finally to the auditory area of the temporal lobe of the cerebral cortex. In the “resting” position of stereocilia the transduction channels are partially open, leading to a small release of transmitter. This, in turn, generates a spontaneous activity in the auditory nerve and the ascending auditory pathways, even in the absence of sound. The cells are thought to recover from the stimulus by pumping out the potassium through gap junctions (Connexin channels) and voltage gated potassium channels (Petit.2001).

As clear from the above complexity of hearing process; a large ensemble of proteins act in concert to orchestrate the function of the sensory cells in the cochlea, through which we hear, and the vestibular apparatus of the inner ear, the organ that senses gravity and acceleration. Defects in any one of these proteins results in disturbance of the auditory pathway which in turn can cause deafness. High proportions of hearing loss cases are due to outer hair cell abnormalities (Avarham. 1998, Kossal. 1997).

THE GATING-SPRING MODEL FOR MECHANOELECTRICAL TRANSDUCTION

The prevailing theory for the gating of mechanoelectrical transduction channel in hair cells is embodied in the gating spring model. The molecular gate of a channel is attached to an elastic element, the gating spring, tension in which favours channel opening (Fig 1:6). When the bundle is undisturbed, “channel opening probability” is only 15% or so. However, when the bundle is pushed all the way in the positive direction towards its tallest edge, the probability rises near unity. Motion of the bundle in the negative direction lowers the open probability to zero. It is suggested that the tip links is the gating spring and it pulls directly on the ion channel (Corey and Hudspeth. 1983, Howard and Hudspeth. 1988).
Search for a new DFNB locus in Pakistan Population

Fig 1:4 Events involved in Hearing mechanism

Fig 1:5 A. Showing shearing action of the stereocilia causes rush of K+ ions and depolarization. B. Showing Ca+ ions enter the cell through voltage-gated channels, Ca+ ions activate the K+ channels, as a result K+ exits the cell and repolarizes the cell to original state.
Fig 1.6 Representative model for sound mechanoelectrical transduction in Hair Cell. 

a. Deflection of hair cells bundle of stereocilia, in to sound or movement of head, causes the stereocilia to bend and tip links between them to tighten. 

b. Gating spring model of mechano-sensory transduction. Tip link are elastic and are attached to ion channels at each end, which open in response to the tension on the tip links. Myo1c is a motor protein that sets a resting tension on the tip link and channels; it is in turn anchored with actin filaments within the cell.
DEAFNESS

Deafness is defined as partial or complete hearing impairment (HI) which leads to an impaired ability to develop speech, language and effective communication skills. Many genetic and environmental causes have been recognized for HI. Hearing loss present at birth is known as congenital deafness, while one that occurs after birth is called adventitious deafness. Hearing loss can appear at any age and with any degree of severity. Deafness is classified by the degree of severity of the hearing loss (mild, moderate, severe, profound) for the better-hearing ear and by the site of the defect. Conductive hearing loss refers to external and/or middle ear defects, and sensorineural hearing loss to the other defects, i.e., anywhere from the inner ear to the cortical auditory centers of the brain. Most cases of sensorineural hearing loss are due to inner ear defects (Petit et al. 2001). Detailed classification of deafness is given below.

CLASSIFICATION OF HEARING LOSS

Deafness is classified into two main categories, depending upon time of onset, and on the basis of portion of ear damaged. The two main classification is as follows;

1. (a) Congenital. (b) Acquired.

2. (a) Conductive. (b) Perceptive (sensorineural). (c) Mixed-conductive & perceptive.

1. (a) CONGENITAL DEAFNESS

It is the type of deafness in which the patient is deaf at the time of birth. There are three types of causes for it:

1. Hereditary i.e. deaf offspring of deaf parents. The cause is generally agenesis of essential organ of hearing in the inner ear or due to congenital arteria of the external when hearing loss is conductive and can be corrected through surgical treatment

2. The fetus may suffer from accidents and diseases during gestation and thus results in deafness at birth (causes include maternal rubella, incompatibility of the blood of mother & fetus due to rhesus factor, congenital syphilis etc)

3. The newborn, at the time of birth may have undergone difficult or prolonged labour or sustained injuries during forceps or surgical delivery.
Search for a new DFNB locus in Pakistan Population

(b) ACQUIRED DEAFNESS

It is acquired during the lifetime of an individual due to wax, fungus, boil foreign bodies, atresia etc in the external ear, or due to problems in the middle ear like rupture of the tympanic membrane, acute otitis media, chronic otitis media, otosclerosis etc. On the other hand acquired perceptive deafness occurs due to senile Menieres disease, trauma from noises that exceed threshold of pain (Fig1:7), toxins, infectious fevers in infancy and childhood (like meningitis, typhod fever, measles, mumps, whooping cough, pneumonia), exogenous toxins ( tobacco and alcohol) or endogenous toxins (found in diabetes and septic foci, malnutrition, vitamin deficiency, and nerve tumour etc).

2. (a) CONDUCTIVE DEAFNESS

In conductive deafness hearing loss is caused by diseases or obstruction in the outer or middle ear and usually is not severe. Since it’s commonest cause is in middle ear (due to suppurative otitis media, otosclerosis or adhesive process) thus it is often referred as middle ear disease. A person with a conductive hearing loss generally can be helped by a hearing aid. Often conductive hearing losses can also be corrected through surgical or medical treatment (Table 1:1).

(b) PERCEPTIVE DEAFNESS

Perceptive deafness results from damage to the sensory hair cells or the nerves of the inner ear and can range in severity from mild to profound deafness. Such loss occurs in certain sound frequencies more than in others, resulting in distorted sound perceptions even when the sound level is amplified. A hearing aid may not help a person with a sensorineural loss. There are two types of perceptive deafness and often both of them are called as Sensorineural hearing loss. These types are; 1. Cochlear deafness, it is due to the defect in the sensory end-organ i.e. cochlea, usually the organ of Corti. 2. Retrocochlear or neural deafness, it is due the defect in the nervous elements connected with hearing, from the nerve terminals in the Corti to the cortical centre of hearing.

(c) MIXED HEARING LOSS

It is caused by problems in both the outer or middle ear and the inner ear.
### Search for a new DFNB locus in Pakistan Population

**Clinical feature** | **Conductive Deafness** | **Perceptive Deafness**
--- | --- | ---
**Concept** | Lesion between the external auditory meatus and the oval window. | Lesion between the cochlea and cortical hearing center.
**Usual history** | Below 10 some prolonged fever | Between 10-40 Drugs e.g quinine.
**Behaviour** | Patient speak in a low tone but requests others to speak loudly. | Patient speaks loudly but requests others to speak in a low tone.
**Tuning fork test** | Rinne negative, Weber to affected side, ABC prolonged. | Weber to healthy side, ABC shortened.
**Audiogram** | Low tones affected first and most. Air bone gap appreciable. | High tones affected first and most. Air bone gap poor.
**Hearing aid** | Accepted well by patient. | Not accepted well by patient due to recruitment phenomenon.

Table1;1 Differential diagnosis between conductive and perceptive hearing loss.

![Fig 1:7](image-url) Sound Intensities measured in decibels (dB). Threshold of pain leading to trauma at 120db.
SECTION-II

DEAFNESS AS A GENETIC DISORDER
Search for a new DFNB locus in Pakistan Population

**HISTORY.**

Documentation of deafness as an inherited disorder can be traced back to the sixteenth century. Goldstein (1933) reported that Johannes Schenck (1531–1598) was the earliest known author to have recognized that some forms of deafness may be hereditary. Johannes noted a family in which several children were born deaf. Stephens (1985) includes a pedigree drawing of a sixteenth century family of the Spanish aristocracy in which deaf members were spanning over three generations. In 1621 Paolus Zacchias (1584–1659) recommended that the deaf refrain from marriage because of evidence that their children will also be deaf (Cranefield and Federn.1970), indicating his conviction that heredity is important in deafness. Reardon (1990) ascribed to Sir William Wilde (1815–1876) assessment that deafness shows different patterns of inheritance, that consanguinity is a relevant factor, and that there is an excess of males among the congenitally deaf. Hartmann (1881) confirmed these findings by his extensive studies in schools for the deaf in Germany. More recently, Konigsmark (1969), Konigsmark and Gorlin (1976), and Fraser (1976) provided comprehensive reviews of hereditary hearing impairment, and emphasized the pronounced heterogeneity.

The phenotypic and genetic heterogeneity was underscored by Gorlin et al. (1995) who listed 427 forms of syndromic and nonsyndromic hereditary hearing impairment. The earliest report of syndromal hearing loss is probably that of mandibulofacial dysostosis by Thomas in 1846. Von-Graefe in 1858 noted retinitis pigmentosa with hearing loss, which was later referred as “Usher syndrome” after Scottish ophthalmologist Charles Usher (1914). Combined euthyroid goiter and congenital hearing loss was described by Pendred in 1896 and its recessive pattern by Brain in 1927. Several studies were carried out to estimate the number of loci for deafness in various populations (Stevenson and Cheeseman.1956, Chung et al.1959, Sank.1963, Chung and Brown.1970, Costeff and Dar.1980, Brownstein et al.1991) with the results ranging from less than ten to several thousand. As far as pattern of inheritance is concerned, the general agreement is that 77% are autosomal recessive, 22% are autosomal dominant, and the remainder are X-linked and mitochondrial (Gorlin et al. 1995).

In the last decade of the 20th century there has been rapid development and extensive studies of the genetic and molecular basis of deafness due to availability of large numbers of genetic markers. In the late 1980's a sex linked form of nonsyndromic hearing deafness locus was mapped to Xq13-q21.1 in a Mauritian (Wallis et al. 1988) and in a Dutch (Brunner et al. 1988) kindred. Four years later, in a large Costa Rican kindred, an autosomal dominant deafness locus was mapped to 5q31 (Leon et al. 1992). The third locus, a mitochondrial
mutation was recognised in a large Arab-Israeli pedigree (Prezant et al. 1993). In 1994 three types of autosomal early childhood deafness were recognized as being linked to chromosomes. The first nonsyndromic recessive deafness locus (DFNB1) was linked to chromosome 13 (Guilford et al. 1994a), the DFNB2 to chromosome 11 (Guilford et al. 1994b) and the DFNB3 to chromosome 17 (Friedman et al. 1995). Till 1996, no nonsyndromic genes had been cloned whereas presently 57 autosomal dominant and 77 autosomal recessive and 8 X-linked loci of deafness have been mapped and 49 genes involved in non-syndromic autosomal recessive/dominant deafness have been identified (Hereditary Hearing Loss Home Page: http://www.uia.ac.be/dnalab/hhh.). Over 400 forms of syndromic deafness have been mapped. Details about these loci are presented in the succeeding sections. Fig 1:8 represents the cytogenetic position of the non-syndromic loci and some of the syndromic loci, mapped to various chromosomes.
Search for a new DFNB locus in Pakistan Population

Fig 1:8 Cytogenetic map positions of human nonsyndromic deafness loci. Loci with published, statistically significant support for linkage are shown with a solid black font. Shown with a gray font are loci for which there are reserved symbols but no published data. DFN is the root of the locus symbol for deafness. An A or B suffix indicates that the mutant allele is segregating as an autosomal dominant or autosomal recessive, respectively. Sex-linked nonsyndromic hearing loss is designated with a DFN symbol and a numerical suffix. DFNM1 A deafness locus is underlined when the gene is known.
GENETIC EPIDEMIOLOGY

Estimates of prevalence of congenital and early childhood hearing impairment vary, and in many cases, are underestimated. Universal neonatal screening programs are perhaps the most accurate ones. Mason and Herrmann have reported bilateral hearing loss >35 dB in 1.4:1000 live births in Hawaii (Mason et al. 1998); other US studies have shown rates of 2.2:1000 and 3:1000 live births (Mhatre et al. 1996, White et al. 1993). European rates, mainly obtained from retrospective studies, are similar with ranges between 1.4–2.1:1000 live births (Parving 1999, Das 1996, Parving 1996). More than 50% of these cases are estimated to be inherited (Marres 1998).

Genetic hearing loss can be classified in many ways, including the mode of inheritance, the age of onset, audiologic characteristics, presence or absence of vestibular dysfunction, and the location and/or identity of the causative gene(s). The analysis of large population study suggested that, approximately 77–88% is transmitted as autosomal recessive traits, 10–20% as dominants, and 1–2% as X-linked traits (Rose et al. 1977). The frequency of mitochondrial deafness is quite variable and can range from less than 1% to more than 20% in some populations. Some forms of genetic deafness have distinctive audiologic findings including conductive, low, mid-tone, or high-frequency hearing losses, or evidence for vestibular dysfunction. In 20–30% of deafness cases, there may be other associated clinical findings that permit the diagnosis of a specific form of syndromic deafness (Walter 2003). It has been estimated that the prevalence of profound bilateral hearing loss is 1.6 per 1000 individuals in Pakistan and 70% of the hearing loss arises in consanguineous families (Elahi et al. 1998, Jaber et al. 1998).

CLINICAL MANAGEMENT OF CONGENITAL DEAFNESS

Clinical management of genetic disorders involves the use of many same techniques of diagnosis and treatment practiced by medical specialists, however these techniques are focused mainly on prevention of the diseases (Edwards 1977). The approaches used in this context are as follows;
1. DETECTION OF GENETIC DISORDERS/GENETIC SCREENING

Clinical geneticists and other health professionals use several screening tests and procedures to determine whether a person has a genetic disorder or is at risk of having a child with a disorder. These tests may be performed at various times in a person’s life. Some genetic screening tests are routinely performed on newborns. Among adults, genetic screening is always voluntary.

1. Preimplantation Diagnosis

This test is performed at the earliest possible stage of life. Before an embryo is created using in vitro fertilization it's surgically implanted into the mother’s uterus, physicians remove a cell from the developing embryo and analyze its DNA to learn if abnormalities associated with deafness or any other genetic disorders are present.

2. Prenatal Screening

Prenatal screening—genetic screening performed during a pregnancy—is used to identify fetuses at risk for certain genetic disorders. Amniocentesis is a prenatal screening test that is offered primarily to women who are 35 years or older at the time of pregnancy. The cells in the amniotic fluid can also be used to check for the presence of certain DNA mutations leading to deafness and to determine whether enzymes present in the fluid are characteristic of certain genetic disorders. Similarly for chorionic villus sampling, doctors isolate fetal cells from the chorionic villus and analyze them in the laboratory to determine if certain genetic abnormalities are present.

3. Carrier Screening

Carrier screening tests can determine if an otherwise healthy person carries a single copy of a mutated recessive gene for an autosomal disorder like deafness, that is common in certain ethnic groups. For example, Pakistani people are at high risk of deafness and should be screened for it. The client usually provides a blood sample or buccal swab sample. These cells are examined in a laboratory to determine if the person has a mutated gene condition, more common in persons children even if both partners are known to carry the same recessive gene. Such screening helps parents make reproductive choices, like people learn in advance
the health status of their child so that they can prepare for a special treatment or rehabilitation measures the infant may need.

2. Predictive Testing

Predictive testing identifies if a person has one or more altered genes whose effects typically do not appear until later in life.

Family History Screening

A client’s family medical history is gathered to determine if an inherited disorder exists within a family and helps identify healthy individuals at risk of developing a genetic disorder themselves, or of having a child with a genetic condition. In obtaining a family history, a health professional asks questions about the health of family members over a span of three or more generations. The information is recorded as a graphic image, to form a family tree that incorporates symbols, such as squares (that indicate males), circles (represent females), triangles, and diamonds, to present a shorthand record of the medical family history. Horizontal and vertical lines connect the circles and squares to show how family members relate to each other. This image, called a pedigree, can reveal the multigenerational pattern of a genetic disorder. For example, a dominant disorder affects at least one family member in each generation, whereas a recessive disorder may cluster in a single generation. The pedigree details significant family and health information for each family member, including birth and death dates, cause of death, miscarriages, stillbirths, and medical conditions, such as cancer, diabetes, heart disease, birth defects, genetic conditions, and mental retardation. Pedigrees also note the ethnic, racial, or geographic origins of the client’s ancestors to alert the counselor to genetic disorders prevalent among certain groups of people (Gelehrter.1908).

