SCREENING FOR DEAFNESS CAUSING MITOCHONDRIAL DNA MUTATIONS AMONG TWO UNIQUE SUBGROUPS OF HEARING IMPAIRED FAMILIES FROM TAMILNADU.

A PROJECT REPORT

Submitted by

VIJAY BARATHI.E

in partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE

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BONAFIDE CERTIFICATE

Certified that this project report " **SCREENING FOR DEAFNESS CAUSING MITOCHONDRIAL DNA MUTATIONS AMONG TWO UNIQUE SUBGROUPS OF HEARING IMPAIRED FAMILIES FROM TAMILNADU**" , is the bonafide work of E.VIJAY BARATHI who carried out the project work under my supervision.

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CERTIFICATE

This is to certify that the project entitled " **Screening for deafness causing mitochondrial DNA mutations, among two unique subgroups of Hearing Impaired families from Tamil Nadu**" was carried out by E.Vijay Barathi in the Department of Genetics at Dr.ALMPG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai – 600 113 during the period from December 2007- April 2008 under my supervision and guidance.

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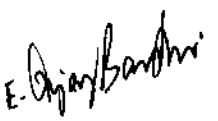

E.VIJAY BARATHI

The human mitochondrial genome is extremely small compared with the nuclear genome.and mitochondrial genetics presents unique clinical and experimental challenges.Despite the diminutive size of the mitochondrial genome.mitochondrial DNA mutations are an important cause of inherited disease.Recent years have witnessed considerable progress in understanding basic mitochondrial genetics and the relationship between inherited mutations and disease phenotypes, and in identifying acquired mtDNA mutations .Genetic causes of deafness are estimated to represent 75% of congenital deafness. Mitochondrial DNA(mtDNA)mutations have been implicated in non- syndromic hearing loss either as primary or as predisposing factors .As only a part of the mitochondrial genome is usually explored in deafness, its prevalence is probably underestimated. Some of the known mtDNA mutations are in 12SrRNA and in the tRNASer(UCN).Screening for these mutations have been carried out in several countries,consisting of samples of their local ethnic group.This is the first report that has attempted in screening for this mutation in the South Indian population.

Two subgroups were taken for mtDNA mutation screening.One subgroup (19subjects) consisting of a multigenerational family with a history of progressive postlingual deafness expressed across nearly four generations, was screened for A1555G mutation.Another subgroup(15 Subjects),consisting of subjects with congenital Non-Syndromic Sensorineural Hearing Impairment (NSHI) was screened for A1555G, A7445G, T7510C , T7511C mutations.The study involves the investigation of the Genotypic and phenotypic expression of the individuals screened for the mitochondrial DNA mutations.In subgroup 1 some subjects phenotypically found to be normal tested positive for A1555G mutation,hence being highly predisposed to hearing impairment which can be aggravated with Aminoglycoside antibiotics usage.In Subgroup 2 the distribution of probands based on parental mating showed the frequency of consanguinity to be 60%.All subjects except one were tested negative for A1555G , A7445G, T7510C , T7511C and Connexin26 mutations.It is found that consanguinity increases the chances of mitochondrial mutations among the offspring.Further, investigation on the role of Autosomal Recessive genes should be carried out.Genetic background should be tested before aminoglycosides are used in patients.

LIST OF ABBREVATIONS

DNA	-	Deoxyribonucleic Acid
RNA	-	Ribonucleic Acid
ATP	-	Adenosine Triphosphate
NSHL	-	Non Syndromic Hearing Loss
NSSNHL	-	Non Syndromic Sensorineural Hearing Loss
mtDNA	-	Mitochondrial Deoxyribo Nucleic Acid
OXPHOS	-	Oxidative Phosphorylation pathway
GTP	-	Guanosine Triphosphate
SNHL	-	Sensory Neural Hearing Loss
Bp	-	Base Pair
DFNB	-	Deafness Neurosensory Autosomal Recessive
dB	-	Decibel

INTRODUCTION

1. INTRODUCTION

Hearing impairment or hearing loss is a full or partial decrease in the ability to detect or interpret sounds. Ironically, Hearing impairment or Deafness is the most common sensory disorder in humans . Epidemiological studies have estimated that deafness affects 1 in 1000 newborns, 3 in 1000 by the age of 9 and as much as 10% by the age of 65years or older . Apart from various environmental factors , biological factors can also cause hearing impairment.The study of hereditary hearing impairments provides a unique opportunity to identify the causative genes and the underlying pathogenic process in each form of deafness, and to elucidate the molecular and cellular mechanisms of hearing.

Hereditary hearing impairment is a heterogeneous class of disorders showing different pattern of inheritance involving multitude of genes. Being a complex multifactorial disorder, nuclear genes as well as mitochondrial genes are involved in the various functions of the auditory system.As of 2007, more than 130 genetic loci have been described for nonsyndromic deafness that accounts for about 60-70% inherited hearing impairment.

Mutations in mitochondrial genome contribute less than 1% of all hereditary hearing loss.Genes of mitochondrial DNA are dedicated entirely to bioenergy functions and the body's organs and the tissues that are most dependent on bioenergy include the brain,the heart, muscles, the sensory organs of the eye and the inner ear.Genetic errors in mitochondrial DNA result in various kinds of defects in energy supply to the tissues of these organs . Cochlear function depends on a very high rate of ATP production, and mitochondrial DNA (mtDNA)-dependent dysfunctions have often been found to cause hearing defects either in syndromic or nonsyndromic form. mtDNA-linked deafness exhibits in high degree the characteristics of tissue specificity and variable degree of penetrance which are a hallmark of mtDNA mutation-dependent disorders.

This is a part of the ongoing study at the host institute aimed at generating a generic resource for deafness in Tamilnadu and to understand the contribution of various genes in its etiology.

- To understand the role of deafness causing mitochondrial DNA mutations by first reviewing the literature upto date.
- To account for the contribution of mtDNA mutation, in two unique cohorts of hearing impaired.
  i. Sporadic childhood hearing loss
  ii. A large extended family with 22 hearing impaired showing matrilineal transmission.

REVIEW OF LITERATURE

2.1 Anatomy of ear

The ear can be classified into three parts namely the Outer ear , Middle ear and the Inner ear.

- The outer ear consists of the ***pinna, or auricle*** and the ear canal.
- The middle ear begins with the *eardrum* at the end of the ear canal. The middle ear contains three tiny bones called the ***ossicles***. The first bone, the hammer (*malleus*) is connected to the eardrum. The hammer connects to the second ossicle, the anvil (*incus*), and then the anvil connects to the third bone, the stirrup (*stapes*).
- The inner ear contains the sensory organs for hearing and balance. The ***cochlea*** is the hearing part of the inner ear. The *semicircular canals*, the *utricle* and the *saccule* are the balance part of the inner ear.

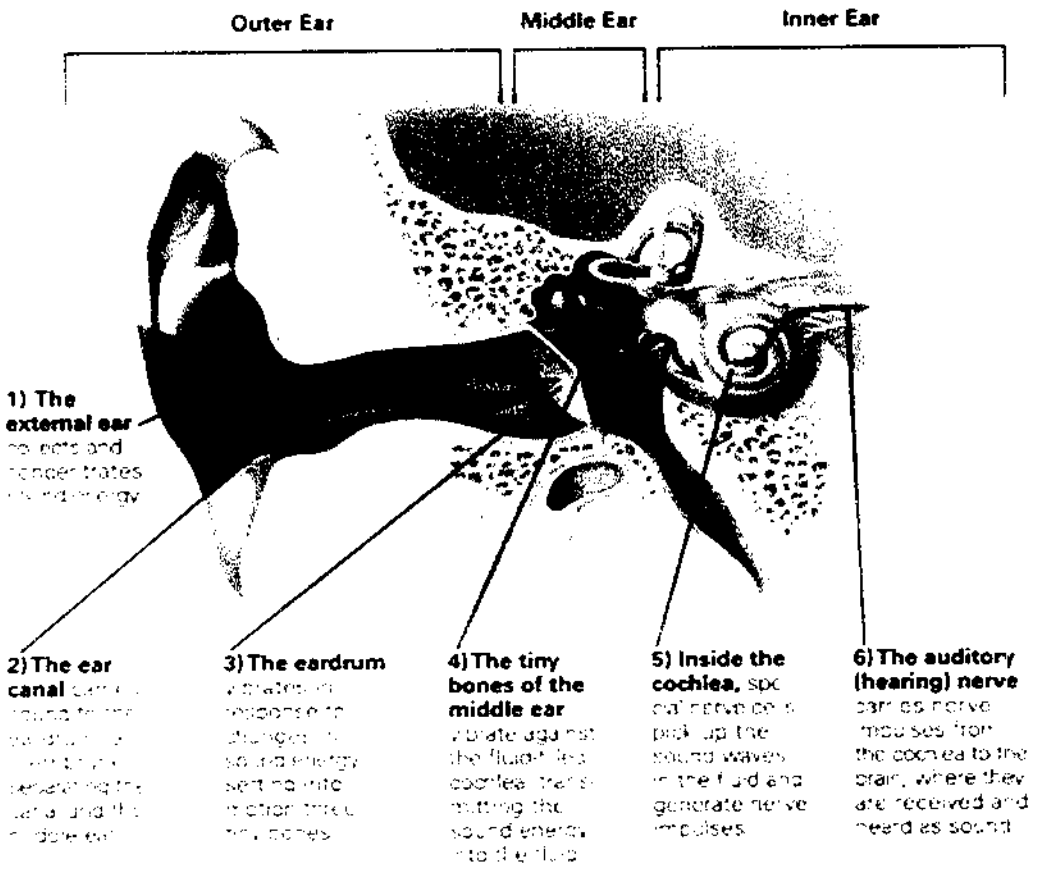

Fig 2.1: Structure of ear

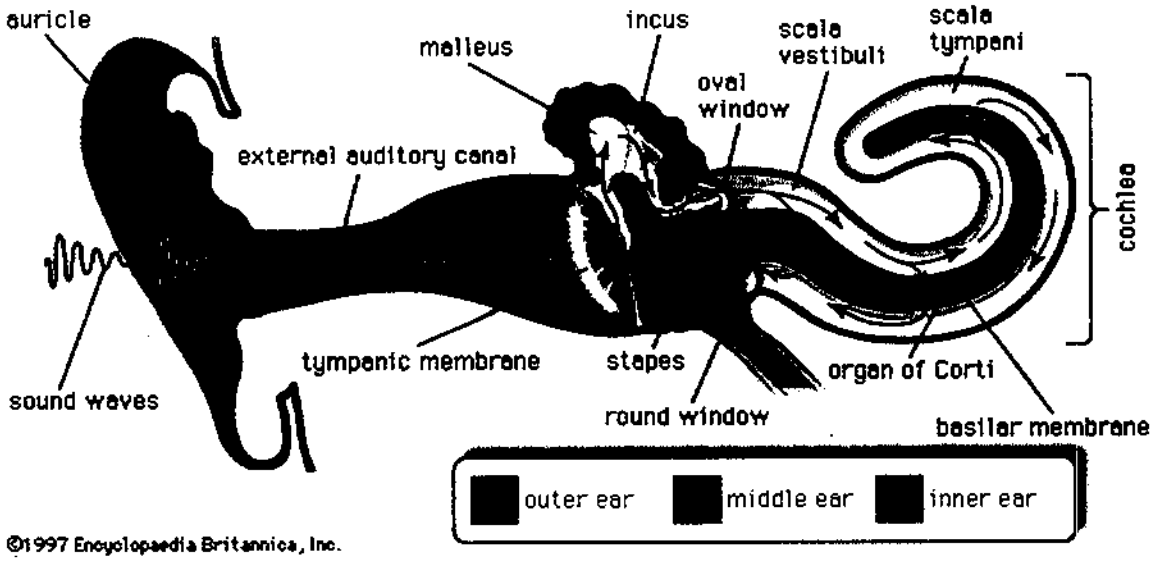

Fig.2.2 Normal Mechanism Of Hearing

The hearing organ is a sensory apparatus that converts the mechanical stimulation of sound into electrical energy in the cochlea, and then into a neural code in the central auditory pathway.An external sound produced , sends sound waves through the air which hits the ear drum. The eardrum sends the vibrations to the three smallest bones in our body namely, the hammer, the anvil, and the stirrup. The stirrup passes those vibrations along a coiled tube called the cochlea. The inner walls of the cochlea in the inner ear are lined with tiny hairs. These move in line with the wave motion set up in the liquid in the inner ear by vibrations coming from outside. Inside the cochlea there are thousands of hair-like nerve endings, cilia. When cochlea vibrates, the cilia moves. The sensory cells in cochlea are called the hair cells. (Robertson *et al.*, 1999)

The sensory hair cell in the cochlea is the site of auditory transduction, where the mechanical energy of sound vibration is converted into an action potential in the cochlear nerve. Several of the molecules involved in genetic deafness are located in sensory hair cells and must therefore have a vital role in hair cell function. During transduction, potassium floods into the hair cell, depolarizing the cell and triggering an action potential in the associated cochlear neurons. This potassium is recycled, and deafness can result from mutations affecting key steps in this process. Connexin 26, which is often involved in human non-syndromic deafness, is a component of the gap junctions between cells involved in potassium recycling. The observation that mutations in many different genes involved in potassium recycling can lead to deafness emphasize the importance of maintaining the correct ionic balance within the cochlea.