GENETIC COUNSELING

After identifying a disease or estimating the probability that a disease will develop, the individuals and their families are guided to choose a course of action to cope with the genetic condition. Genetic Counseling as defined by American Society of Human Genetics in 1975, is a medical specialty that helps parents and prospective parents evaluate and cope with their risk of passing hereditary disorders to their children.
Search for a new DFNB locus in Pakistan Populatoin

Genetic counseling requires sensitivity to a client’s needs and belief systems. Individuals have varying life experiences, social attitudes, and religious values that will influence their thinking about testing and medical issues. For instance, a couple may not mind coping with the disabilities of a child with an inherited disease, or their religious values may prevent them from considering abortion. These couples may decide against prenatal testing. In other cases, a couple who has already had a child with a terminal illness may not want to have any more children. The couple may choose to undergo sterilization procedures that will permanently prevent pregnancy (Gelehrter.1908).

Genetic counseling is provided by a team of health experts which consists of counselors who have graduate training in genetics, psychology, population statistics, and medical education, along with physicians who have advanced training in human genetics. The counselor gathers information about the client’s family medical history. The counselor may discuss options for diagnostic tests that could help determine if a person is at risk for passing an inherited disorder on to children or is susceptible to a particular genetic disease. When testing is completed, the genetic counselor analyzes the family history and test results to determine whether a genetic disorder exists within a family and, if so its pattern of inheritance. The counselor then discusses those conclusions with the client and help the client cope with the emotional repercussions of the diagnosis as well as any practical concerns. Genetic counselors respect the privacy of the individual and family, keeping all information exchanged during counseling sessions confidential.

Many prospective parents use genetic counseling to help resolve issues like it’s helpful to parents who already have a child with a genetic disorder so they can learn more about the condition and the chance that their other children may be affected. Expecting couples concerned about the health of their unborn baby may request genetic counseling to learn if the baby is at risk for a disease. A pregnant woman may worry that she has been exposed to factors that can hurt the development of her fetus, such as infectious diseases, medications, alcohol, non-medical drugs, or radiation. Parents interested in adopting a child whose biological parent or grandparent has an inherited condition may seek counseling to determine if this family history is likely to affect the child. Others seek genetic counseling to determine the likelihood that they will develop a disease that occurs more frequently in their ethnic group.
Search for a new DFNB locus in Pakistan Population

The parents of a newborn diagnosed with a genetic deafness can ask a genetic counselor to explain the progression of the disorder and describe the types of treatments required to keep their baby healthy. People with a family history of deafness may consult a genetic counselor to learn the probability that their children will develop this disorder.

REHABILITATION FOR THE DEAF COMMUNITY

Rehabilitation, is a term signifying any programmed ameliorative exercise, guidance, or instruction afforded to those with a particular disability, whether physical, psychological, or social. People who can profit from rehabilitation include convalescents, deaf-mutes or blind people, amputees or paralytics, emotionally disturbed people, alcoholics, criminals, and juvenile delinquents. In all cases the purpose of such beneficial treatment is the restitution of positive skills or attitudes in a person to provide him or her with a more contributive and fulfilling role in society. Until the Middle Ages, most people believed that deaf persons were incapable of learning language or of being educated in any way. By the 16th century, however, a few philosophers and educators began to reconsider the condition of deaf persons. A Spanish Benedictine monk, Pedro de Ponce, is considered the first teacher of deaf students, and in 1620 Juan Paulo Bonet, another Spaniard, wrote the first book on educating deaf persons (the book contained a manual alphabet similar to the one used today).

Deafness does not affect a person’s intellectual capacity or ability to learn. A child who sustains a hearing loss early in life, however, may lack the language stimulation experienced by children who can hear. A delay in learning language may cause a deaf child’s academic progress to be slower than that of hearing children and he may be four or more grade levels behind his or her hearing peers. However deaf children who receive early language stimulation through sign language, however, generally do well academically.

Today, more than 1 million school-aged children are hearing impaired. Approximately one-third of the population of school-age deaf children attends private or public residential schools. Deaf children in public schools may receive instruction exclusively with other deaf children, but increasing numbers of deaf children are being placed in regular classes with hearing children for physical education and vocational training. Some deaf children do most or all of their schoolwork in regular classes, occasionally with the use of interpreters and with periodic assistance from special teachers of the deaf.
MOLECULAR BASIS OF GENETIC DEAFNESS
HEARING IMPAIRMENT LOCI

Congenital deafness is genetically heterogeneous and over 300 genes are predicted to cause this disorder in humans (Friedman and Griffith.2003). It can occur with other pleiotropic manifestations to form a recognized phenotype (syndromic hearing loss, SHL) or appear in isolation (nonsyndromic hearing loss, NSHL). Syndromic hearing loss, associated with other recognizable phenotypic traits, is found in approximately 30% of the subjects and may be conductive, sensorineural or mixed, while nonsyndromic deafness, in which inner ear abnormalities are the only clinical feature, is found among the other 70% of the subjects and is almost exclusively sensorineural (Gorlin et al. 1995).

NONSYNDROMIC HEARING LOSS (NSHL)

NSHL is the kind of deafness with no other associated symptoms except deafness and is more prevalent mode of hearing loss than syndromic deafness. It seems to account for 70% of all the genetically determined cases of deafness.

NONSYNDROMIC AUTOSOMAL DOMINANT HEARING LOSS

Autosomal dominant deafness is passed directly through generations. It is often possible to identify an autosomal dominant pattern through simple inspection of the family tree .57 loci for autosomal dominant deafness have been mapped (Hereditary hearing loss homepage: http://dnalab-www.uia.ac.be/dnalab/hhh/). Mapped loci for non-syndromic autosomal dominant hearing impairment are symbolized as DFNA1, DFNA2 and so on in the order in which they are reported or reserved. Some of the DFNA and DFNB loci share the same chromosomal localizations (Petit.2001) hence when all deafness genes will be identified many more dominant and recessive loci might be found to be allelic forms of each other.

NONSYNDROMIC AUTOSOMAL RECESSIVE HEARING LOSS

Autosomal recessive disorders require a gene from both the mother and father. It is estimated that for NSRHL (autosomal recessive NSHL) there are between 30-100 genes (Chung et al.1959, Morton.1991). Non-syndromic autosomal recessive hearing loss loci are symbolized as DFNB1, DFNB2 and so on in the order in which they are first reported or reserved. To date, 77 non-syndromic recessive deafness loci have been mapped (Table 1:1).
### Autosomal Recessive Loci

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Search for a new DFNB locus in Pakistan Population

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Table 1: Loci for Nonsyndromic Autosomal Recessive Deafness (DFNB), attained from Hereditary hearing loss homepage
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SYNDROMIC HEARING LOSS

London dysmorphology database has identified over 400 syndromes associated with hearing loss and musculoskeletal, cardiovascular, urogenital, nervous, endocrine, digestive or integumentary systems (Gorlin et al. 1995). It may accounts for 30% of all genetically determined deafness cases. Syndromic deafness can be either dominant (Wardenberg syndrome, Branchio-oto-renal syndrome and Stickler syndrome) recessive (Usher syndrome and Pendred syndrome), X-linked (Alport syndrome, Nance syndrome and Hunter syndrome) or mitochondrial (Table 1:1).

SYNDROMIC AUTOSOMAL DOMINANT LOCI

Some of the common autosomal dominant syndromes are as under:

Waardenburg Syndrome is the most common type of autosomal dominant syndromic hearing loss with an incidence of 1 in 4000 live births, and a total of 2.3% of children with congenital hearing loss are suspected to have the syndrome (Tomaski and Grundfast 1999). This syndrome was named after Petrus Johannes Waardenburg, a Dutch ophthalmologist (1886-1979) who was the first to notice that people with two different coloured eyes frequently had hearing problems. The clinical features usually include dystopia canthorum (lateral displacement of the inner canthus of the eyes to give an appearance of a widened nasal bridge), pigmenory abnormalities of the skin, iris, and hair, and sensorineural hearing loss. Waardenburg syndrome is both clinically and genetically heterogenous, while four subtypes of Waardenburg syndrome Type 1, Type 2, Type 3 and Type 4 are known. Mutations of PAX3 gene are associated with type 1 and type3 phenotypes, while Type 2 has been linked to MITF and SLUG gene mutations. Three genes EDN3, EDNRB, and SOX10 have been reported to be associated with type 4 phenotypes (Friedman et al. 2003)

Branchial-oto-renal syndrome (BOR) is an autosomal dominant disorder that affects branchial, ear, and kidney structures. Branchial anomalies include branchial cysts and fistulas and preauricular pits; renal abnormalities are remarkably varied, ranging from mild hypoplasia to bilateral aplasia, even in the same family. Hearing is most often affected, with ~80% penetrance. Pure conductive loss being more common (30%) than pure sensorineural (20%). The prevalence of BOR is estimated to be 1 in 40,000, or 2% of all children with profound hearing loss. The EYA1 gene, located at 8q13.3, has been identified as the responsible gene for BOR. A recently described EYA1 knockout mice reveals the absence of ears and kidneys from apoptotic regression of the organ primordia implicating EYA1 as the early inductive tissue interaction signal specifically involved in ear and kidney formation. It
has been noted that 70% of families with the BOR phenotype have no mutations in the coding
sequence of *EYA1*. This provides an opportunity to identify new genes and to further elucidate
the pathophysiology of BOR (Tseng and Lalwani.2000).

**Syndromic Autosomal Recessive Loci**

Pendred syndrome and Usher syndrome are the most common autosomal recessive
syndromes having deafness as one of the phenotype in Pakistan. Pendred Syndrome is an
autosomal recessive disorder comprised of hearing loss associated with thyroid hormone
organification defect, resulting in a euthyroid goiter (Pendred.1896). *PDS* is estimated to be
responsible for 4 to 10% of hereditary prelingual deafness worldwide (Park *et al.* 2003). An
enlargement of the vestibule is found nearly in all patients. Hearing loss is characteristically
prelingual (though not necessarily congenital), sensorineural or, rarely, mixed, and severe to
profound (Cremers *et al.* 1998; Fraser, 1976; Phelps *et al.* 1998). Mutations of gene
*SLC26A4 (PDS)* are known to cause Pendred syndrome (Everett *et al.* 1997). Approximately
80 pathogenic mutations of PDS are known till now. Defects in the same gene underlie
nonsyndromic deafness *DFNB4* and many cases of enlarged vestibular aqueduct syndrome
(Li *et al.* 1998). *SLC26A4* encodes pendrin, an anion transporter found in the inner ear,

Usher Syndrome is an autosomal recessive disorder characterized by bilateral
sensorineural deafness associated with loss of vision due to retinitis pigmentosa. It is
estimated to be responsible for more than 50% of deaf and blind individuals and 8-33% of
patients with retinitis pigmentosa and 3-6% of congenitally deaf individuals (Vernon.1969,
Boughman *et al.* 1983). Based on studies of Scandinavian, Columbian, British and American
populations, its prevalence is estimated to be between 1/16,000 and 1/50,000 (Grondahl.
the severity and onset of deafness, retinitis pigmentosa and vestibular dysfunction. Various
classifications were proposed due to clinical heterogeneity (Hammersschlag.1907, Hallgren.
1959, Merin *et al.* 1974). Usher syndrome is classified into three main clinical subtypes i.e
Type1, Type2 and Type3 on the basis of variability in the onset of RP and on the presence or
absence of areflexia (Davenport and Omenn.1977).

At least seven distinct genetic loci for Usher syndrome type 1 (*USH1A-1G*), three for
Usher syndrome type2 (*USH2A-2C*), and two for Usher syndrome type3 have been mapped
to different chromosomes. Genes for seven usher syndrome loci have been identified as
unconventional myosin VIIa encoded by *MYO7A* (*USH1B*, Weil *et al.*1995), harmonin
PREVALENT DEAFNESS LOCI/GENES IN PAKISTANI POPULATION

To date twenty four non-syndromic loci and fifteen genes have been reported in Pakistani population.

DFNB1/DFNA3

The first locus for nonsyndromic, recessive deafness (NSRD), DFNB1 was mapped to chromosome 13q12 in two Tunisian families affected with profound prelingual deafness (Guilford et al. 1994). Subsequently, dominant form of deafness, DFNA3, was localized to the same chromosomal region (Chaib et al. 1994) and led to the hypothesis that Cx26 is the causative gene for both forms of deafness. Two distinct nonsense mutations were identified in three consanguineous Pakistani DFNB1 families and established that Cx26 is the causative gene (Kelsell et al, 1997). Authentic missense mutations, W44C and C202F, were eventually detected in two families, including the family described originally, thus establishing Cx26 as the causative gene for DFNA3 (Denoyelle et al. 1998, Morle et al. 2000). Cx26 missense mutations have been reported in two forms of dominant syndromic deafness with skin anomalies (Heathcote et al. 2000, Kelsell et al. 2000, Maestrini et al. 1999). GJB2 and other members of the connexin gene family have simple genomic structures composed of two exons.

High prevalence of mutations at DFNB1 locus became apparent, as these gene mutations were found to be the most common cause of both familial and sporadic cases of NSRD from many parts of the world (Carrasquillo et al. 1997, Scott et al. 1998). Over 70 different mutations within the Cx26 gene are known (Rabionet et al. 2000, Pandya et al. 2003). DFNB1 accounts for 20% in Japan (Abe et al. 2000, Kudo et al. 2000), 13.3% in India (Maheshwari et al. 2003), 16.7% in Iran (Najmabadi et al. 2005), while is less prevalent in Pakistan as compared to India (Santos et al. 2005).

Furthermore it was concluded that mutations in the complex DFNB1 locus, which contains 2 genes (GJB2 and GJB6), can result in a monogenic or in a digenic pattern of inheritance of prelingual deafness (del Castillo et al. 2002, Lerer et al. 2001, Feldmann et al. 2004).

One model that postulate the pathophysiology of DFNB1, suggests Cx26 gap junction system in ear plays a role in K+ recycling, facilitating the rapid transport of K+ ions through
the supporting cell network to the stria vascularis, where the ions can be actively pumped in to the endolymph through voltage-gated potassium channels thereby maintaining the unique K+/Na+ endolymph balance (Tekin et al. 2001).

The genomic knockout of connexin 26 is lethal in the mouse, (Gabriel et al. 1998) so to study gene expression in vivo, a targeted, tissue-specific knockout of connexin 26 had to be created that eliminated the expression in the epithelial cells of the inner ear (Cohen-Salmon et al. 2002) and concluded that Cx26-containing epithelial gap junctions are essential for cochlear function and cell survival and that prevention of cell death in the sensory epithelium is essential in restoring auditory function in DFNB1 patients.

**DFNB2/DFNA11/USH1B**

*DFNB2*, the second reported locus responsible for an autosomal recessive form of deafness, was localized to 11q13.5 by linkage studies in a large consanguineous Tunisian family (Guilford et al. 1994b), and it was noted that the region overlapped that of *USH* syndrome 1B (*USH1B*) (Kimberling et al. 1992, Smith et al. 1992)

Positional cloning of sh-1 in the mouse led to the identification of a gene, *MYO7A*, predicted to encode an unconventional myosin (myosin VIIA) (Gibson et al. 1995). Subsequently *MYO7A* gene (49 exons) was tested and mutations were identified in individuals with Usher 1B (Weil et al. 1995) and in two Chinese families linked to *DFNB2* (Liu et al. 1997a), and in a *DFNA11* family (Liu et al. 1997b).


Ophthalmological reevaluation of original Tunisian *DFNB2* family revealed mild retinal degeneration and retinitis pigmentosa (Zina et al. 2001). Moreover, Astuto et al. (2002) noted that there is no discernable difference between mutations that can cause usher syndrome and those that are nonsyndromic, and questioned whether the cases of *DFNB2* are truly nonsyndromic as it is difficult to explain the absence of a retinal phenotype.

The predicted human protein encoded by *MYO7A* is a member of the family of unconventional myosins, which do not assemble into filaments like conventional myosins. Unconventional myosins are motor molecules with structurally conserved heads that move
Search for a new DFNB locus in Pakistan Population

along actin filaments using their actin-activated ATPase activity. Myosin VIIA is expressed in a variety of tissues, is a common component of motile and sensory cilia, and is distributed along the entire length of stereocilia of inner ear hair cells (Hasson et al. 1995).