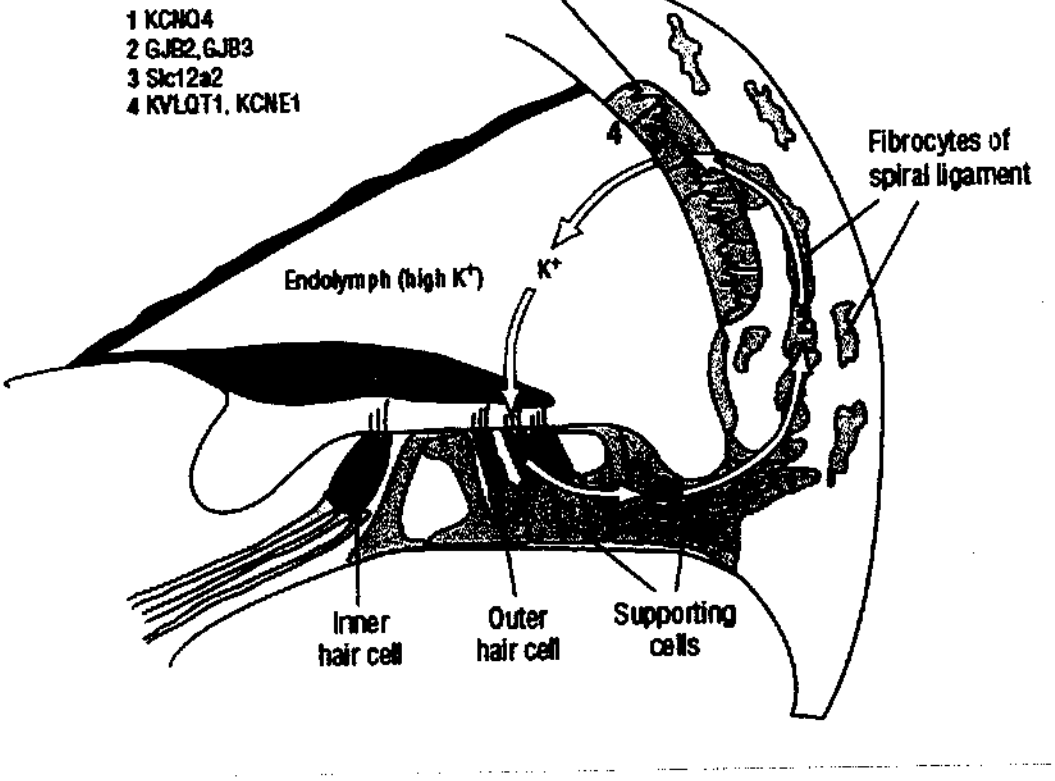

Fig 2.3 . Movement of Potassium in the inner ear(*Karen*, 2000)

The deflection of the hair-cell stereocilia opens mechanically gated ion channels that allow any small, positively charged ions (primarily potassium and calcium) to enter the cell. Unlike many other electrically active cells, the hair cell itself does not fire an action potential. Instead, the influx of positive ions from the endolymph in scala media depolarizes the cell, resulting in a receptor potential. This receptor potential opens voltage gated calcium channels; calcium ions then enter the cell and trigger the release of neurotransmitters at the basal end of the cell. The neurotransmitters diffuse across the narrow space between the hair cell and a nerve terminal, where they then bind to receptors and thus trigger action potentials in the nerve. In this way, the mechanical sound signal is converted into an electrical nerve signal. The repolarization in the hair cell is done in a special manner. The perilymph in scala tympani has a very low concentration of positive ions. The electrochemical gradient makes the positive ions flow through channels to the perilymph.

Conductive due to abnormalities of the external ear and/or the ossicles of the middle ear.

Sensorineural due to malfunction of inner ear structures (i.e.. cochlea).

Mixed due to a combination of conductive and sensorineural hearing loss.

Central auditory dysfunction results from damage or dysfunction at the level of the eighth cranial nerve, auditory brain stem, or cerebral cortex.

2.3.1 Onset

Prelingual hearing loss is present before speech develops. All **congenital** (present at birth) hearing loss is prelingual, but not all prelingual hearing loss is **congenital.**

Postlingual hearing loss occurs after the development of normal speech.

2.3.2 Severity of hearing loss.

Hearing is measured in **decibels** (dB). The threshold or 0 dB mark for each frequency refers to the level at which normal young adults perceive a tone burst 50% of the time. Hearing is considered normal if an individual's thresholds are within 15 dB of normal thresholds. Severity of hearing loss is graded as:

- Mild (26-40 dB)
- Moderate (41-55 dB)
- Moderately severe (56-70 dB)
- Severe (71-90 dB)
- Profound (90 dB)

Percent hearing impairment. To calculate the percent hearing impairment, 25 dB is subtracted from the pure tone average of 500 Hz, 1000 Hz, 2000 Hz, 3000 Hz. The result is multiplied by 1.5 to obtain an ear-specific level. Impairment is determined by weighing the better ear five times the poorer ear.

100%	91 dB	0%
80%	78 dB	20%
60%	65 dB	40%
30%	45 dB	70%

. Pure tone average of 500 Hz, 1000 Hz, 2000 Hz, 3000 Hz

Frequency of hearing loss. The frequency of hearing loss is designated as:

- Low (<500 Hz)
- Middle (501-2000 Hz)
- High (>2000 Hz)

2.3.3 Establishing the Diagnosis

Physiologic tests include:

- Auditory brainstem response testing (ABR, also known as BAER, BSER).
- Auditory steady-state response testing (ASSR).
- Evoked otoacoustic emissions (EOAEs).
- Immittance testing (tympanometry, acoustic reflex thresholds, acoustic reflex decay).

Audiometry

Audiometry is the testing of hearing ability. Typically, audiometric tests determine a subject's hearing levels with the help of an audiometer, but may also measure ability to discriminate between different sound intensities, recognize pitch, or distinguish speech from background noise. Acoustic reflex and otoacoustic emissions may also be measured. Hearing loss is diagnosed using the results of the audiometric tests. Audiometry consists of behavioral testing and pure tone audiometry.

reinforcement audiometry (VRA). **BOA** is used in infants from birth to age six months, is highly dependent on the skill of the tester, and is subject to error. VRA is used in children from age six months to 2.5 years and can provide a reliable, complete audiogram, but is dependent on the child's maturational age and the skill of the tester.

Pure-tone audiometry (air and bone conduction) involves determination of the lowest intensity at which an individual "hears" a pure tone, as a function of frequency (or pitch). Octave frequencies from 250 (close to middle C) to 8000 Hz are tested using earphones. Intensity or loudness is measured in decibels (dB), defined as the ratio between two sound pressures. 0 dB HL is the average threshold for a normal hearing adult; 120 dB HL is so loud as to cause pain. Speech reception thresholds (SRTs) and speech discrimination are assessed.

Air conduction audiometry presents sounds through earphones; thresholds depend on the condition of the external ear canal, middle ear, and inner ear.

Bone conduction audiometry presents sounds through a vibrator placed on the mastoid bone or forehead, thus bypassing the external and middle ears; thresholds depend on the condition of the inner ear.

Conditioned play audiometry (CPA) is used to test children from age 2.5 to five years. A complete frequency-specific audiogram for each ear can be obtained from a cooperative child.

Conventional audiometry is used to test individuals age five years and older; the individual indicates when the sound is heard.

The presence and the severity of hearing loss can be ascertained by means of audioprofiling. Audioprofile refers to the recording of several audiograms on a single graph.These audiograms may be from one individual at different times, but more frequently they are from different members of the same family segregating deafness usually in an **autosomal dominant** fashion. By plotting numerous audiograms with age on the same graph, the age-related progression of hearing loss can be appreciated within these families. Often the composite picture is characteristic of specific genetic causes of **autosomal dominant** non-syndromic hearing loss.

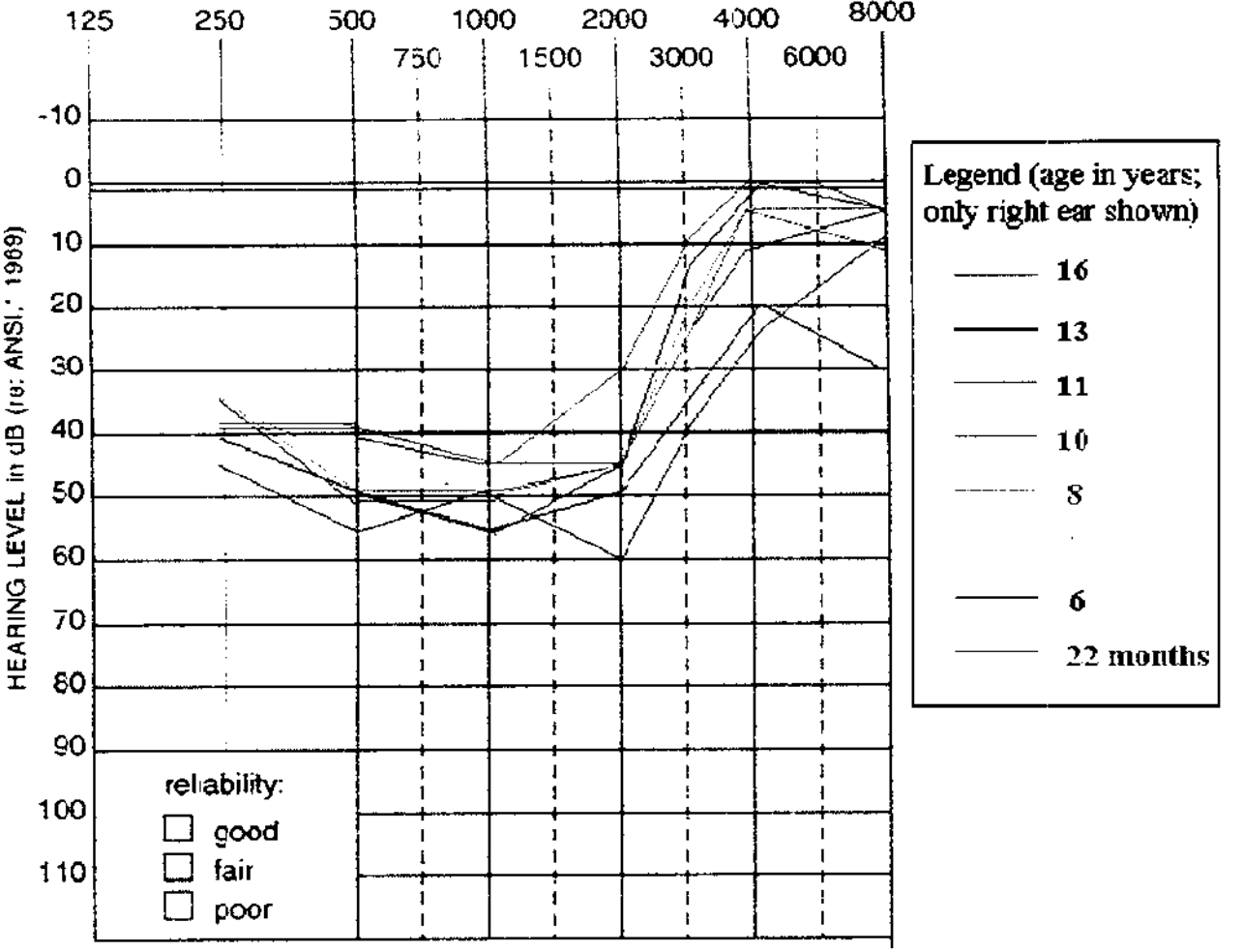

Fig2.4:Audioprofile

Congenital hearing loss can be identified through universal **screening** of newborns, which has been advocated by the National Institutes of Health and is available in most states although requirements and implementation strategies vary. Parental concerns about possible hearing loss or observed delays in speech development require auditory **screening** in any child.