Myosin VIIA has also been implicated in endocytosis in hair cells. The inner ears of wild-type mice take up aminoglycoside antibiotics, which are ototoxic. However, homozygous sh1 mice are protected from gentamicin toxicity presumably because a step in the endocytotic pathway is disrupted (Richardson et al. 1999). Moreover, myosin VIIA participates in opsin transport through the connecting cilium to the outer segment of the photoreceptor cell (Liu et al. 1999), which may be the critical cellular process disrupted by USH1B mutations of MYO7A. Yet another proposed role for myosin VIIA is in transduction channel adaptation of inner ear hair cells. A myosin motor has long been favored as the source of the resting tension on the gating spring(s) of the transduction channel. In mice homozygous for either of two hypomorphic alleles, MYO7A6J or MYO7A4626SB, hair cell stereocilia bundles transduce a mechanical stimulus (Richardson et al. 1997), but require a larger force than is necessary in the wild-type bundles to open the transduction channel (Kros et al. 2002). Myosin VIIa may thus be a component of the adaptation motor complex. Alternatively, myosin VIIA may provide tension on the stereocilia plasma membrane (Gillespie 2002) and/or transport proteins to the stereocilia that are required for transduction indirectly affecting adaptation (Boeda et al. 2002).

El-Amraoui et al. (1996) analyzed the expression of myosin VIIA in retinal and cochlear cells during development in mouse and human. Analysis of myosin VIIA distribution in mouse retina showed that the pigment epithelium cells expressed myosin VIIA throughout murine development and post-natal life, while myosin VIIA is expressed in the cochlear sensory hair cells during mouse embryonic development and that myosin VIIA expression is restricted to sensory hair cells in the developing human otic vesicle. They noted that this expression pattern correlated to the vestibular and cochlear dysfunctions resulting in balance problems and hearing impairment observed in both USH1B patients and shaker-1 mouse mutants.

It has been anticipated that the shaping of the hair bundle relies on a functional unit composed of MYO7A, harmonin b, and CDH23 and that the interaction of these proteins ensures the cohesion of the stereocilia (Boeda et al. 2002). Furthermore, Adato et al. (2005) proposed that SANS via its binding to myosin VIIa and/or harmonin, controls the hair bundle cohesion and proper development by regulating the traffic of USH1 proteins to the stereocilia.
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Interestingly, in the zebrafish myosin VIIa, five of eight different circler mutants, designated mariner, segregate two missense and three nonsense mutations. Mariner fish have inner ear hair cell abnormalities, lack acoustic vibrational sensitivity and reduced or abolished microphonic potential (Ernest et al. 2000) and is likely to be a good model system to more fully explore the function of this unconventional myosin in the auditory system of vertebrates. Thus, this study demonstrated the striking conservation of the function of myosin VIIA throughout vertebrate evolution.

**DFNB3**

*DFNB3* was identified on chromosome 17p11.2 for NSRD segregating in 2% of the 2,200 residents of Bengkala (Friedman et al. 1995). On the basis of conserved synteny, shaker 2 (sh2) was proposed as a mouse model of *DFNB3* (Liang et al. 1998). Affected mice exhibit no auditory brainstem responses to sound pressure levels up to high levels, indicating profound deafness and associated head-tossing and circling behavior due to vestibular defects. Families with deafness linked to *DFNB3* were then screened for mutations of *MYO15A* and missense and nonsense mutations cosegregating with the hearing phenotype were found (Wang et al. 1998, Liburd et al. 2001).

The largest of several splice isoforms of *MYO15A* has 65 exons encoding 3530 amino acids (365-kDa). Myosin XVa, an unconventional myosin is suggested to have a role in the formation of stereocilia. Absence of staircase organization of sh2 mouse indicates that Myosin XVa is required for the elongation and formation of the stereocilia-bundle staircase (Belyantseva et al. 2003). Localization of myosin XVa to the extreme tips of stereocilia raises the possibility that it is tethered there by integral membrane proteins (Belyantseva et al. 2003a). Although the proteins that interact with myosin XVa are not known, it has two predicted FERM domains that could mediate interactions with ERM proteins. Perhaps, more interestingly, myosin XVa has a PDZ ligand sequence that could interact with PDZ domain-containing proteins to coordinate a macromolecular complex at the tips of stereocilia (Frolenkov et al. 2004). It has been recently demonstrated by Belyantseva and co workers that whirlin is transported to the tips of stereocilia by myosin XVa in the hair cells (Belyantseva et al. 2005).

**DFNB4**

Pendred syndrome (autosomal recessive deafness with goiter) and nonsyndromic deafness *DFNB4* are allelic disorders caused by mutations of the SCL26A4 (PDS) gene on
Search for a new DFNB locus in Pakistan Population.

chromosome 7q22-31.1 (Everett et al. 1997, Li et al. 1998). Enlargement of the endolymphatic duct and/or sac (EVA) is a sensitive and fairly specific radiological marker for Pendred syndrome or DFNB4 deafness (Phelps et al. 1998).

Over 60 mutations have been found in nearly every coding exon and protein domain throughout SLC26A4 and account for as much as 10% of hereditary deafness in diverse populations that include east and south Asians. Each ethnic population has a different and diverse mutant allele series, with one or a few prevalent founder mutations (Everett et al. 1997, Li et al. 1998, Coyle et al. 1998, Van Hauwe et al. 1998, Reardon et al. 2000, Campbell et al. 2001, Park et al. 2003).

SLC26A4 is composed of 21 exons encoding an 86-kDa polypeptide called Pendrin that is expressed in thyroid and kidney, as well as the cochlea (Everett et al. 1997). Pendrin is a multipass transmembrane protein predicted to have at least nine membrane-spanning domains, but its topology has not been experimentally determined. Recent studies by Scott et al. 2000, demonstrated that PDS mutations in individuals with Pendred syndrome differ functionally from PDS mutations in individuals with non syndromic hearing loss. They found that mutations associated with pendred syndrome have a complete loss of pendrin induced chloride and iodide transport, while alleles unique to people with DFNB4 are able to transport both iodide and chloride, although at much lower level than a wild type Pendrin (Scott et al. 2000).

A Pds-/- knockout mouse generated and characterized by Everett and coworkers has provided fascinating insights into the function of pendrin in the inner ear and the pathogenesis of hearing loss in Pendred syndrome (Everett et al. 2001). Homozygous Pds-/- mice manifest variable degrees of vestibular dysfunction as evidenced by gait unsteadiness, circling behavior, head-tilting, and abnormal performance in rotarod balance testing. Auditory brainstem response analyses demonstrated that Pds-/- mice are deaf, whereas Pds_-/- heterozygotes have normal hearing. The endolymphatic duct of Pds-/- mice is anatomically normal until E15, which begin to enlarge in comparison to control mice afterwards. Interestingly, no thyroid abnormalities have been detected in the Pds-/- mice. Although serum thyroid function tests and macroscopic and histologic studies could detect no abnormalities, it is possible that a subtle iodination defect is still present. Since these phenotypic features are incompletely penetrant in human Pendred syndrome, and since the auditory/ vestibular phenotype is very similar to that observed in human patients, the pds...
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knockout mouse should continue to provide an outstanding mouse model for further studies of pendrin and hearing loss in Pendred syndrome.

**DFNB6**

*DFNB6* was first localized by homozygosity mapping to chromosome 3q21 in a consanguineous Indian family (Fukushima *et al.* 1995b). Because of chromosomal homology with the linked region, the mouse mutant spinner (sr) is a candidate for *DFNB6*. Naz *et al.* (2002) cloned the human *TMIE* ortholog and identified five different *TMIE* mutations cosegregating with *DFNB6* deafness in five consanguineous families, including the original *DFNB6* family. There are no published data on the location of *TMIE* mRNA or protein expression in the auditory system, which should provide clues to its function and the pathogenesis of *DFNB6* deafness.

**DFNB7/11/DFNA36**

*DFNB7* and *DFNB11*, were mapped to chromosome 9q13-q21 in two consanguineous Indian families and two inbred Israeli Bedouin kindreds, respectively (Jain *et al.* 1995, Scott *et al.* 1996). *DFNA36* was also mapped in a large five-generation US family to same region (Kurima *et al.* 2000), suggesting they might be allelic disorders. Kurima *et al.* (2002) identified eight different mutations in a novel gene, transmembrane channel-like gene 1 (*TMC1*), in the *DFNA36* family and in 11 large families segregating *DFNB7/B11* deafness from Pakistan and India, including the original *DFNB7* family (Jain *et al.* 1995). The function of *TMC1* is unknown, but it is predicted to encode a multipass transmembrane protein and it is likely to be involved in ion transport. *TMC1* mutations were also identified in the recessive deafness (dn) and dominant Beethoven (Bth) mouse mutant strains segregating hearing loss and postnatal hair cell degeneration, indicating that *TMC1* is required for postnatal hair cell development or maintenance (Kurima *et al.* 2002, Vreugde *et al.* 2002). Moreover, a D572N mutation in *TMC1* gene was found to be associated with *DFNA36* (Makishima *et al.* 2004). Meyer *et al.* (2005), recently sequenced *TMC1* in Sudanese deaf individuals and identified a new mutation 1165C>T in exon 13, leading to the stop codon Arg389X, and the splice-site variant 19+5G>A.
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**DFNB8/B10**

DFNB8/B10, an autosomal recessive deafness locus, was independently mapped in two consanguineous families from Palestine (DFNB10) and Pakistan (DFNB8) to chromosome 21q22.3 (Bonne-Tamir et al. 1996, Veske et al. 1996). Haplotype and gene sequence analyses of individuals in these two families led to the identification of mutations in a gene encoding a serine protease, TMPRSS3 (Scott et al. 2001, Ben-Yosef et al. 2001). TMPRSS3 is the only protease reported thus far to be involved in nonsyndromic deafness.

The TMPRSS3 gene, spanning approximately 24 kb on chromosome 21, contains thirteen reported exons (Scott et al. 2001). In humans there are alternatively spliced transcripts (TMPRSS3a, b, c and d), encoding predicted polypeptides of 454, 327, 327 and 344 amino acids, respectively (Scott et al. 2001). A fifth isoform, TMPRSS3e, which has a longest open reading frame is recently identified, it encodes 538 amino acid residues and is the only isoform of this gene with a predicted signal sequence (Ahmed et al. 2004). Although the in vivo substrate (s) of TMPRSS3 have not been reported in the auditory system, TMPRSS3 is thought to regulate the activity of the epithelial amiloride sensitive sodium channel (ENaC) in vitro, which was suggested to control critical signaling pathway(s) in the inner ear and may have a role in the maintenance of the low sodium concentration of endolymph (Guipponi et al. 2002).

**DFNB12/USH1D**

The nonsyndromic recessive deafness locus DFNB12 was mapped to chromosome 10q21–q22 in consanguineous kindred from Syria (Chaib et al. 1996). The Usher syndrome type 1D (USH1D) locus was subsequently mapped in Pakistani kindred to 10q, that colocalize DFNB12 interval (Wayne et al. 1996).

Allelic mutations of CDH23 encoding cadherin 23 cause both nonsyndromic deafness DFNB12 (Bork et al. 2001, Astuto et al. 2002) and USH1D (Bolz et al. 2001, Bork et al. 2001). A genotype-phenotype relationship for USH1D and DFNB12 was proposed where some amino acid substitutions in cadherin 23 were presumed to be leaky or hypomorphs, causing partial loss of function and nonsyndromic deafness, whereas more disabling mutations and functional null alleles of CDH23 cause RP and vestibular dysfunction as well as deafness (Bork et al. 2001, Astuto et al. 2002, Pennings et al. 2004). All reported CDH23 alleles identified in nonsyndromic deafness patients are missense mutations (Bork et al. 2001, Astuto et al. 2002). Nonsense mutations, insertions, deletions, splicing variants, and other

Cadherin 23 is membrane of cadherin superfamily of integral membrane proteins (Jamora and Fuchs, 2002, Nelson and Nusse 2004). Homophilic interaction of these proteins might form links that interconnect stereocilia within a bundle. Cadherin 23 is located at the tips of the bundle in frog and zebrafish hair cells and has been proposed as an essential component of tip links (Siemens et al. 2004, Sollner et al. 2004). It is suggested that CDH23 and PCDH15 play an essential long-term role in maintaining the normal organization of the stereocilia bundles (Zheng et al. 2005).

DFNB18/USH1C

The DFNB18 locus was mapped in a consanguineous Indian family at chromosome 11p15.1-p14 (Jain et al. 1998), overlapping within a region of USH1C (Smith et al. 1992, Keats et al. 1994) and it was postulated that DFNB18 and USH1C are allelic variants of the same gene (Jain et al. 1998). Mutations in USH1C gene have been identified as the primary defect in USH1C patients (Verpy et al. 2000, Bitner-Glindzicz et al. 2000). A mutation in the USH1C gene was also found, associated with nonsyndromic recessive deafness (DFNB18) segregated in the original family from India, (Ahmed et al. 2002).

USH1C has 20 primary and 8 alternatively spliced exons encoding several isoforms (Verpy et al. 2000). Depending upon the harmonin splice isoform, there are either two or three PDZ domains and one or two coiled coil regions in the encoded protein. The USH1C gene produces at least eight isoforms in the mouse inner ear, with all but two of the longer isoforms also expressed in eye. This has led to the conjecture that mutations that selectively affect those isoforms would cause nonsyndromic hearing loss without retinal abnormalities. Longer isoforms have an additional PDZ domain, and is expressed only in the ear.

Mutation analysis of harmonin in the Indian family with DFNB18 revealed homozygosity for an intronic mutation that causes skipping of exon 12 with a resulting frameshift producing a stop codon in exon 13. This should disrupt isoforms in the retina as well as the ear. However expression studies have shown that normally spliced protein is also produced indicating that this is a “leaky” mutation. It is possible that enough product is formed to sustain activity in the eye but not in the ear (Ahmed et al. 2002). A splice-site mutation, 216G>A, in exon 3 of
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USH1C is associated with Acadian Usher type IC. This mutation was reported to create an in-frame deletion of 39 base pairs, resulting in an unstable transcript (Lentz et al. 2005).

**DFNB21/ DFNA8/A12**

DFNB21 locus was mapped in a Lebanese family to chromosome 11q23-25, and mutation in TECTA gene was identified (Mustapha et al. 1999). Mutations in the TECTA gene encoding α-tectorin are also associated with both dominant DFNA8/A12 hearing loss provide an unusual robust correlation of auditory phenotype with TECTA genotype. TECTA encode α-tectorin, major noncollagenous glycoprotein component of the tectorial membrane, which is an extracellular matrix that overlies the stereocilia of the outer hair cells in the organ of Corti.

Homozygosity for functional null alleles of TECTA at the DFNB21 locus causes recessive, prelingual, severe-to-profound stable hearing loss with a flat or shallow U-shaped audiometric configuration (Naz et al. 2003). In contrast, heterozygous carriers of missense mutations in TECTA at the DFNA8/A12 locus have dominant hearing loss with phenotypic features dependent on the type and location of the amino acid substitution within TECTA (Alloisio et al. 1999, Balciuniene et al. 1999, Moreno-Pelayo et al. 2001, Verhoeven et al. 1998, Iwasaki et al. 2002).

**DFNB23/USH1F**

DFNB23 was mapped to an interval of chromosome 10q21-22 (Van Camp and Smith 2002) that colocalizes USH1F (Wayne et al. 1997). Recessive mutations in the human PCDH15 gene are identified in the affected members of families segregating USH1F (Ahmed et al. 2001, Alagramam et al. 2001b, Ben-Yosef et al. 2003). PCDH15 mutant alleles were also found to cause nonsyndromic hearing loss (DFNB23) in three families from Pakistan (Ahmed et al. 2003). A genotype-phenotype correlation was suggested, in which hypomorphic alleles of PCDH15 are associated with nonsyndromic hearing loss-DFNB23, while more severe mutations of this gene result in USH1F (Ahmed et al. 2003).

PCDH15 belongs to the cadherin superfamily of calcium-dependent cell-cell adhesion molecules (Alagramam et al. 2001b). Precise cellular localization of protocadherin 15 showed its expression in the retina of mouse, human and monkey and along the entire stereocilia length (Ahmed et al. 2003). The R245X mutation of the PCDH15 gene was found to be the most common cause of USH1 in the Ashkenazi Jewish population (Brownstein et al. 49
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2004). A study showed that 5601-5603delAAC is a common mutation of PCDH15 (USH1F) in US and UK deaf individuals and accounts for 33% of mutant alleles (Ouyang et al. 2005).