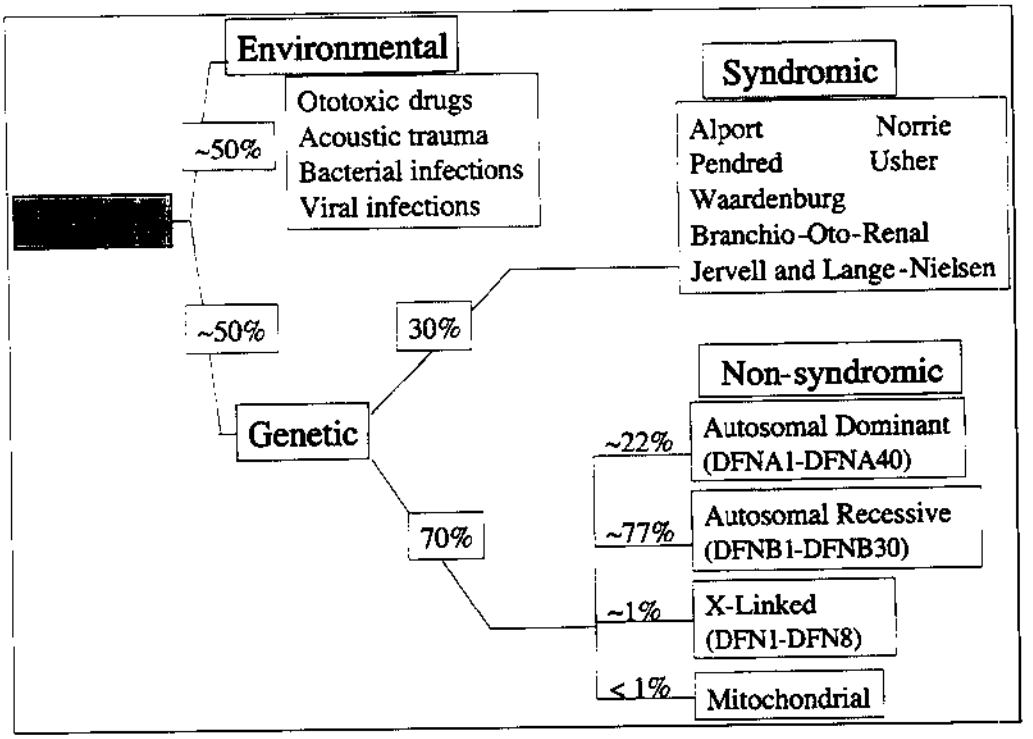

Fig 2.5 Classification of deafness based on cause.

Heritable Causes

Single-Gene Disorders

Syndromic hearing impairment is associated with malformations of the external ear or other organs or with medical problems involving other organ systems. **Nonsyndromic hearing impairment** has no associated visible abnormalities of the external ear, nor are there any related medical problems; however, it can be associated with abnormalities of the middle ear and/or inner ear.

Syndromic Hearing impairment

Syndromic hearing impairment may account for up to 30% of prelingual deafness, but its relative contribution to all deafness is much smaller, reflecting the occurrence and diagnosis of postlingual hearing loss.

I. **Autosomal Dominant Syndromic Hearing Impairment**

i. Stickler syndrome
i. Waardenburg syndrome
i. Branchiootorenal syndrome
v. Neurofibromatosis 2 (NF2)

I. **Autosomal Recessive Syndromic Hearing Impairment**

i. Usher syndrome
ii. Biotinidase deficiency
ii. Refsum disease

I. **X linked Syndromic Hearing Impairment**

i. Alport syndrome
ii. Mohr-Tranebjaerg syndrome (deafness-dystonia-optic atrophy syndrome)

V **Mitochondrial Sydromic hearing impairment**

i. MELAS
ii. MERRF
ii. NARP

Non Syndromic Hearing Impairment

More than 70% of hereditary hearing loss is nonsyndromic.Disorders discussed in this section are organized by mode of inheritance. The different **gene loci** for nonsyndromic deafness are designated DFN (for DeaFNess). Non syndromic hearing loss can be categorized into

i. Autosomal dominant hearing impairment
ii. Autosomal recessive hearing impairment
ii. Mitochondrial Non- syndromic hearing impairment.

mapping and/or discovery.

50% of persons with **autosomal recessive** nonsyndromic hearing loss have **mutations** in *GJB2* . The other 50% of cases are attributed to **mutations** in numerous other **genes**, many of which have been found to cause deafness in only one or two families . Extensive genotype-phenotype studies have shown that it is possible to predict the hearing loss associated with *GJB2* **mutations** based on the specific **genotype**. Family studies of **autosomal dominant** nonsyndromic hearing loss have shown that heterogeneity is high. Unlike **autosomal recessive** nonsyndromic hearing loss,(which is also extremely heterogeneous but in which the majority of cases are caused by **mutations** in a single **gene** in many world populations), a single **gene** responsible for the majority of cases of **autosomal dominant** nonsyndromic hearing loss has not been identified. In spite of this limitation, the audioprofile can be distinctive and useful in predicting candidate **genes** for **mutation screening**. For example, **mutations** in *WFS1* are found in 75% of families segregating **autosomal dominant** nonsyndromic hearing impairment that initially affects the low frequencies while sparing the high frequencies.

2.4 The Mitochondrial Structure and Function

The mitochondrion is an essential cytoplasmic cell organelle that provides most of the energy necessary for a cell , for which it is called as the POWER HOUSE OF THE CELLS. It is present in all eukaryotic cells, and typical human cell has several hundred mitochondria. There are 1000-2000 mitochondria in a single liver cell, occupying roughly a fifth of its total volume. It is widely accepted that the present mitochondrion is a remnant of a prokaryotic organism that had become a vital symbiotic partner to the eukaryotic cell early in evolution.

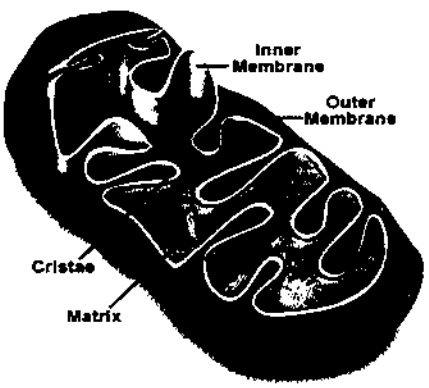

Fig 2.6 Structure of Mitochondria

composed of five multipolypeptide enzyme complexes and is located in the inner membrane, which surrounds the matrix space of the mitochondrion. The matrix contains mitochondrial DNA (mtDNA) molecules, ribosomes, tRNAs and various enzymes needed in protein synthesis, the oxidation of pyruvate and fatty acids and the citric acid cycle, for example. (Alberts *et al.*, 1994, Wallace *et al.*, 1997) there are about 1000 proteins in a mitochondrion, most of which are nuclear-encoded

The mitochondrion converts energy derived from chemical fuels by an oxidative phosphorylation process that is more efficient than anaerobic glycolysis. In mitochondrion the metabolism of one molecule of glucose produces about 30 molecules of ATP (adenosine triphosphate), while only two molecules of ATP are produced by glycolysis alone.The oxidative phosphorylation pathway (OXPHOS) is composed of ETC and ATPase. The main function of the system is the coordinated transport of electrons and protons and the production of ATP. This passage of electrons releases energy, which is largely stored in the form of a proton gradient across the inner mitochondrial membrane and is used by the last OXPHOS complex (F_1F_O-ATPase) to generate ATP from ADP and inorganic phosphate. Oxidative metabolism in the mitochondria is fuelled by pyruvate produced from carbohydrates by glycolysis and fatty acids produced from triglycerides. These are selectively imported into the matrix of the mitochondria and broken down into acetyl CoA by the pyruvate dehydrogenase complex or the β -oxidation pathway. The acetyl group then participates in the citric acid cycle, which produces molecules of NADH and FADH. Electrons generated from NADH are passed along a series of carrier molecules called the electron transport chain (ETC), the products of this process being H_2O and energy.