**DFNB26**

Riazuddin et al. reported the localization of a novel recessive non-syndromic deafness locus DFNB26 on chromosome 4q31 segregating in a large consanguineous Pakistani family. The family defining DFNB26 is unique as a dominant modifier DFNM1 is also present in some members that can suppress the expression of deafness in its carriers (Riazuddin et al. 2000).

**DFNB29**

Mutations in the gene encoding tight junction Claudin 14 causes autosomal recessive deafness DFNB29 and mapped on chromosome 21q22 (Wilcox et al. 2001). The phenotype of Cldn 14-null mice is similar to the human phenotype; homozygotes for the null allele have profound hearing loss, with normal vestibular function (Ben-Yousef et al. 2003). CLDN14 mutations are a relatively infrequent cause of nonsyndromic recessive deafness in the Pakistani population while the contribution of CLDN14 mutation to recessive deafness in other populations is unknown, and may significantly differ from Pakistani population.

**DFNB36**

A novel autosomal recessive deafness locus DFNB36 was mapped to chromosome 1p36.3 in two consanguineous families. Mutational analysis showed two frameshift mutations, 1988delAGAG and 2469delGTCA, in ESPN, which encodes a calcium-insensitive actin-bundling protein called espin (Naz et al. 2004). Furthermore, it has been demonstrated that dominant mutation of ESPN can cause nonsyndromic deafness (Donaudy et al. 2005).

**DFNB37/DFNA22**

A novel locus DFNB37 was mapped on chromosome 6q13 in large consanguineous kindred from Pakistan (Ahmed et al. 2003). On the other hand two recessive null mutations of mouse MYO6 were known to cause deafness and vestibular dysfunction in Snell's waltzer mice (Avraham et al. 1995). Moreover, the DFNA22 locus was defined by a missense allele (C422Y) of MYO6 cosegregating with nonsyndromic, dominantly inherited, progressive hearing loss in a single family (Melchionda et al. 2001). Mutational analysis has shown three different mutations in MYO6 gene: a homozygous single-base-pair insertion (36-37insT), a
Search for a new DFNB locus in Pakistan Population

transition mutation, 3496C→T, and a transversion mutation, 647A→T segregated in families linked to DFNB37 (Ahmed et al. 2003).

DFNB39
A new autosomal recessive non-syndromic deafness locus DFNB39 was mapped on chromosome 7q11.22-q21.12 in consanguineous Pakistani family (Wajid et al. 2003).

DFNB48
A novel autosomal recessive non-syndromic deafness locus DFNB48 was mapped to chromosome 15q23-q25.1 in five large consanguineous Pakistani families (Ahmad et al. 2005).

DFNB49
A novel autosomal recessive non-syndromic deafness locus DFNB49 was mapped on chromosome 5q12.3-14.1 in two consanguineous families from Pakistan (Ramzan et al. 2004).

DFNB51
A novel autosomal recessive non-syndromic deafness locus DFNB49 was mapped on chromosome 11p13-p12 in two consanguineous families from Pakistan (Shaikh et al. 2005).

DFNB67
Four consanguineous families segregating recessive deafness were linked to markers on chromosome 6p21.1-p22.3, defining a novel locus DFNB67. Mutations of human TMHS gene causes DFNB67 deafness (Shabbir et al. 2006).

DFNB68
A novel autosomal recessive non-syndromic deafness locus DFNB68 was mapped on chromosome 19p13.2 in two unrelated consanguineous families from Pakistan (Santos et al. 2006).

DFNB72
A novel autosomal recessive non-syndromic deafness locus DFNB72 was mapped on chromosome 19p13.3 in three consanguineous families from Pakistan (Ain et al. 2007).
SECTION –III

MOUSE MODELS FOR DEAFNESS
MOUSE AND HUMAN GENOME HOMOLOGY

Advancement of comparative genomics has made increasingly clear how far structural and functional homologies extend across even distantly related species. Stretches of 1 to 50cM of chromosomes are conserved between humans and mice, with orthologous genes present along each chromosome. There are approximately 200 such shared homologous regions between human and mouse chromosomes, termed “syntenic” regions. Recently obtained drafts sequence of the human and mice genome revealed that both genomes contain about 30,000 genes and less than 1% of mouse genes have no human orthologue. In fact, encoded proteins between mouse and human have a median amino acid identity of 78.5% (Avraham.2003). Existing mouse models for some of these genes have allowed predictions of candidate genes for human disease and disorders. One of the truly remarkable advances for using the mouse as a model for human disease is the ability to determine the chromosomal localization of a mouse gene and correlate it with the human chromosomal location and when available, mouse mutant.

DEAF MOUSE MODELS

Hearing mechanism in mice have long been known to be genetically similar to humans. Human-mouse phenotype homology provide valuable clues to identify human deafness genes because orthologous gene mutations are likely to produce similar phenotype in both species.

NON-SYNDROMIC DEAFNESS GENES FOUND THROUGH DEAF MOUSE MODELS

Deaf mouse models are powerful tools for advancing the understanding of hearing loss and to identify novel genes or novel functions of known genes that underlie deafness and to develop innovative treatment strategies for deafness in humans using mouse genetic models. Many Mouse models are present for corresponding humans genes ,and they either helped identify a gene or its involvement in hearing mechanism in humans (Table1:3). Human non-syndromic deafness genes like OTOA (DFNB22), TMIE (DFNB6), TMC1 (DFN B7/11), MYO6(DFNA22),POU3F4 (DFN3), POU4F3 (DFNA15) and EYA4 (DFNA10) were identified using corresponding mice genes namely Otoa, Tmie, Tmc1, Myo6sv, Pou3f4, Pou4f3, and Eya4 respectively (Hereditary Hearing Loss Home Page).
### Human gene, Human disorder, Human Reference, Human Chr, Mouse Chr, Mouse gene, Mouse mutation

<table>
<thead>
<tr>
<th>Human gene</th>
<th>Human disorder</th>
<th>Human Reference</th>
<th>Human Chr</th>
<th>Mouse Chr</th>
<th>Mouse gene</th>
<th>Mouse mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD7</td>
<td>CHARGE syndrome</td>
<td>Vissers et al. (2004) Nat Genet 36:955</td>
<td>8q12</td>
<td>4 (2)</td>
<td>Chd7</td>
<td>Wheels, Whi, and multiple additional mutants</td>
</tr>
<tr>
<td>COL11A1</td>
<td>STL3, Stickler syndrome type III</td>
<td>Richards et al. (1996) Hum Mol Genet 5:1339</td>
<td>1p21</td>
<td>3 (53)</td>
<td>Coll1a1</td>
<td>chondrodysplasia, cho</td>
</tr>
<tr>
<td>COL11A2</td>
<td>STL2, Stickler syndrome type II (also DFNA13)</td>
<td>Vikkula et al. (1995) Cell 80:431</td>
<td>6p21</td>
<td>17 (19)</td>
<td>Coll1a2</td>
<td>targeted null</td>
</tr>
<tr>
<td>COL2A1</td>
<td>STL1, Stickler</td>
<td>Williams et al. (1996) Am J</td>
<td>12q13</td>
<td>15 (55)</td>
<td>Coll2a</td>
<td>disproportionate</td>
</tr>
</tbody>
</table>
Search for a new DFNB locus in Pakistan Population

<table>
<thead>
<tr>
<th>Syndrome Type I</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Reference</th>
<th>Mouse Gene</th>
<th>Mouse Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL4A3 Alport syndrome</td>
<td>Mochizuki et al. (1994) Nat Genet 8:77</td>
<td>2q37-q37</td>
<td>1 (51)</td>
<td>Col4a3</td>
<td>targeted null</td>
</tr>
<tr>
<td>GPR98 (MASS1, VLGR1) USH2C, Usher syndrome type 2C</td>
<td>Weston et al. (2004) Am J Hum Genet 74:357</td>
<td>5q14</td>
<td>13 (40)</td>
<td>Gpr98</td>
<td>BUB/BnJ and Frings inbred strains targeted null</td>
</tr>
<tr>
<td>KCNE1 (ISK) JLNS2, Jervell and Lange-Nielson syndrome</td>
<td>Schulze-Bahr et al. (1997) Nat Genet 17:267; Tyson et al. (1997)</td>
<td>21q22</td>
<td>16 (64)</td>
<td>Kcne1</td>
<td>targeted null</td>
</tr>
<tr>
<td>Locus</td>
<td>Description</td>
<td>Reference</td>
<td>Chromosome</td>
<td>Position</td>
<td>Gene</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>------------</td>
<td>--------------</td>
<td>--------</td>
</tr>
<tr>
<td>locus 2</td>
<td></td>
<td>Hum Mol Genet 6:2179</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>KIT</strong></td>
<td>PBT, piebald trait</td>
<td>Giebel and Spritz (1991) Proc Natl Acad Sci USA 88:8436</td>
<td>4q12</td>
<td>5 (42)</td>
<td>Kit</td>
</tr>
<tr>
<td><strong>MYO7A</strong></td>
<td>USH1B, Usher syndrome type IB (also DFNB2, DFNA11)</td>
<td>Weil et al. (1995) Nature 374:60</td>
<td>11q13</td>
<td>7 (48)</td>
<td>Myo7a</td>
</tr>
<tr>
<td><strong>PCDH15</strong></td>
<td>USH1F, Usher syndrome</td>
<td>Ahmed et al. (2001) Am J Hum Genet</td>
<td>10q21-q22</td>
<td>10 (40.2)</td>
<td>Pcdh15</td>
</tr>
<tr>
<td>Locus</td>
<td>Description</td>
<td>Chromosome</td>
<td>Position</td>
<td>Size (cM)</td>
<td>Reference</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------</td>
<td>------------</td>
<td>----------</td>
<td>-----------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><strong>type 1F</strong></td>
<td>(also DFNB23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PMP22</strong></td>
<td>CMT1A, Charcot-Marie-Tooth disease, type 1A</td>
<td></td>
<td>17p11-p12</td>
<td>11 (34)</td>
<td>Kovach et al. (1999) Am J Hum Genet 64:1580</td>
</tr>
<tr>
<td><strong>SALL1</strong></td>
<td>TBS, Townes-Brocks syndrome</td>
<td></td>
<td>16q12</td>
<td>8 (41)</td>
<td>Kohlhase et al. (1998) Nature Genet 18:81</td>
</tr>
<tr>
<td><strong>SIX5</strong></td>
<td>BOR2, bronchiootorenal syndrome 2</td>
<td></td>
<td>19q13</td>
<td>7 (4)</td>
<td>Hoskins et al. (2007) Am J Hum Genet 80:800</td>
</tr>
<tr>
<td><strong>SLC26A4</strong></td>
<td>PDS, Pendred syndrome (also DFNB4)</td>
<td></td>
<td>7q31</td>
<td>12 (~15)</td>
<td>Everett et al. (1997) Nat Genet 17:411</td>
</tr>
<tr>
<td><strong>SNAI2</strong></td>
<td>WS2, Waardenburg syndrome, type II</td>
<td></td>
<td>8q11</td>
<td>16 (9)</td>
<td>Sanchez-Martin et al. (2002) Hum Molec Genet 11:3231</td>
</tr>
<tr>
<td><strong>SPTBN4</strong></td>
<td>CMT4F, Charcot-Marie-Tooth disease, type 4F</td>
<td></td>
<td>19q13</td>
<td>7 (7.5)</td>
<td>not confirmed</td>
</tr>
</tbody>
</table>
Search for a new DFNB locus in Pakistan Population

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Chromosome Location</th>
<th>Mutation Details</th>
<th>Corresponding Mouse Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBX1</td>
<td>DGS, DiGeorge syndrome</td>
<td>22q11</td>
<td>16 (11)</td>
<td>Tbx1 overexpression targeted null</td>
</tr>
<tr>
<td>THRB</td>
<td>thyroid hormone resistance</td>
<td>3p24</td>
<td>14 (1.5)</td>
<td>Thrb targeted null</td>
</tr>
<tr>
<td>USH1C</td>
<td>USH1C, Usher syndrome type 1C (also DFNB18)</td>
<td>11p15</td>
<td>7 (23.5)</td>
<td>Ush1c deaf circler, dfcr</td>
</tr>
<tr>
<td>USH1G</td>
<td>USH1G, Usher syndrome, type 1G</td>
<td>17q24-25</td>
<td>11 (77)</td>
<td>Ush1g Jackson shaker, js</td>
</tr>
<tr>
<td>USH2A</td>
<td>USH2A, Usher syndrome type 2A</td>
<td>1q41</td>
<td>1 (106)</td>
<td>Ush2a targeted null</td>
</tr>
<tr>
<td>USH3A</td>
<td>USH3A, Usher syndrome type 3A</td>
<td>3q21-q25</td>
<td>3 (30)</td>
<td>Clrn1 targeted null</td>
</tr>
<tr>
<td>WFS1</td>
<td>WF, Wolfram syndrome (also DFNA6, DFNA14, DFNA38)</td>
<td>4p16</td>
<td>5 (21)</td>
<td>Wfs1 targeted null, but hearing not assessed</td>
</tr>
<tr>
<td>WHRN</td>
<td>USH2D, Usher syndrome type 2D (also DFNB31)</td>
<td>9q32-q34</td>
<td>4 (31)</td>
<td>Whrn whirler, wi</td>
</tr>
</tbody>
</table>

Table 1:3 Human syndromic deafness genes and corresponding mouse genes and mutations (Hereditary hearing loss homepage).
Hundreds of mouse models are now known to have hearing defects, and each one provides a piece of the puzzle in our understanding of inner ear biology. Some are directly relevant to human deafness and others provide key elements in the development and function of sensory structures of the ear. Using mouse models to study the human inner ear has and will continue to make a tremendous impact on the field, via providing clues to map new deafness loci or genes.
SECTION-IV
LINKAGE ANALYSIS;
POWERFUL TOOL FOR
MAPPING DISEASE GENES
Search for a new DFNB locus in Pakistan Populatoin

BASIS OF LINKAGE ANALYSIS

The human genome is very large and complex containing thousands of genes, panned over 23 chromosomes therefore, finding a particular gene or genes responsible for any human disease has always been tricky, and daunting task, quite literally like finding a needle in a haystack. Traditionally, the search for a disease gene begins with linkage analysis. Linkage analysis is a technique of developing a relationship between the loci; i.e. two loci on the same chromosome are said to be linked if the phenomenon of crossing over does not separate them. At the stage of meiosis homologous chromosomes exchange segments as the basis for crossing over or recombination. The original arrangements of alleles on the two chromosomes are called the parental combinations whereas the new combinations that are formed due to crossing over are known as recombinants (Fig 1:9). If two loci are physically close to each other on the same chromosome then there are rare chances that they will be separated by a recombination event. Sets of alleles for different markers or genes on the same chromosome are termed as haplotypes. Alleles on the same haplotype are passed on in pedigrees as a block. These blocks can only be broken by a recombination event. Moreover the term linkage refers to the loci, not to specific alleles at these loci. The most common application of linkage analysis is to try and find the location, in the genome, for a gene responsible for a certain mendelianly-inherited disease (Ott.1991).

Fig 1:9 Recombination event during meiosis.
RECOMBINATION FRACTION REFLECTS GENETIC DISTANCE

Alleles at loci on same chromosome for different genes co-segregate at a rate that is associated to the physical distance between them on the chromosome. This rate is the probability or recombination fraction ($\theta$), of a recombination event occurring between two loci. Two loci are said to be genetically linked when recombination fraction is less than 0.5. One of these loci is the disease locus while the other is a polymorphic marker like microsatellite repeats (Strachan et al.1996). The recombination fraction ranges from $\theta = 0$ for loci right next to each other through $\theta = 0.5$ for loci apart (or on different chromosomes), so that it can be taken as a measure of the genetic distance or map distance between gene loci. Two loci which show 1% recombination are defined as being 1 centiMorgan (cM) apart on the genetic map. And a genetic distance of 1cM represents 0.9 Mbp on the sex averaged physical map (Foroud et al.1997).