2.5 Mitochondrial DNA (mtDNA)

The human mtDNA is a 16,569 nucleotide pair (np) closed, circular molecule located within the cytoplasmic mitochondria. Each of the several thousand mtDNAs per cell encodes a control region encompassing a replication origin and the promoters, a large (16S) and small (12S) rRNA, 22 tRNAs and 13 polypeptides. All of the mtDNA polypeptides are components of the mitochondrial energy generating pathway, oxidative phosphorylation (OXPHOS), which is functionally essential.

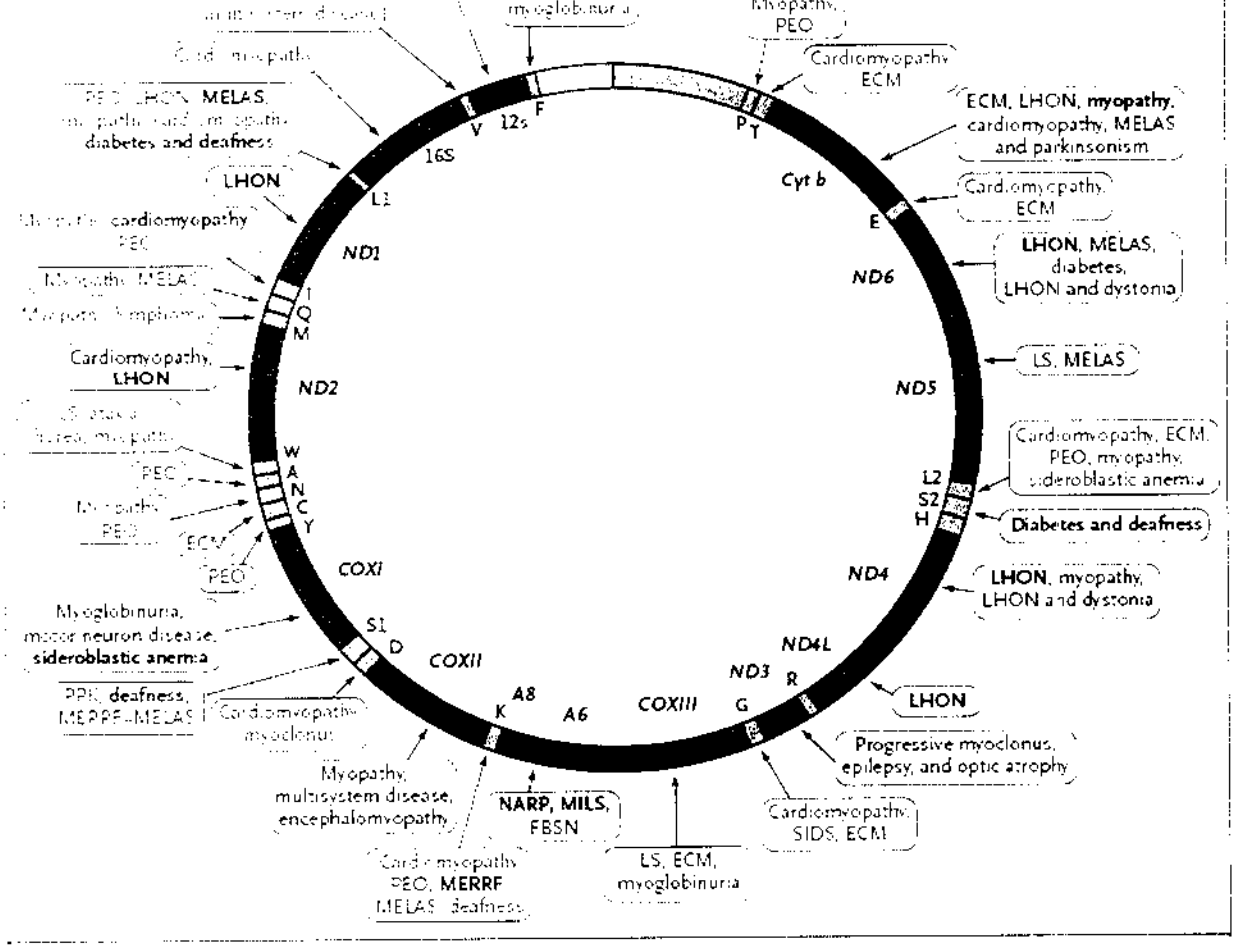

Fig 2.7 Structure of Mitochondrial DNA

i) The protein-coding genes **(shown in red)**

Seven subunits of complex I (ND):

- three subunits of cytochrome *c* oxidase (*COX*),
- the cytochrome *b* subunit of complex III (*Cyt b*), and
- two subunits of adenosine triphosphate (ATP) synthase (*A6* and *A8*)

ii) The protein-synthesis genes **(shown in blue)**

The 12S and 16S ribosomal RNAs and the 22 transfer RNAs (three letter amino acid symbols)

iii) The D -loop region (shown in green) controls the initiation of replication and,

iv) Transcription of mtDNA.

Nuclear DNA encodes protein subunits of oxidative phosphorylation and the myriad macromolecular compounds required for mitochondrial structure and function.

- Mitochondrial DNA mutates more than 10 times as frequently as nuclear DNA and has no introns, so that a random mutation will usually strike a coding DNA sequence.

- Mitochondrial DNA has neither protective histones nor an effective repair system, and it is exposed to oxygen free radicals generated by oxidative phosphorylation.

- Mitochondrial DNA is inherited maternally and does not recombine; mutations thus accumulate sequentially through maternal lineages. Each mitochondrion contains 2 to 10 DNA molecules, and each cell contains multiple mitochondria. Thus, normal and mutant mitochondrial DNA can coexist within the same cell.

- The proportion of mutant mitochondrial DNA required for the occurrence of a deleterious phenotype, known as the threshold effect, varies among persons, among organ systems, and within a given tissue. The threshold effect depends on the delicate balance between oxidative supply and demand.

2.5.1 Maternal Inheritance of the mtDNA

The inheritance of mtDNA is from mother to child, but never from father to child. Men and women are equally affected by mitochondrial mutations, but only women can pass it on. A mother carrying an mtDNA mutation passes it on to all her children, but only her daughters will transmit it to their progeny. Recent evidence of paternal transmission of mtDNA in skeletal muscle (but not in other tissues) in a patient with a mitochondrial myopathy serves as an important warning that maternal inheritance of mtDNA is not an absolute rule, but it does not negate the primacy of maternal inheritance in mtDNA-related diseases.