LOD-SCORE CALCULATION

When parametric linkage analysis methods are used, a quantity known as lod score (logarithm of the odds) is typically calculated. The score provides the strength of evidence in favour of linkage.

\[
\text{lod score} = \log_{10} \left( \frac{\text{Probability of the data if disease and marker are linked}}{\text{Probability of data if disease and marker are unlinked}} \right)
\]

In a lod score calculation the numerator is the probability of data in the family if the disease and marker are linked and therefore not segregating independently and the denominator is the probability if the disease and the marker are unlinked and therefore segregating independently (null hypothesis). If the marker and the disease gene are unlinked then the numerator is no more than the denominator and the ratio will be less than or equal to 1. However, when the marker and the disease gene are linked, the numerator will be greater than the denominator and the ratio will be greater than 1. A score of +3 or a positive score is an indication of linkage while a score of −2 or a negative score denotes absence of linkage. It is carried out by various computer programs (Ott.1991, Terwillger and Ott.1994).
MULTIPOINT MAPPING

Linkage analysis can be more efficient if the data for more than two loci are analysed simultaneously. Multipoint mapping is particularly useful for finding the chromosomal order of a set of linked markers. Usually the starting point in mapping a disease locus is to find a two point score which gives linkage between a specific marker and a disease locus. A multipoint score is calculated to find the location of disease gene between two or more markers.

DNA POLYMORPHISM: TOOL FOR LINKAGE ANALYSIS

Polymorphic markers are necessarily required, which can be checked for inheritance with the disease locus in question, for linkage analysis. Genotyping is carried out by a genetic marker defined as an observable polymorphism within the population. Prior to 1960s a limited source of genetic markers was obtained from blood group antigens (Conneally and Rivas. 1980). After 1980s RFLPs were introduced as a new class of genetic markers (Botstein et al. 1980). The RFLPs detect genome sequence differences that results in the presence or absence of a restriction enzyme cutting site. Subsequently, VNTRs (Variable number of tandem repeats) and SSLPs (simple sequence length polymorphism) were identified as a new source for genetic markers. The most useful class of polymorphisms for the purpose of genomic screening and fine genetic mapping are the SSLPs. The main advantage of SSLPs is their ubiquitous presence across the genome and a small amount of DNA is required as compared to RFLPs or VNTRs.

The simple sequence repeats also known as microsatellite, have revolutionised the world of genotyping. SSRs are hyper variable tandem sequence repeats which consist of di- tri- or tetra-nucleotide repeats. The most widely used SSRs to be developed for genotyping are the simple (CA)n and (GT)n repeats. The (CA)n repeats are extremely abundant and can be found, on average, once every 30-60 kb. (CA)n repeats are generally polymorphic if the repeat length is greater than 10. By isolating and sequencing DNA fragments containing the microsatellite, PCR primers that flanked the SSRs can be created and used to amplify it.
CHAPTER-2
MATERIALS AND METHODS
MATERIALS AND METHODS

For molecular and genetic study of hearing impairment the work plan was categorized into two main phases.

1. FIELD WORK
2. LABORATORY WORK

FIELD WORK

Prior to commencement of the work ascertained here, approval for this study was obtained from the Institutional Review Board (IRB) at the National Centre of Excellence in Molecular Biology, Lahore, Pakistan (FWA00001758) and our collaborator NINDS/NIDCD IRB at the National Institutes of Health, USA (OH-93-N-016).

IDENTIFICATION AND ENROLLMENT OF DEAF FAMILIES

Family identification

Families segregating sensorineural hearing loss (either syndromic or nonsyndromic) with three or more deafness affected individuals were identified through special education schools and centre present in different cities of Pakistan. Principals were contacted and briefed about deafness research program. Especially designed performa in Urdu was provided to them in order to obtain information about the history of deafness and number of affected in the family of the affected student. In addition consent forms were given to the families to inform them about the risks, discomfort and various facts that might influence them, if they willingly volunteer to participate in the research.

Pedigree analysis

Families were visited and multiple family members were interviewed for confirmation of cousin marriages and pedigree drawing. Pedigree is the most important step while studying human disease as it helps inferring mode of inheritance of the trait. Once transmission of deafness is evident to be in recessive mode of inheritance (as assessed from the family structure) blood samples from all participants were obtained after proper signing.
Search for a new DFNB locus in Pakistan Population

of informed consent. If a family had other affected relatives with hearing loss, they were also included in the study depending upon their willingness and availability.

Macromedia® FreeHand® MX and CYRILLIC® 3.1 (for Windows) software’s were used to draw pedigree structures of the enrolled families. Pedigree are made up to many generations, with the help of elders of the concerned family, using standard method described by Benett et.al, 1995). Males and females were represented by squares and circles respectively while affected persons of either sex were represented by filled symbols. The case history, the associated defects, the onset of disorder, number of generations involved, number of affected individuals, deceased persons, miscarriages in the family and especially no of consanguineous marriages were carefully recorded.

Blood sampling

For molecular studies blood samples of families affected with non-syndromic hearing loss were collected. Blood samples of 10ml were taken in 50 ml silicon falcon tubes containing 400µl of 0.5M EDTA. The samples were kept at -70°C for 20-30 mins otherwise for long storage the samples are frozen at -20°C.

Clinical Examination

Detailed history was taken from each family to minimize the presence of other abnormalities and environmental causes for deafness. Families were specially questioned about skin pigmentation, hair pigmentation, and problems relating to balance, vision, night blindness, thyroid, kidneys, diabetes, heart, and infectious diseases like meningitis, antibiotic usage, injury, and typhoid.
LABORATORY WORK

DNA EXTRACTION FROM BLOOD

Total genomic DNA was prepared using modern non-organic extraction method (Grimberg,et.al.,1989). White blood cells (WBC) are the only nucleated cell in the blood therefore it provides an easy source for extraction of genomic DNA.

Day1

Blood samples were thawed for the red blood cells (RBC) lyses.
35 ml of TE buffer (10 mM Tris HCl, 2 mM EDTA, pH 8.0) was added for washing of blood samples.
Samples were centrifuged at 3000 rpm for 20 min and supernatant was discarded to wash out the lysed RBC. Washing was repeated for three to four times till the WBC pellet is free of hemoglobin.
Digestion of proteins in the pellets of WBC was carried out by adding 0.5 mg of proteinase K along with 200 µl of 10% SDS in the presence of 6 ml TNE buffer (10 mM Tris HCl, 2 mM EDTA, 400 mM NaCl).
The samples were left overnight in an incubating shaker at a temperature of 37oC and a speed of 250 rpm.

Day2
Proteins were precipitated by adding 1ml of super saturated NaCl, followed by vigorous shaking and chilling on ice for 15 min before centrifugation at 2400 rpm.
Supernatant is shifted to another Sterilin® falcon tube and DNA was extracted from the supernatant by adding equal volume of Isopropanol.
After washing the DNA pellet with 70% ethanol, DNA was dissolved in TE buffer (10 mM Tris HCl, 0.2 mM EDTA) and heated at 70oC in a water bath for 1 h to inactivate any remaining nucleases.

DNA EXTRACTION FROM BUCCAL SWABS

Buccal swabs were collected in case of very young children or elderly people, where it was difficult to obtain blood sample. Cheek cells were obtained by means of MasterAmpTM Buccal Swab Brushes (EPICENTRE® Biotechnologies WI, Medical Package Co-operation, CA, USA). Subjects were asked to restrain from drinking, smoking, or eating for 1 hour before sample collection to reduce the possibility that food particles or other
Search for a new DFNB locus in Pakistan Populatoin

exogenous materials would compromise the sample and they were instructed to thoroughly rinse their mouth with water. Two swabs were taken from an individual by swirling each brush firmly on the oral mucosa for 30 sec, air dried and then stored in the original packaging at room temperature.

DNA was extracted from these buccal cells using MasterAmpTM DNA Extraction Solution from EPICENTRE® Biotechnologies (Walker et al. 1999). 500µl of the MasterAmpTM DNA Extraction Solution was added into an appropriate number of 1.5 ml ependroff tubes and placed them on ice. Buccal brush was placed into a tube containing DNA extraction solution and was rotated a minimum of 20 times. The brush was pressed against the side of the tube and rotated while removing it from the tube to ensure most of the liquid remains in the tube. The cap was closed on the tube tightly, vortex for 10 seconds and was incubated at 60°C for 30 minutes. Vortex mixed for 15 seconds. The tube was transferred to 98°C and incubated for 8 minutes. Vortex mixed for 15 seconds. The tube was returned to 98°C and incubated for an additional 8 minutes. Again vortex mixed for 15 seconds. Chilled the tube on ice briefly to reduce the temperature and cellular debris was pellet down by centrifugation at 4°C for 5 minutes at 14000 rpm. The supernatant containing the DNA was transferred carefully to a sterilized properly labeled screw tube without including any of the beads. The yields of the DNA are usually 2-8 ng/µl and were kept at -20°C, or at -70°C for longer term storage.

**ESTIMATION OF DNA CONCENTRATION**

DNA concentrations were obtained by measuring the optical density (OD). The optical density of samples were taken using “spectrophotometer”, which gives absorbance of UV light by DNA at 260nm and by protein at 280nm. First of all absorbance of the dilatant (distilled water) was measured and the spectrophotometer was set at zero. One cuvet having the dilatant, as standard, and the other having the DNA samples and the dilatant in the above-mentioned ratio was placed in the spectrophotometer. The optical density of each individual samples was measured.
DNA was diluted in low T.E Working DNA concentrations were kept at 25 ng/µl and 100 ng/µl for single marker and multiplex PCR amplification, respectively. The DNA was kept at -20°C for long storage.

The total amount of DNA was calculated by the following formula:

\[
\text{Absorbance at 260nm x correction factor x dilution factor} = \text{ug DNA/ul}
\]

i.e. \((\text{WL2}) \times 50 \times 50 = \text{ug DNA/ul}\)

1 optical density is taken as standard and it represents 50ug of DNA/ul (i.e. the correction factor). For pure DNA, the ratio A260:A280 should be between 1.7 to 2.0. After taking optical density of all the samples.
AUTOMATED FLUORESCENT GENOTYPING
TYPING STR MARKERS BY PCR FOR EXCLUSION OF KNOWN LOCI

Three-four short tandem repeat (STR) markers were genotyped for all the known recessive deafness loci (Table 2:1) as preliminary exclusion studies. DNA templates were amplified by PCR; using fluorescently labeled primers (forward primers labeled with one of the fluorescent dyes, FAM, NED, VIC). The markers used for linkage analysis encompassed the chromosomal locations as mentioned by hereditary hearing loss homepage (http://dnalab-www.uia.ac.be/dnalab/hhh) and were obtained commercially from either Applied Biosystems (ABI) or Integrated DNA Technologies (IDT). For the purpose of automated fluorescent genotyping; initially a 96 well master plate was made by assigning individual DNA of a particular family to each well. Replicates of this master plate were made with 50ng of DNA dispensed into each well overlaid with a 10 µl mineral oil.

PCR master mix (total conc 100ul) was prepared by mixing the contents mentioned below in the following order;

**REACTION MIXTURE FOR AMPLIFICATION OF STR MARKERS**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Final Conc.</th>
<th>Stock</th>
<th>Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>50 ng</td>
<td>25 ng/µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>Primer Forward</td>
<td>2.4 pM</td>
<td>8.0 pM</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>Reverse</td>
<td>2.4 pM</td>
<td>8.0 pM</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>dNTPs (dATP, dTTP, dCTP, dGTP)</td>
<td>200µM</td>
<td>1.25 mM</td>
<td>0.8 µl</td>
</tr>
<tr>
<td>PCR Buffer*</td>
<td>1X</td>
<td>10X</td>
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* 10X PCR buffer (100 mM Tris HCl pH 8.4, 500 mM KCl, 15-25 mM MgCl2 and 1% Triton)

The microsatellite markers were amplified on an ABI 2700 or ABI 9700 thermocycler. The thermo cycling programs used for amplification of single markers were touch down programs of either 67°C→57°C or 64°C→54°C and for amplification of primers in form a multiplex annealing of 54°C along with extension of 2 min was used as shown in
Search for a new DFNB locus in Pakistan Population

Fig 2: Thermocycling profiles used for the amplification of markers. A. Thermocycler programme touchdown 67°C→57°C. B. Thermocycler programme, touchdown 64°C→54°C. C. Multiplex PCR reaction thermocycler programme (with annealing at 54°C) for panel amplification.
SAMPLE PREPARATION FOR ABI 3100 /ABI 3730 GENETIC ANALYZER

The fluorochromes labelled PCR products were pooled together in such a way that none of the PCR products had the same size or fluorochrome in common. 1-2 µl of the PCR products together with 11.8 µl deionized Hi-DiTM Formamide (ABI) and 0.2 µl of one of the internal size standard ROX® or LIZ® (ABI) were pooled by using 12 capillary Hamilton® Syringe. The samples were denatured at 95°C for 5 min followed by chilling in the ice and loaded for genotyping in ABI 3100 /3730 Genetic Analyzer according to the manufacturer’s instructions given in technical manuals.

The phenomenon behind the automated genotyping is that when the dye labeled DNA fragments electrophorese through the capillaries filled with acrylamide gel, they are separated according to their size. At lower end of the capillaries the dye labelled fragments pass through a region where a laser beam continuously scans the capillaries. The laser excites the fluorescent dyes attached to the fragments and they emit light at a specific wavelength for each dye. A spectrograph collects and separates the lights according to wavelength, thus all four types of fluorescent emissions can be detected with one pass of the laser. With the help of data collection software light intensities are collected and stored as electrical signals (Lee et al. 1997). Automated allele assignment was performed using the ABI PRISM® Gene Scan Analysis Software Version 3.7 for Windows NT® Platform. The Gene Scan analysis software uses the automated fluorescent detection capability of the ABI PRISM® 3100 Genetic Analyzer instrument to size and quantitate DNA fragments and displays the result of the experiment as a reconstructed gel image, electropherogram or tabular data or a combination of electropherogram and corresponding tabular data. ABI PRISM® Genotyper ® 3.7 NT is data analysis software and transformation tool that converts data from Gene Scan result files into user application results. After obtaining a printout of the genotypic results the alleles (smallest allele was called as 1) were called manually (Fig 2:2).
Search for a new DFNB locus in Pakistan Population

FIG 2: Representative electropherogram of alleles, showing father, mother, and normal individual as heterozygous while all the deaf individuals are homozygous for allele “2”. The alleles were called manually; smaller allele was called as “1”.

1. FATHER

2. MOTHER

3. DEAF

4. DEAF

5. DEAF

6. DEAF

7. DEAF

8. NORMAL
LINKAGE ANALYSIS BASED ON CANDIDATE GENE APPROACH

Twenty four STR markers were selected (from UCSC Genome Database) for the eleven candidate genes (Table3:1). Multiplex PCR were standardized for all the markers, segregating them in appropriate set according to their base-pairs range. All the markers were labeled with FAM. PCR amplification methodology and thermal cycler profile used in this context is same as used for linkage analysis for exclusion studies.

GENOME WIDE SCAN

Genome wide scan was carried out with ABI PRISM® Linkage Mapping Set version 2.5 MD10 having 411 microsatellite markers (28 panels) spaced at ~10cM intervals across the whole human genome (Fig 3:1), to map new loci on families which remained unlinked to known loci. Multiplex PCR were standardized for the 388 markers of first 27 panels (for autosomes) by dividing them into appropriate sets (Table2:2). PCR fragments were amplified from 200 ng of genomic DNA in 5µl reaction containing 0.04-0.08 pM of each primer, 200µ M of dATP, dTTP, dCTP and dGTP, 0.8 units of Taq polymerase, .0.5µl of 10 X PCR reaction buffer (750mM KCl; 100mM Tris HCl PH: 8.3. 25 mM MgCl2) and 10µl overlay of mineral oil. Thermal cycling profile is same as above (Fig 2:1).
## Search for a new DFNB locus in Pakistan Population

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Table 2: Genome Wide Panels Sets for Multiplex PCR.
DATA ORGANIZATION AND ANALYSIS

Microsoft Excel macro was specially developed by Bioinformatics Lab, CEMB. This macro has different modules and help in integrating different excel sheets to analyze data. Different excel sheets are named: Data Sheet, Ranges Sheet, Basic Information Sheet, and Code Sheet.

Data Sheet Genotyping data of individuals in shape of alleles sizes were called and entered manually into the data sheet, it contain further information like: Panel ID, Marker’s name, cM distance, Labeling dye, ASR, Person name, Roman ID, Disease status / relation. Markers were listed column wise while individuals were arranged horizontally and assigned 2 columns per individual for a set of alleles.

Ranges Sheet Data entered in the data sheet were subjected to different analysis by using various modules of the macro, like Parentage, Coloring, Coding, and Filing. To run specific module different ranges were adjusted in the ranges sheet.

USAGE OF “MACRO SOFTWARE”

Data sheets act as a backbone to run different modules of software. To run the macro it is selected from the Tools present in the Menu bar. A window with the list of modules will be opened; relevant module was selected and Run command is given. The whole procedure is depicted below:
MODULES

They provide a computerized format for the enhanced management of data and related information. The macro package is provided with five dynamic modules as under:

1. Parentage (Confirmation of inheritance pattern); this module compares the given alleles of siblings with parental alleles. If any deviation regarding inheritance pattern is observed, the relevant cell was highlighted as RED.

2. Coloring (Coloring of homozygous alleles); this module highlights all the homozygous alleles by changing their background color. For each marker, if there were more than one homozygous pairs of alleles, different colors were assigned to different sets of values and same color to same set of values.

3. Coding: This module analyzes all the alleles appearing against a marker and assigns them a numeric codes starting from the lowest number. Finally, it generates a new version of data sheet having all information of original data sheet except alleles are replaced by its numeric code.

4. Filing; This module compiles the allelic data for a given set of markers (as adjusted in the ranges sheet) in the form of concatenated alleles. The output of the module is to populate the column labeled “alleles” on a different sheet named Basic Info which is further used to make pre file for lod score calculation. Other columns of this sheet are filled manually according to the information of subjected pedigree.

5. Create Pre ; This module picks the data from “Basic Info” sheet; arrange it in a specific pattern recommended by Linkage programme and saves it in a text-formatted file with a “pre” extension. This pre file is used as starting point while calculating LOD Scores.

LOD SCORE CALCULATIONS

Morton (1995) demonstrated that lod scores represent the most efficient statistical proof of evaluating pedigrees for linkage. Lod scores were calculated using FASTLINK (v4.1p) (Schaffer et al. 1994,1996). Two point lod scores were performed with MLINK program while multipoint was performed with LINKMAP.

Two files, .pre and .dat act as backbone to calculate lod scores. Pre file include genotypic data of the family, while dat file contains population allele frequencies for the markers whose lod score has to be calculated. Deafness was assumed to be inherited in an autosomal recessive manner with complete penetrance.
Recombination frequencies were assumed to be equal in both males and females. The frequency of deafness alleles for all the markers, in all genotyped individuals was assumed to be same. Genetic distances were based on Marshfield human genetic map (www.marshmed.org/genetics).
CLINICAL STUDIES OF ENROLLED FAMILIES

PEDIGREE ANALYSIS

Analysis of a family medical history, known as pedigree analysis, is used to track the transmission of a condition through generations. Enrolled families medical family history was recorded to trace the inheritance of a genetic deafness among multiple generations. The information was placed in pedigree, which resembles a traditional multigenerational family tree but includes information about individuals who were diagnosed with congenital deafness who suffered from certain medical symptoms. All pedigrees were analyzed to recognize mode of inheritance of deafness that is whether it is expressed in dominant or recessive form. Dominant disorders affect every generation. Recessive disorders may cluster in a single generation, reflecting when two parents who both carry a recessive allele for a disease have one or more children who develop the disease. A pedigree can also identify diseases that show X-linked inheritance.

CLINICAL EVALUATION

Detailed clinical histories were obtained for all of the individuals of the enrolled families to investigate the presence of other clinical abnormalities segregating with deafness and environmental causes for hearing loss. Families were questioned regarding any medical problems likely skin pigmentation, hair pigmentation, and problems relating to balance, vision, diabetes, night blindness, thyroid, kidneys, heart and infectious diseases like meningitis, mumps, rubella, typhoid, injury, chronic otitis media and, antibiotic/ototoxic drug usage. Parents as well as other members of the family were asked about the onset of deafness for each affected individual to confirm that deafness was congenital. Pure tone audiometry tests for air and bone conduction were performed at frequencies 250 to 8,000 Hz on many affected and unaffected members of these families. Vestibular function was evaluated by testing tandem gait ability and by using the Romberg test. Ocular funduscopy and electroretinography (ERG) was performed to detect the presence of retinopathy. Thyroid function was evaluated by performing perchlorate discharge test while goiter was observed physically in case of Pendred syndrome.
AUDIOLOGICAL TESTING

Audiometry

Audiometry is a method used to determine the degree of hearing loss and it provides a means of classifying deafness according to the scale of severity as shown in Fig 2:3 (Mazeas and Bourguet.1975). Audiometric studies were carried out on deaf individuals and their normal hearing relatives. Hearing sensitivity using air borne pure tones and bone conducted pure tones were measured by Siemens SD 28 Audiometer. The results of representative audiograms from an affected individual are presented in Fig 2:4. The affected individuals of all the families included in the present study had severe to profound hearing loss at sound frequencies from 250Hz to 8000Hz. Normal individuals had hearing 25-30dB from 250Hz to 8000Hz, which is considered as normal hearing.

1. THRESHOLD SENSITIVITY USING AIR BORNE PURE TONES

Threshold sensitivity is measured by using right and left earphones, allowing each ear to be examined independently. Tones are reduced in intensity until a just detectable threshold of hearing is determined. This is repeated at frequencies from 250 to 8000 kHz within the audible range and the results are plotted as an audiogram. The shape of the curve is a measure of the frequency sensitivity of both the middle ear and the inner ear. To differentiate between the middle ear (conductive) and inner ear (sensorineural) components, additional tests are conducted.

2. THRESHOLD SENSITIVITY USING BONE CONDUCTED PURE TONES

When a person speaks, he hears his own voice through air as well as through the bones of his own skull (the “feedback phenomenon”). This helps the person in keeping his voice modulated and at appropriate tone level. In conductive deafness, the bone conduction assumes precedence over air conduction and may become the main route of transmitting sound to the internal ear. In this method, testing is done by means of a vibrator which is placed somewhere on the skull, usually the mastoid bone. The testing and plotting procedures are the same as with air conduction testing. Sound at various frequencies and sound pressure leads directly to the cochlea via bone conduction bypassing the middle ear. Audiograms obtained using bone and air conducted sounds provide information about the integrity of both the middle and inner ears.
Search for a new DFNB locus in Pakistan Population

**Tympanometry**

Tympanometry is a method to measure the mobility or compliance of the tympanic membrane and provides information about the function of the middle ear including the tympanic membrane, ossicles and the eustachian tube. The instrument used is known as tympanometer.

**OTOAcoustic Emission (OAE)**

Otoacoustic emissions are widely used in human and animals to study the cochlear function. The origin of OAE is ascribed to the process associated with the mechanical motion of the outer hair cells. Thus the otoacoustic emissions OAEs are the sounds that the activity of the outer hair cell generate and can be measured with a microphone. A probe containing both a speaker and a microphone is sealed in the ear canal and a stimulus (sound of two different frequencies) is provided to the ear and the emissions produced by the outer hair cell in response to the stimulus are recorded.
Search for a new DFNB locus in Pakistan Population

Fig 2:3 Showing the Severity of Hearing Loss

Fig 2:4 Representative audiogram showing a normal hearing and a profound hearing loss
VESTIBULAR TESTING

Body orientation is controlled by the vestibular system, which consists of three semi-circular canals, the utricle and saccule. Each of these semi-circular canals lie automatically in a different plane. Each plane is at a right angle to the other and deals with different movement up and down, side-to-side and tilting from one side to the other. As the head moves, hair cells in the semicircular canals send nerve impulses to the brain by way of the vestibular portion of the acoustic nerve. Vestibular testing is used to determine the vestibular apparatus of the inner ear. In this regard, three tests Romberg tandem gait and ENG are usually performed on the affected individuals.

Romberg and Tandem Gait Test

The Romberg test is a physical examination in which the patient is asked to stand with their feet together (touching each other) and to close their eyes. An observer remains close to the patient if the patient begins to fall. With closed eyes, visual input is removed and instability can be apparent. If there is a more severe vestibular lesion, or a midline cerebellar lesion, the patient will be unable to maintain this position even with their eyes open and may fall (Blumenfeld.2001).

In case of tandem gait test, the patient is asked to walk with their hand attached with the body, each foot has to place adjacent with the other foot and have to walk. If there is any problem with the vestibular system, then the person may not walk properly.

Electronystagmography Test (ENG)

Electronystagmography (ENG) is another clinical test used to evaluate patients with dizziness and balance problems. It is a graphic recording of eye movements. ENG consists of an oculomotor evaluation, positioning testing, and caloric stimulation of the vestibular system. Comparison of results obtained from various subtests of ENG tests assist in determining whether a disorder is central or peripheral. In peripheral vestibular disorders, the lesion can be inferred from results of caloric stimulation and, to some degree, from positional findings (Levy and Arts.1996).
Search for a new DFNB locus in Pakistan Population

RETINITIS PIGMENTOSA

Retinitis pigmentosa is a progressive retinal degeneration (Fig 2:5) that begins with loss of peripheral vision and night blindness, and often leads to total blindness in later life. Two tests were performed on the affected individuals of each family for the diagnosis of retinitis pigmentosa.

Fig 2:5 Normal human retina and retina with retinitis pigmentosa
CHAPTER 3
RESULT AND DISCUSSION
SECTION-I

RESULTS OF LINKAGE ANALYSIS BASED ON “CANDIDATE GENE APPROACH”
PREAMBLE

Hearing impairment being the most prevalent sensory defects in humans, ranging in severity from modest difficulty with speech comprehension through profound hearing loss. Recently obtained drafts of human and mouse genomes suggested to have 85% homology, in addition hearing mechanism in mice have long been known to be genetically similar to humans, therefore the modern “candidate gene approach” was introduced. According to it human-mouse phenotype homology provides valuable clues to identify human disease genes because orthologous gene mutations are likely to produce similar phenotype in both species. Human non-syndromic deafness genes like OTOA (DFNB22), TMIE (DFNB6), TMC1 (DFNB7/11), MYO6 (DFNA22), POU3F4 (DFNB3), POU4F3 (DFNA15) and EYA4 (DFNA10) were identified using corresponding mouse genes namely Otoa, Tmie, Tmc1, Myo6sv, Pou3f4, Pou4f3, and Eya4 respectively. Recessively inherited deafness is relatively common in geographical remote areas with high consanguinity like Pakistan therefore our local population offers a powerful genetic resource for mapping and identifying novel deafness loci due to high consanguinity, via homozygosity mapping (Hussain and Bittles. 1998, Jaber et al. 1998, Elahi et al. 1998).

In order to identify new DFNB locus “Candidate gene approach” was used. Eleven candidate genes in humans for which corresponding deaf mice models were reported, were selected, namely MCOLN3, ITGA8, KLOTHO, UROCORTIN, NEUROG, PMCA2, SLC12A2, SLC12A7, KCNJ10, Bronx Waltzer and TASMANIAN DEVIL (Friedman et al. 2002, Hereditary hearing loss home page). For each candidate gene upto three polymorphic (STR) markers were selected. Either markers were selected from candidate gene intronic region or from genes flanking regions (Table 3:1). Seven hundred deaf families present in CEMB DNA bank were screened to identify orthologous gene in order to localize novel DFNB locus.

RESULT

Unfortunately all deaf families which were screened for the above mentioned candidate genes were found unlinked to these candidate gene markers. The reason might be that these selected genes are expressed in mice ear but they are not expressed in human ear or due to the probability that these gene mutations might be rare in Pakistani population. Moreover there might be some error during screening or linkage analysis of these screened families.
DISCUSSION

Over the past decade it has become increasingly clear how structural and functional homologies extend across even distantly related species. Therefore discovery of defects in a mice ear genes has led to identification of similar genetic defects in hearing impaired people and also helps to intricate choreography of genes and proteins involved in normal development of human hearing and the tiny missteps that disrupts normal hearing leading to congenital deafness. In addition if there is a mouse model for a human hearing loss, different therapeutic measures can be tested on mouse.

In our study in order to find a novel DFNB locus through “candidate gene approach”, eleven deaf mice models (genes) for which corresponding orthologous genes were present in humans were selected (Table 3:1). These candidate mice (ear) genes Mcoln3, Itga8, Klotho, Urocortin, Neurog, Pmca2, Slc12a2, Slc12a7, Kcnj10, Bronx waltzer (bv), and Tasmanian devil (Piourette) were all found to be involved in ear morphogenesis or in normal hearing mechanism of mice (Friedman and Griffith, 2003, Hereditary Hearing loss homepage: http://dnalab-www.uia.ac.be/dnalab/hhh/). For all these deaf mice genes, orthologous genes in humans are mapped to chromosome 1p22.3, chromosome 10p13, chromosome 13q13.1, chromosome 2p23.3, chromosome 5q31.1, chromosome 3, chromosome 5, chromosome 1, chromosome 22q12.1, and chromosome 4p12 respectively (http://www.enome.ucsc.edu/). These genes in humans were excellent candidate genes for screening families segregating autosomal recessive deafness. Moreover DFNB40 was mapped to chromosome 22q11.21-12.1, and since Bronx waltzer (bv) mouse mutant has been mapped to syntenic region on murine chromosome 5, it was suggested that DFNB40 and bv might result from orthologous genetic mutations (Demaghani et al, 2003). Linkage and haplotype analysis found that the seven hundred deaf families screened for these candidate genes remained unlinked. There are a number of reasons for not finding linkage; first possible reason might be that the selected genes are expressed in mice ear but their orthologous genes are not expressed in human ear, secondly due to the probability that these gene mutations might be rare in Pakistani population, furthermore there might be some error during screening or linkage analysis of these screened families.
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<tr>
<td></td>
<td></td>
<td>D5S649</td>
<td>133.65</td>
<td>171-185bps</td>
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<tr>
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<td>5p15.33</td>
<td>D5S1981</td>
<td>1.72</td>
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<td></td>
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<td>D5S2005</td>
<td>1.72</td>
<td>169-187bps</td>
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<tr>
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<td>D1S1167</td>
<td>168.52</td>
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<td>D1S2707</td>
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<td>135-159bps</td>
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<td>1p22.3</td>
<td>D1S401</td>
<td>110.0</td>
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<td>D1S2889</td>
<td>116.72</td>
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<td>D1S2766</td>
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<td>D13S260</td>
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<td>D2S2247</td>
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<td>D2S223</td>
<td>45.83</td>
<td>233-253bps</td>
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Table 3: Screened candidate genes; their chromosomal location, STR markers genotyped for candidate genes along with their genetic interval (cM) and range.

<table>
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<th>Gene</th>
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<th>STR Markers</th>
<th>Genomic Interval</th>
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<tbody>
<tr>
<td>NEUROG</td>
<td>5q31.1</td>
<td>D5S2115</td>
<td>138.64, 251-277bps</td>
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<tr>
<td></td>
<td></td>
<td>D5S396</td>
<td>139.33, 122-136bps</td>
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<td>BRONX-WALTZER</td>
<td>22q12.1</td>
<td>D22S1154</td>
<td>23.37, 218-268bps</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D22S1167</td>
<td>24.74, 266-278bps</td>
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<td></td>
<td>D22S310</td>
<td>23.37, 181bps</td>
</tr>
<tr>
<td>TASMANIAN DEVIL</td>
<td>4p12</td>
<td>D4S396</td>
<td>60.79, 227-235bps</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D4S401</td>
<td>60.16, 187-193bps</td>
</tr>
</tbody>
</table>
SECTION-II

RESULTS OF LINKAGE ANALYSIS BASED ON THE “CLASSICAL APPROACH”
Search for a new DFNB locus in Pakistan Population

PREAMBLE

Majority of congenital and prelingual deafness cases with genetic causes are autosomal recessive whereas postlingual hearing disorders have autosomal dominant or mitochondrial mode of inheritance (Peterson et al. 2006, Morton 1991, Readon 1992). Presently 142 NSHL loci have been localized [57 autosomal dominant loci (DFNA), 77 autosomal recessive loci (DFNB) and 8 X-linked loci (DFN)] through linkage studies and forty nine genes have been identified through positional cloning efforts (Hereditary Hearing Loss Homepage, http://www.uia.ac.be/dnalab/hhh). Recessively inherited diseases are common in population with cousin marriages therefore Pakistan population provides a valuable source for mapping and isolation of deafness genes. DFNB16, DFNB20, DFNB26, DFNB29, DFNB35, DFNB36, DFNB37, DFNB38, DFNB39, DFNB42, DFNB44, DFNB46, DFNB47, DFNB48, DFNB49, DFNB51, DFNB55, DFNB56, DFNB62, DFNB63, DFNB65, DFNB67, DFNB 68 and DFNB72 locus were mapped in Pakistani families.