2.5.2 Heteroplasmy and the Threshold Effect

There are thousands of mtDNA molecules in each cell, and in general, pathogenic mutations of mtDNA are present in some but not all of these genomes. As a result, cells and tissues harbor both normal (wild type) and mutant mtDNA, a situation known as heteroplasmy. Heteroplasmy can also exist at the organellar level: a single mitochondrion can harbour both normal and mutant mtDNAs. In normal subjects, all mtDNAs are identical (homoplasmy).Not surprisingly, a minimal number of mutant mtDNAs must be present before oxidative dysfunction occurs and

to the effects of pathogenic mutations in mtDNA.

2.5.3 Mitotic Segregation

The random redistribution of organelles at the time of cell division can change the proportion of mutant mtDNAs received by daughter cells; if and when the pathogenic threshold in a previously unaffected tissue is surpassed, the phenotype can also change.

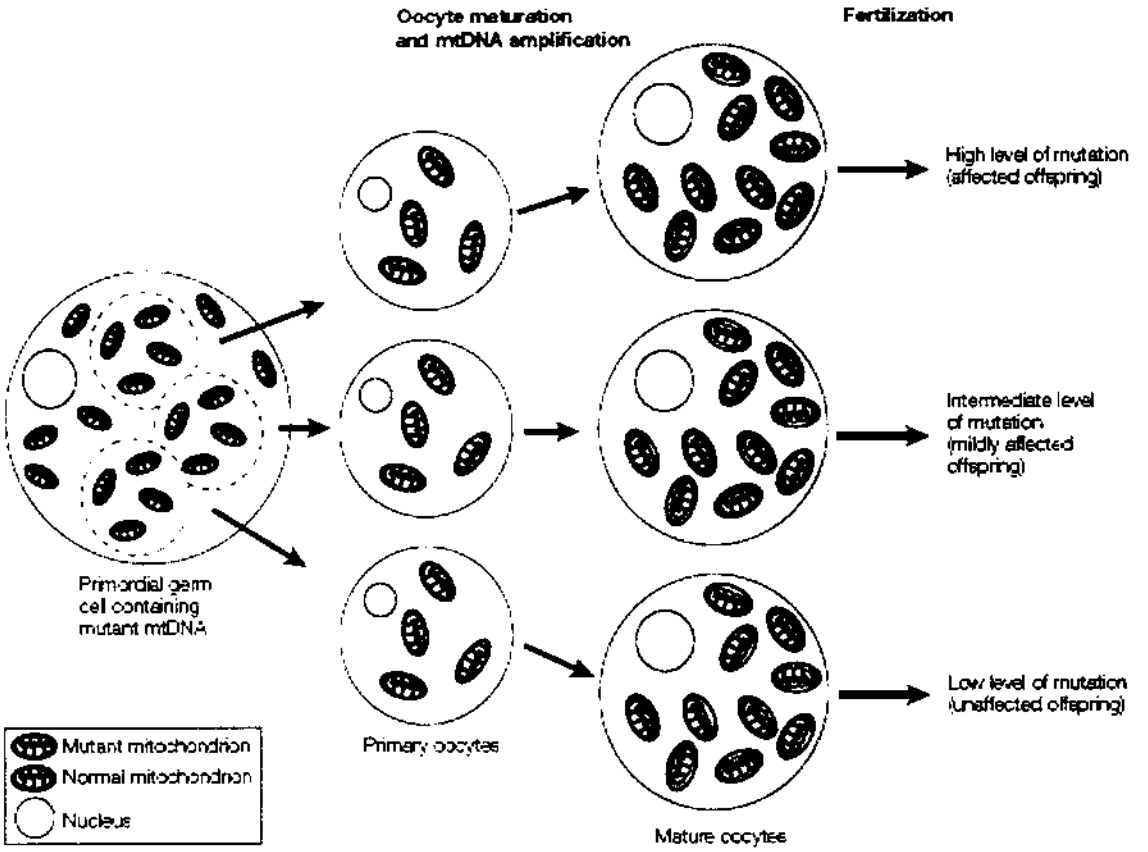

Fig 2.8 Mitotic segregation during formation of primary oocytes

A selected number of mtDNA molecules are transferred into each oocyte. Oocyte maturation is associated with the rapid replication of this mtDNA population. This restriction-amplification event can lead to a random shift of mtDNA mutational load between generations and is responsible for the variable levels of mutated mtDNA observed in affected offspring from mothers with pathogenic mtDNA mutations.

The maintenance of normal hearing mechanism is highly dependent on the ATP produced by mitochondrial oxidative phosphorylation. The cells most likely involved by a mitochondrial defect are the sensory hair cells and those of the stria vascularis in the cochlea. It is still unclear how mtDNA mutations induce hearing loss. Deficiencies in mitochondrial OXPHOS appear to be the main pathogenic factors, although the reactive oxygen species generation and altered apoptotic signaling may also play a role.

One possibility could be the heavy dependence of the energy metabolism of the organ of Corti and the stria vascularis on mitochondrial OXPHOS. The progressive accumulation of the mutant mtDNA with age causes a decline in the OXPHOS capacity. Energy-dependent ATPase and the release of neurotransmitters in the cochlea are then suppressed by reduced ATP production

Another possibility is a disturbance in ion transport, leading to a reduction in the efficiency of acoustic transduction. As we know, the stria vascularis is the most metabolically active site in the cochlea, and its primary function is to maintain the ionic environment of hair cells. This requires ATP-dependent pumps to secrete potassium ions back into the endolymph against an ionic gradient. The declined production of ATP owing to mitochondrial dysfunction may slow down these pumps, which in turn leads to an imbalance of ionic environment in the inner ear, and a pronounced reduction of the capacity for the cochlea to detect and transmit sound waves. Also, the reduced ATP production would activate both nonselective cation channels and ISK channels in the strial marginal cells and inactivate the Ca2+-ATPase in the outer hair cells. (Guangqian Xing *et al.*, 2007)

The mechanism of hair cell death in non-hypersensitive individuals was proposed where the hair cell death is ultimately the result of inhibition or mistranslation at the level of mitochondrial protein synthesis. All mitochondrial proteins are involved in ATP production, an interference of mitochondrial protein synthesis would likely lead to a fall in ATP production. Ionic gradients within the ear are maintained by ATP-driven pumps; therefore a reduction in ATP levels in the cochlea might result in an imbalance in ionic concentrations in the stria vascularis, endolymph or the hair cells themselves. Eventually, intracellular accumulation of toxic levels of ions such as Ca2+ might lead to hair cell death by a mechanism such as excitotoxicity. (Hutchin *et al.*, 1993)

NONSYNDROMIC HEARING IMPAIRMENT

Gene	Mutation	Remark/ Additional Symptom	References
12S rRNA	A1555G	Aminoglycoside induced/worsened	Prezant *et al.*., 1993 Usami *et al.*, 2000 Estivill *et al.*, 1998
12S rRNA	C1494T	Aminoglycoside induced/worsened	Zhao *et al.*, 2004
12S rRNA	(different mutations)	Aminoglycoside induced/worsened	Bacino *et al.*, 1995, Casono *et al.*, 1998
tRNASer(UCN)	A7445G	Palmoplantar keratoderma	Reid *et al.*.,1994 Sevior *et al.*, 1998
tRNASer(UCN)	7472insC	Neurological dysfunction, including ataxia, dysarthria and myoclonus	Tiranti *et al.*,1995 Jaksch *et al.*,1998 Schuelke *et al.*,1998 Verhoeven *et al.*, 1999
tRNASer(UCN)	T7510C	no additional symptoms	Hutchin *et al.* (2000)
tRNASer(UCN)	T7511C	no additional symptoms reported	Sue *et al.*, 1999

Problems invclved in determining the pathogenicity of any mitochondrial-DNA mutation.