Fifty highly consanguineous families with three or more multiple affected individuals in a single or multiple sibships were identified through special children schools from different cities of Punjab, Sindh, and Balochistan, out of them eleven families were enrolled for further studies. Families were visited and asked for the presence of any obvious symptoms regarding skin pigmentation, night blindness, balance, vision, thyroid, kidney, and heart problems in affected individuals of the family. Furthermore, involvement of any environmental factor was ruled out by asking questions regarding injuries, the usage of antibiotics, infectious disease like meningitis or typhoid. Parents as well as other members of the family were asked about the onset of disease to confirm the congenital nature of deafness. Audiometric studies were performed on the affected individuals of the family. After obtaining written informed consent from Institutional Review Board (IRB), 10cc blood samples were collected from each of the affected individual, normal siblings, parents and grandparents, if alive. DNA was extracted from blood samples of all enrolled families and linkage analysis studies for all the known recessive deafness loci were performed by typing at least three STR markers. If the deafness phenotype in a family showed potential linkage to a known locus, additional available members of that family were genotyped for those markers as well as additional markers to confirm the results.

During exclusion studies one family was found linked to DFNB7/11, which is a very common locus in Pakistani population whereas another family was linked to DFNB32, refining its region from 16Mb to 13.8Mb. These families will be discussed in the proceeding
part. Nine families remained unlinked to known loci which further augment the conjecture that a large repertoire of human genes associated with deafness remains to be mapped and identified (Friedman and Griffith, 2003). The genetic heterogeneity of deafness is further established by our lab data which represents that more than 50% of the families segregating deafness remain unlinked to known loci.

The deaf families which remained unlinked during exclusion studies were an excellent genetic resource to map novel deafness loci and genes. Genome wide scan was pursued on seven selected families as an advancing step to map novel deafness loci. Panels 1 to 27 of the ABI PRISM® Linkage Mapping Set version 2.5 MD10 .Panel containing 388 fluorescently labeled microsatellite markers spaced at an average interval of 10 cM across the human genome , was used for genome wide scan (Fig 3:1). STR’s were amplified by the polymerase chain reaction (PCR) according to standardized conditions (Table 2:1 ) and were analyzed on ABI PRISM® 3100 Genetic Analyzer. For intervals in which only a single informative marker was homozygous, the entire family was retyped for the same marker as well as additional closely spaced markers to distinguish between a false positive and a real positive chromosomal interval in which the novel locus resides thereby confirming and defining proximal and distal boundaries of the novel locus. LOD SCORE was calculated for these markers showing linkage to this locus.

DFNB75 (as designated by HUGO Gene Nomenclature Committee) was mapped on chromosome 5q23.3-q31.1 in highly consanguineous family PKDF365 (Fig 3:6) segregating recessively inherited, profound congenital deafness. No other clinically allied feature was observed in all the four families except deafness. Two hundred families segregating deafness from the CEMB repository were screened for the novel locus (DFNB75 ) but unfortunately no more family was found linked with this locus, bringing up the possibility that this locus might be rare in Pakistani population. The remaining six families on genome scan showed no visual homozygosity, probably due to the reason that there might be the slippage of a small linkage interval between the two markers placed apart.

Indepth and more precise fundamental understanding of molecular mechanisms of hearing impairment are unveiled with every upcoming discovery in the auditory system. Localisation of non-syndromic hearing loss DFNB75 and refinement of DFNB32 region are progressive steps towards identification of a new gene that effects development or mechanism of normal hearing and thereby will help in eradication of deafness.
Search for a new DFNB locus in Pakistan Population
Search for a new DFNB locus in Pakistan Populatoin

Fig 3:1  Schematic representation of the ABI PRISM® Linkage Mapping Set version 2.5. Bolded are the markers of MD10 panel set arranged 10cM apart, while plain are markers of HD5 panel set arranged 5cM apart.
RESULT OF EXCLUSION STUDIES
LINKAGE OF FAMILY PKDF153 TO DFNB7/11

PEDIGREE ANALYSIS

1. FAMILY DESCRIPTION

The family reported under the present study, a large consanguineous Pakistani family spanning up to five generations was found to be suffering from deafness (Fig 3.2). The kindred under study belonged to a remote area of Pakistan - district “Chakwal” in Punjab province. The family is “Sheikh” by caste. Most of the family members were located in Chakwal while some were settled in Karachi. The pedigree was made up to five generations with the help of elders of the concerned family. It is a highly in-bred family having six consanguineous marriages; yielding eight congenitally deaf individuals dispersed over two generations (Fig 3:2). Among the eight affected members of the family three were females (IV:6, IV:7, V:12) and five were males (V:4, V:5, V:8, V:11, V:15). The five affected individuals which were genotyped are V:4, V:8, V:11, V:12, IV:7 and the three normal individuals genotyped are V:13, IV:4 and IV:7. Their ages ranged from 10 to 36 year. The patients were clinically diagnosed for prelingual bilateral moderate to profound sensorineural hearing loss.

From the pedigree analysis it was concluded that there was an increased incidence of parental consanguinity which could account for all the affected individuals being homozygous, whereas affected children were usually born to unaffected parents who were usually asymptomatic carriers and individuals of either sex were affected. Therefore the mode of inheritance was considered to be in autosomal recessive manner. In addition as more than one member of the family was affected therefore it was considered as “familial disorder”.

2. CLINICAL ASSESSMENT

Audiometric studies on the affected individuals, showed severe to profound level of hearing loss.

Prenatal and Postnatal history
All patients’ medical history showed no pre-natal illness i.e. infections (CMV, rubella), diabetes, hypertension (eclampsia & pre eclampsia) and no drugs intake.
Search for a new DFNB locus in Pakistan Population

All the patients were born with normal birth weight (i.e. not less than 1.5 Kg) and did not suffer from postnatal diseases such as neonatal jaundice, meningitis, typhoid, septisemia, pneumonia, high grade fever, unconsciousness, and trauma.

**Age of onset and Parents observation**

All the affected individuals of the family were congenitally deaf.

Parents observed lack of response to sounds after few years of birth in the affected individuals. The affected individuals were considered stone (i.e. they were not able to hear sounds even of highest frequency) and mute.

**Complications other than deafness**

The affected individuals were in good general health, and were of normal intelligence. All the affected individuals were mute. Syndromes associated with deafness like cardiac rhythms, thyroid goiter, nephritis, ocular abnormalities (blindness-retinitis pigmentosa), vestibular symptoms, and diabetes were not observed.

**CLINICAL DESCRIPTION**

The affected individuals were clinically diagnosed with pure-tone audiometer with air and bone conduction. All affected individuals showed auditory response at 90db and 100db at frequencies 1000, 2000, 4000, 6000, 8000 from both the ears and thus was found to be suffering from bilateral profound (stone) hearing loss. All affected individuals did not show any sign of air conduction.

The patients were diagnosed to be suffering from non-syndromic, prelingual non-progressive bilateral severe to profound sensorineural hearing loss.

**HAPLOTYPE ANALYSIS**

The haplotype of the entire family PKDF153 was found to be linked to *DFNB7/11* locus at chromosome 9q13-q21q31. The affected individuals V:4, V:8, V:11, V:12, and IV:7 were homozygous for markers D9S1124, D9S1837, D9S769 and D9S175 (Fig 3:2). This chromosomal region harbors two overlapping loci i.e. *DFNB7* and *DFNB11*. Therefore family PKDF153 was considered to be linked with *DFNB7/11*. 
Search for a new DFNB locus in Pakistan Population

**LOD SCORE CALCULATION**

A two-point LOD score of 3.30 at $\theta = 0$ was observed for all the markers D9S1124, D9S1837, D9S769, and D9S175 in family PKDF153.

Previous studies strongly suggested that DFNB7/11 is genetically homogeneous in Pakistani population (Kitajiri et al. 2007). Linkage of PKDF153 to DFNB7/11 confirms the fact that this locus is common in Pakistani population.
FIG 3:2 PEDIGREE DRAWING OF PKDF153 ALONG WITH HAPLOTYPES FOR MARKERS ON CHROMOSOME 9, SHOWING LINKAGE TO DFNB7/11
LINKAGE AND REFINEMENT OF DFNB32 LOCUS IN PKDF539

FAMILY PKDF539 LINKED TO DFNB32

Through exclusion studies family PKDF539 was found linked to DFNB32 (a 16Mb region with causative gene unknown). All affected individuals of PKDF539 showed linkage to DFNB32 locus. Recombination event in PKDF539 affected individual (V:I) at marker D1S248 defined the distal limit of DFNB32 locus thus refining the linkage interval from 16Mb to 13.8Mb.

Cloning and identification of the causative gene is difficult, as mapped regions are usually very large for positional cloning efforts. Narrowing down of linkage interval, with a different recombination event in another affected family than the original family will facilitate isolation of mutative genes responsible for impairing hearing mechanism.

PEDIGREE ANALYSIS

1 FAMILY DESCRIPTION

PKDF539 a consanguineous Pakistani family spanning upto six generations was found to be suffering from deafness (Fig; 3.4). This family belonged to a village in Pakistan-“Tando Adam” in “Sindh” province. The family is “Khaskheli” by caste. The pedigree was drawn upto six generations with the help of elders of the concerned family. According to the information gathered two consanguineous marriages were contracted, yielding five congenitally deaf individuals dispersed over two generations (Fig 3:4). Eleven individuals of the family were sampled ,among whom four were affected members V:2, V:3, V:4 (all are females) and V:1(male) while V:6,VI:1,VI:2, VI:3,VI4 VI:6 and VI:7 were normal. These individuals were from 6years to 36 years of age. The patients were clinically diagnosed for prelingual bilateral moderate to profound sensorineural hearing loss.

Pedigree analysis concluded that parental consanguinity could account for all the affected individuals being homozygous, whereas affected children were usually born to unaffected asymptomatic carrier parents. In addition pedigree showed that individuals of either sex were affected therefore the mode of inheritance was considered to be “familial autosomal recessive disorder”.

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2 CLINICAL ASSESSMENTS

Audiometric studies on the affected individuals, showed severe to profound level of hearing loss.

Prenatal and Postnatal history

All patients’ medical history showed no pre-natal illness. All the patients were born with normal birth weight (i.e. not less than 1.5 Kg) and did not suffer from any postnatal diseases as well.

Age of onset and Parents observation

All the affected individuals of the family were deaf by birth. Parents observed lack of response to sounds after few years of birth in the affected individuals. The affected individuals were considered deaf and mute.

Complications other than deafness

The affected individuals were in good general health, and were of normal intelligence. All the affected individuals were mute. Syndromes associated with deafness like cardiac rhythmias, thyroid goiter, nephritis, ocular abnormalities (blindness-retinitis pigmentosa), vestibular symptoms, and diabetes were not observed.

CLINICAL DESCRIPTION

The affected individuals were clinically diagnosed with pure-tone audiometer with air and bone conduction. All affected individuals showed auditory response at 100db and 110db at frequencies 1000, 2000, 4000, 6000, 8000 from both the ears and thus was found to be suffering from bilateral profound (stone) hearing loss. All affected individuals did not show any sign of air conduction.

Family PKDF539 was found to segregating severe to profound non-syndromic deafness.

HAPLOTYPETE ANALYSIS

The deafness phenotype of this family was found linked to DFNB32 locus on chromosome 1. DFNB32 locus was mapped on chromosome 1p13.3-22.1 between markers D1S2868 and afmb014zb9 defining a region of 16Mb (Masmoudi et al., 2002). In PKDF539 all the four affected individuals V:2, V:3, V:4 and V:1 were homozygous for markers D1S2739, D1S206, D1S495 and D1S248 except individual V:1 was found heterozygous to marker D1S248 (Fig 3:4). This cross in deaf individual V:1 with marker D1S248 narrowed down
Search for a new DFNB locus in Pakistan Population

DFNB32 locus critical region from 16Mb to 13.8Mb, limiting the locus to 1p21.1-p22.1. (Fig 3:5).

**LOD-SCORE CALCULATION**

A maximum two-point LOD score of 2.58 at $\theta = 0$ was observed for marker D1S206. Allele frequencies of the polymorphic markers were assumed to be equal.

**IDENTIFICATION OF CANDIDATE GENES IN REFINED REGION OF DFNB32**

DFNB32 was reported to overlap DFNA37 locus, Marshall and Stickler syndromes locus. COL11A1 responsible for the later syndromes was sequenced but no mutation was found. Genes (Calopnin 3, Loc50999, Eukaryotic translation elongation factor1, Alpha1 type XI collagen) corresponding to ESTs in this region are also being screened for isolation DFNB32 gene (Masmoudi et al. 2002).

There are approximately seventy nine candidate genes in this refined critical region of 13Mb (UCSC genome browser: http://www.ename.ucsc.edu/) (Fig3:3). Among these candidate genes, depending upon that genes tissue expressivity and function some strong candidate genes can be isolated and screened for identification of DFNB32 gene which will in turn help in understanding mechanism of hearing.

![UCSC Genome Browser](http://www.ename.ucsc.edu/)

**Fig 3:3** Genes inside the DFNB32 refined region as derived from data available on the UCSC Genome Browser web page.
Fig 3:4 Pedigree drawing of family PKDF539 showing linkage to DFNB32 locus. Haplotype analysis of Individual V:1 gives a cross at marker D1S248, giving the distal boundary, while D1S2868 gives the proximal boundary thereby narrowing down the DFNB32 region from 16Mb to 13.8Mb.
Fig 3:5 Schematic representation of refinement of *DFNB32* interval on chromosome 1p21.1-p22.1 showing STR markers (●) and meiotic recombinations (____). Solid vertical lines represent the genetic intervals in which affected individuals are homozygous for the STR markers. Based on haplotype analysis of family PKDF539, markers D1S2868 and D1S248 define the proximal and distal boundaries of critical region of DFNB32 locus thereby refining DFNB32 locus region from 16Mb to 13.8Mb. There are ~79 genes annotated in the refined *DFNB32* interval (UCSC Genome Browser, http://genome.ucsc.edu).
RESULTS OF GENOME WIDE SCAN
MAPPING OF DFNB75 LOCUS TO CHROMOSOME 5Q23.3-Q31.1

A novel locus *DFNB75* was localized to chromosome 5q23.3-q31.1 following a whole genome wide scan in a highly consanguineous family PKDF365 segregating recessively inherited, profound congenital deafness (Fig 3:6). Haplotype analysis of affected individuals of family PKDF365 mapped the locus to 6.2 Mb region. This region was delimited by marker D5S2078 (134.72 cM) proximally and by marker D5S396 (139.33 cM) distally, delineating a genetic interval of approximately 4.61 cM (Fig3:7). A maximum two-point lod score of 4.7 for marker D5S2110 at recombination fraction $\theta=0$ was obtained (Table 3:2). It is a gene rich region and approximately one hundred and forty candidate deafness genes reside in the *DFNB75* interval (Fig 3:9). The mapping of *DFNB75* is part of our saturating search for human genes that are necessary for the development of the inner ear and the maintenance of normal hearing. Detailed description of the family that helped to map this novel locus *DFNB75* precede below.

FAMILY PKDF365 LINKED TO NOVEL DFNB75 LOCUS

PEDIGREE ANALYSIS

FAMILY DESCRIPTION

PKDF365, a highly inbred family with eight consanguineous marriages was enrolled from Hyderabad (Sindh) and belonged to “Khaskheli” caste (Fig 3:6). Family resides in district “Matriali”. Multiple individuals were interviewed to acquire the clinical history of the family and to draw a detailed pedigree up to nine generations. Twenty eight members of the family were enrolled for detailed study. This family comprised of four affected individuals in two loops, out of which two affected members were male (VIII:10, XI:1) and the other two were female. The affected individuals range in age from 7 years to 20 years. The first affected individuals appeared in the 8th generation i.e. two affected siblings (VIII:5, VIII:10).

Pedigree analysis concluded that normal carrier parents gave birth to deaf children and parental consanguinity could account for all the affected individuals being homozygous, whereas individuals of either sex were affected therefore the mode of inheritance was considered to be “familial autosomal recessive disorder”.

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2 CLINICAL ASSESSMENTS

Audiometric studies on the affected individuals, showed severe to profound level of hearing loss.

**Age of onset**

All the affected individuals of the family were congenitally deaf.

**Prenatal and Postnatal history**

Medical history of affected members showed no pre-natal illness. Birth weight of all the patients were above 1.5 kg. All affected members did not suffer from postnatal diseases such as neonatal jaundice, meningitis, typhoid, septisemia, pneumonia, high grade fever, unconsciousness, and trauma.

**Parents observation**

Parents observed lack of response to sounds after few years of birth in the affected individuals. The affected individuals were considered stone and mute.

**Complications other than deafness**

All the affected individuals were mute. Moreover, no other medical problem (i.e syndromes associated with deafness) was found co-segregating with deafness.

**CLINICAL DESCRIPTION**

The affected individuals were clinically diagnosed with pure-tone audiometer with air and bone conduction. All affected individuals showed auditory response at 100db and 110db at frequencies 1000, 2000, 4000, 6000, 8000 from both the ears and thus was found to be suffering from bilateral profound hearing loss. All affected individuals did not show any sign of air conduction.

All affected individuals of family PKDF365 exhibited prelingual bilateral profound sensorineural hearing loss, with no obvious vestibular or ocular abnormalities. Audiometric profiles of affected individual (VIII: 10) of family PKDF365 are shown in Fig 3:8.

**HAPLOTYPE ANALYSIS**

Genome wide linkage analysis of family PKDF365 showed initial evidence of linkage at markers D5S2055, D5S471 and D5S2115. In order to fine map and confirm the linkage additional eight STR markers D5S505, D5S622, D5S649, D5S2078, D5S2110, D5S2002, D5S2117 and D5S396 were genotyped for all participating family members. Haplotype analysis revealed a homozygous region of 4.61 cM (Fig 3:6), delimited by markers D5S2078.
Search for a new DFNB locus in Pakistan Population

(134.72 cM) and D5S396 (139.33 cM). The proximal recombination at marker D5S2078 was given by a normal individual VII:12 while distal recombination at D5S396 was provided by individuals VII:5, VII:10. However DFNB75 locus overlaps with DFNB60 locus, which was mapped at chromosome 5q22-q31, delimited by markers D5S404 (127.93cM) and D5S1979 (144.06 cM), delineating it to a genetic interval of 16.1cM (no further information is available on URL). In contrast to DFNB60, DFNB75 locus was mapped to chromosome 5q23.3-q31.1, spanning over a critical region of 6.2Mb, with genetic interval of 4.61 cM, therefore excluding DFNB75 locus from DFNB60 region and confirming it to be a novel locus (Fig3:7).

**LOD-SCORE CALCULATION**

A maximum two-point lod score (Z max) of 4.7 at recombination fraction θ=0 was obtained at D5S2110 (Table 3:2).

<table>
<thead>
<tr>
<th>MARKERS</th>
<th>Marshfield Map Position (cM)</th>
<th>PKDF365 Z max at θ = 0</th>
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</thead>
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<tr>
<td>D5S2055</td>
<td>1255.91</td>
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</tr>
<tr>
<td>DD5S471</td>
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</tr>
<tr>
<td>D5S1505</td>
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</tr>
<tr>
<td>D5S622</td>
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</tr>
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<td>D5S649</td>
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</tr>
<tr>
<td>D5S396</td>
<td>139.33</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 3:2 Two-Point Lod Score for markers used to map the novel DFNB75 locus.

**HUGO GENE NOMENCLATURE**

Fine mapping in PKDF365 revealed that a novel locus of 6.2Mb, lies within a 4.61 cM genetic interval on chromosome 5q23.3-q31.1. HUGO GENE NOMENCLATURE committee designated this locus as DFNB75.
PKDF365

Fig 3.6 Pedigree of family PKDF365 segregating recessive deafness with haplotype of markers at 5q23.3-q31.1. Squares or circles filled with black symbolize individuals affected with NSHL. The core haplotypes are boxed representing the ancestral chromosome harboring DFNB75. Haplotypes of PKDF365 revealed a homozygosity of approximately 4.61 cM delimited by markers DSS2078 and DSS396. Normal individual VII:12 provided the centromeric break point at marker DSS2078 while two affected individuals VIII:5 and VIII:10 provided the telomeric recombination at marker DSS396. STR markers and their relative positions in centimorgan (cM) according to the Marshfield human genetic map are shown on the left side of each pedigree.
Search for a new DFNB locus in Pakistan Population

**LINKAGE ON CHROMOSOME 5**

- **D5S2055** (125.91 cM)
- **D5S471** (129.83 cM) 23.1
- **D5S1505** (129.83 cM)
- **D5S622** (130.38 cM)
- **D5S649** (133.65 cM)

**PKDF365**

- **D5S2078** (134.72 cM)
- **D5S2110** (135.25 cM)
- **D5S2002** (136.33 cM)
- **D5S396** (139.33 cM)
- **D5S2115** (138.64 cM)
- **D5S436** (147.49 cM)

**Region reserved by Smith (DFNB60) 127.93 cM - 144.06 cM**

**Critical region (4.61 cM)**

- **D5S404** (127.93 cM)

**Fig 3:** Schematic representation showing a comparison between DFNB57 and DFNB60 linked to chromosome 5q. The solid vertical line represents the chromosomal intervals in which markers are homozygous for affected individuals and the cross represents meiotic recombination. Fine mapping of family PKDF365 revealed a centromeric recombination at D5S2078 (134.72 cM) in individual VII:12, while a telomeric recombination was detected at D5S396 (139.33 cM) in individuals VIII:5 and VIII:10. Thus the linked region for DFNB75 is 4.61 cM delimited by D5S2078 (134.72 cM) and D5S396 (139.33 cM).
Fig 3:8 Representative pure tone audiogram (of left and right ear respectively) of an affected individual (VIII:10) (20 years) of family PKDF365. The observed threshold in all deaf subjects showed profound hearing loss. “O” denote air conduction while “X” denote bone conduction.
IDENTIFICATION OF CANDIDATE GENES OF DFNB75

Cloning and identification of the causative gene has always been a daunting task, as mapped regions are usually very large for positional cloning efforts. For that reason two methodologies are normally adopted; one way is to have another family with a different recombination event than the original family to reduce the linkage interval, or to screen candidate genes for mutations on the bases of their putative functions. In order to search for the causative gene of DFNB75, for the first strategy a cohort of 200 consanguineous families from CEMB repository were screened for linkage to DFNB75 but no more family was found linked; perhaps it is a rare locus in Pakistani population. Secondly approximately 140 putative and known genes are present on 5q23.3-q31.1 region as searched on UCSC genome Bioinformatics (http://www.ename.ucsc.edu/) (Fig 3:9). Sequencing of these putative candidate genes will facilitate isolation of genetic mutation of DFNB75.

**Fig 3:9** Genes inside the interval of DFNB75 are derived from data available on the UCSC Genome Browser web page.
DISCUSSION

Mechanism of hearing and balance is attributable to conductance of electrical signals via nerve fibers to brain in reaction to stereocilia movements generated by pressure waves of sound. A large ensemble of proteins are involved in normal mechanism of hearing, mutation in any of these proteins can result in deafness (Trussell, 2000). Considering the complexity of the hearing process, it has been estimated that at least 1% of 30,000 human protein-coding genes are involved in the hearing process (Friedman and Griffith 2003). To date, 142 loci for nonsyndromic hearing loss have been reserved and 49 of the genes have been identified. Autosomal recessive nonsyndromic hearing loss (ARNSHL) is the most common form of hereditary hearing impairment (HHI). 77 autosomal recessive nonsyndromic hearing loss loci have been mapped, and 28 genes have been isolated, making ARNSHL an extremely heterogeneous disorder. Of the 77 reported autosomal recessive nonsyndromic hearing loss (ARNSHL) loci, the typical phenotype is prelingual non-progressive severe to profound hearing loss with the exception of DFNB8, which displays postlingual onset and DFNB13, which is progressive (Hereditary Hearing Loss Homepage: http://dnalab-www.uia.ac.be/dnalab/hhh/). Pakistani population provides a valuable genetic resource for mapping deafness loci as recessively inherited diseases are common in populations where cousin marriages are frequent. To date twenty four DFNB loci and fifteen causative genes [GJB2 (DFNB1), TMIE (DFNB6), TMC1 (DFNB7/11), TMPRSS3 (DFNB8), CDH23 (DFNB12), USH1C (DFNB18), PCDH15 (DFNB23), CLDN14 (DFNB29), ESRRB (DFNB35), ESPN (DFNB36), MYO6 (DFNB37), RDX (DFNB24), TRIOBP (DFNB28), MARVELD2 (DFNB49), HFPL5 (DFNB66) ] have been localized in Pakistani population (Hereditary Hearing Loss Homepage: http://dnalab-www.uia.ac.be/dnalab/hhh/).

In order to characterize the identify a novel DFNB locus in Pakistani population, 50 families having three or more deaf individuals were ascertained from different cities of Pakistan. Linkage studies were performed on selected eleven consanguineous families with inherited hearing loss by using highly polymorphic microsatellite markers for reported recessive (DFNB) loci and two families were linked to DFNB7/11/TMC1 and DFNB32 (causative gene unknown).

FAMILY PKDF153 LINKED TO DFNB7/11

DFNB 7/11 is the 6th most common locus in Pakistani population. It accounts for ~2.9% of the reported deafness cases (unpublished lab data). DFNB11 was mapped for the first time in a family of Bedouin origin. To date further nineteen families have been reported.
Search for a new DFNB locus in Pakistan population
to segregate nonsyndromic deafness *DFNB7/11* and all of these nineteen families were of
Pakistani origin (mostly from Punjab Province). In the study ascertained here a large
consanguineous family, PKDF153, segregating NSHL and having five deaf individuals in
two loops was found linked to *DFNB7/11* (Fig3:2). A two-point LOD score of 3.30 at \( \theta = 0 \)
was observed for all the markers D9S1124, D9S1837, D9S769 and D9S175 in family
PKDF153. Sequence analysis showed that mutations of transmembrane channel-like gene 1
(TMC1) cause *DFNB7/11* deafness on chromosome 9q13-q21. TMC1 encodes a
transmembrane protein of unknown function that is expressed in neuro-sensory hair cells of
the mouse cochlea.

Nine different TMC1 mutations were identified in nineteen Pakistani families
segregating severe to profound deafness, while a single TMC1 mutation p.R34X was detected
in normal samples of African-American and northern European origins, raising the possibility
that TMC1 mutations are prevalent genetic contributors of deafness in variant populations
and continents (Kitajiri *et al.* 2007). Audiometric profile of affected individuals of PKDF153
displayed moderate to severe, prelingual hearing loss. Due to similarity in hearing phenotype
in family PKDF153 and already reported nineteen Pakistani families, it can be anticipated that
one of the nine different mutant allele of TMC1 is also segregating in pedigree, PKDF153.
Linkage of family PKDF153 to *DFNB7/11* locus provides convincing evidence of the
existence of nonsyndromic recessive deafness, *DFNB7/11*, and also confirms the fact that this
locus is common in Pakistani population.

**REFINEMENT OF DFNB32 LOCUS REGION**

Autosomal recessive non-syndromic deafness was mapped to DFNB32 locus on
chromosome 1p13.3-22.1, delimited by markers D1S2868 and afmb014zb9, defining a 16Mb
critical region in a Tusnanian family. (Masmoudi *et al.*, 2002). During exclusion studies,
family PKDF539 spanning over six generation with four deaf individual in a single loop was
found to segregate DFNB32 deafness (Fig3:4). Family PKDF539, in contrast to the reported
data, narrowed down critical region of DFNB32 locus from 16Mb to 13.8Mb, by
recombination event at marker D1S248 (139.02 cM) in deaf individual V:1 (Fig3:5). A
maximum two-point LOD score of 2.58 at \( \theta = 0 \) was observed for marker D1S206.
Allele frequencies of the polymorphic markers were assumed to be equal. The critical region of
DFNB32 is narrowed down to genetic interval of \(~13cM\) between markers D1S2868
(126.16cM) and D1S248 (139.02cM), mapping it to chromosome 1p21.1-p22.1 (not
published).
DFNB32 region overlaps with Marshall and Stickler syndrome locus, therefore
DFNB32 region contains COL11A1 gene which is responsible for both these syndromes.
COL11A1 was considered an excellent candidate gene and was sequenced but the results
ruled out their involvement in disease pathogenesis. Moreover EST Database revealed five
ESTs expressed in DFNB32 region, they are being screened for deafness-causing mutation
(Masmoudi et al, 2003). To date DFNB32 causative gene has not been identified, as it is a
daunting task, because mapped region is very large for positional cloning efforts. For that
reason two methodologies are normally adopted; one way is to have another family with a
different recombination event than the original family to reduce the linkage interval, or to
screen candidate genes for mutations on the bases of their putative functions. PKDF539
linkage to DFNB32 not only confirmed the existence of DFNB32 deafness, but also refined
its critical region from 16Mb to 13.8Mb thus making it easy to identify candidate genes in
this region. There are ~79 candidate genes in refined region of DFNB32 (UCSC genome
browser:http://www.enome.ucsc.edu/). Among these candidate genes few strong putative
genes will be selected and sequenced to identify mutations causing deafness. Narrowing
down of critical region of DFNB32 locus is an advance step towards isolation of a novel gene
that is involved in normal hearing mechanism. Thus exploring the nature and location of the
DFNB32 mutations will provide an insight into the molecular basis of this disorder and the
diversity of mutations in Pakistani population.

**MAPPING OF A NOVEL LOCUS DFNB75**

Cosegregation of markers on chromosome5q23.3-q31.1 with profound deafness family
PKDF365, defines a novel recessive deafness locus DFNB75 (Fig 3:6). Family PKDF365
belongs to a remote area of Pakistan: district “Matriali”. This highly consanguineous family
comprised of eight cousin marriages (Fig3:6). A maximum two-point lod score of 4.7 and 3.1
was obtained for markers D5S2110 and D5S2078 respectively at recombination fraction θ=0
(unpublished).

Assuming that the deafness segregating in family PKDF365 is caused by allelic mutations,
haplotype analysis mapped the gene distal to D52078 (134.72 M) and proximal to D5S396
(139.33cM), delineating a genetic interval of approximately 4.61 cM. The proximal limit was
determined by recombination at marker D5S2078 in a normal individual VII:12 while distal
limit was determined by recombination at marker D5S396 in individuals VII:5, VII:10 (Fig
3:7).
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Although several NSHL loci including DFNB49/MARVELD2, DFNB60, DFNA1/DIAPH1, DFNA15/POU4F3, DFNA42, and DFNA32 have been localized on chromosome 5 (HEREDITARY HEARING LOSS HOME PAGE) yet all of these nonsyndromic deafness loci and genes are located outside of the DFNB75 genetic interval. However DFNB75 locus overlaps with DFNB60 locus, which was mapped at chromosome 5q22-q31, delimited by markers D5S404 (127.93cM) and D5S1979 (144.06 cM), delineating it to a genetic interval of 16.1cM (no further information is available on URL). In contrast to DFNB60, DFNB75 locus was mapped to chromosome 5q23.3-q31.1, spanning over a critical region of 6.2Mb, with genetic interval of 4.61 cM, therefore excluding DFNB75 locus from DFNB60 region and confirming it to be a novel locus.

Moreover, to identify gene mutations in DFNB75 locus leading to deafness two methodologies were adopted; for the first methodology a cohort of 200 consanguineous families from CEMB repository were screened for linkage to DFNB75 (to have another family with a different recombination event to reduce the linkage interval) but no more family was found linked; perhaps it is a rare locus in Pakistani population. In addition, to search for the causative gene of DFNB75, approximately one hundred and forty candidate genes are identified in this region (4.61cM) (UCSC genome browser: http://www.ename.ucsc.edu). Identification and sequencing of deaf mouse model mapped to the syntenic region on mouse chromosome 13 and 18 corresponding to human chromosome 5q23.3-31.1, as well as screening of putative candidate genes lying in this region will reveal the gene mutations causing DFNB75 deafness which will in-turn elucidate the molecular basis of hearing mechanism. Intricate knowledge of normal hearing mechanism at molecular level will suggest therapies for prevention and treatment of deafness, due to genetic as well as other causes.
CHAPTER-4
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