It can be difficult to link a mutation to a clinical disease for several reasons: mitochondrial DNA is highly polymorphic, there is dissociation between the genotype and the phenotype, different mutations can be associated with the same phenotype, the same mutation can be associated with different phenotypes, and epigenetic factors can affect clinical manifestations.(Donald, 1995).

The most disappointing area has been the lack of treatment for patients with mtDNA disease; several new experimental approaches are currently under investigation. It is crucial that further work and ideas are forthcoming to treat or prevent the transmission of mtDNA disease to the future generations.

2.7 A1555G Mutation

2.7.1 A1555G Mutation and Hearing Impairment

Mutations in mtDNA have been shown to cause syndromic, nonsyndromic or antibiotic-induced deafness that is maternally inherited. (Fischel-Ghodsian.N, 1999). In countries where this particular class of antibiotics are, or have in the past been very widely used, there would be a much greater occurrence of severe hearing loss associated with the combination of the mutation and the drug.

The A1555G mutation in the 12S rRNA gene is a relatively common mtDNA mutation that is associated with antibiotic-induced and non-syndromic deafness. While most of the mtDNA mutations are associated with a variety of multisystem disorders, many of which include sensorineural deafness as a symptom, the A1555G 12SrRNA mutation causes a tissue specific disorder with no general myopathy or neurological symptom.

Usually, the A1555G mutation occurs in homoplasmy, but in some families the heteroplasmic state was identified. In the absence of aminoglycosides, the A1555G mutation produces a variable clinical phenotype among family members. Also, the penetrance differs between families for this mutation. These findings indicate that the A1555G mutation itself is not sufficient to produce a clinical phenotype but requires the involvement of modifier factors for the phenotypic expression.

As environmental factors play an important part in the development of deafness, it is conceivable that, by interacting with the mutated 12S rRNA or a ribosomal protein binding to the mutation site, the product(s) of a putative nuclear gene(s) could enhance the effect of mutation so as to produce the clinical phenotype, or suppress it so as to make the hearing normal.

The first homoplasmic mutation associated with nonsyndromic deafness was identified in an Arab Israeli pedigree, when the striking pattern of transmission only through mothers was noted (Jaber *et al.*, 1992). The very significant decrease in the rate of mitochondrial protein synthesis observed in their study in transmitochondrial cell lines derived from members of the Arab-Israeli family carrying the A1555G mutation, whether symptomatic or asymptomatic, compared with the average rate measured in transformant cell lines lacking the mutation, has unambiguously shown that the A1555G mutation is the primary factor responsible for the protein synthesis defect observed in original lymphoblastoid cell lines and the resulting substantial reduction in rate of assembly of functional respiratory complex. Cell lines derived from asymptomatic individuals exhibited a lower degree of mitocohndrial dysfunction than those

2.7.2 Ototoxicity Induced by Aminoglycosides

Aminoglycoside antibiotics, such as gentamycin, streptomycin, kanamycin, and tobramycin, are drugs widely used for controlling bacteria-related infections, especially in developing countries. They are known to exert antibacterial effects by directly binding to the 16S rRNA of the bacterial ribosome, causing insufficient protein synthesis. It is evidenced that the A-site of the small ribosomal RNA is the primary target site for aminoglycoside antibiotics. However, when administrated with high doses or for a long period, these drugs may become concentrated in fluids of the cochlea and potentially lead to ototoxicity *(Fischel-Ghodsian., 2005).*

As the human mitochondrial ribosomes share many similarities to bacterial ribosomes, it is proposed that one of the primary targets for the aminoglycoside antibiotics in human cells is 12S rRNA of mitochondrial ribosome *(Guan., 2004).* By now, several mutations including A1555G, C1494T, T1095C, A827G, and 961 mutation in the mitochondrial 12S rRNA gene have been found to be associated with nonsyndromic hearing loss.

In familial cases of toxic deafness, the aminoglycoside hypersensitivity is often maternally transmitted, suggesting the occurrence of a mutation(s) in mitochondrial DNA (mtDNA). Recently, a homoplasmic A→G transition at position 1555 of mtDNA, in a highly conserved region of the 12S rRNA gene, has been found in a number of pedigrees and sporadic patients with aminoglycoside-induced deafness. In the absence of exposure to aminoglycosides, the A1555G mutation has also been observed in many families with maternally inherited non-syndromic deafness. In many families, the onset appears to occur in early adult life. No abnormalities in any other organ, including the vestibular system, have been observed. (Min-Xin Guan *et al.*, 2000)

2.7.3 Aminoglycoside Antibiotics Interfere with Protein Synthesis

Aminoglycoside antibiotics exert their antibacterial effects at the level of the prokaryotic ribosome, inducing errors in protein synthesis. The basis for the selective bactericidal effects of the aminoglycosides is presumably their preferential binding to the bacterial ribosome. Since mitochondrial ribosomes are structurally more similar to their prokaryotic ancestors than either ribosome is to the eukaryotic ribosome, aminoglycosides might be expected to interfere with mitochondrial protein synthesis, which could be the basis of their ototoxicity *(Tim Hutchin and Gino Cortopassi., 1994).*

seen from their secondary structures that position 1555 lies in a highly conserved region, the penultimate stem, which is involved in aminoglycoside binding to the bacterial ribosome. Mutations in this region alter the ribosome's susceptibility to aminoglycosides and have been shown to confer resistance to aminoglycosides in bacteria and the mitochondria of yeast. A guanine at position 1555 would be expected to form a base pair with the cytosine at position 1494 on the opposite strand of the penultimate stem. Molecular dynamics simulation was used to see what effect this extra base-pairing might have on the structure of the ribosome. It was found that the additional pairing resulting from 1555G shrank the volume of RNA at this site This reduction in volume might leave more space for aminoglycosides to enter or bind to the mitochondrial ribosome, thus resulting in increased levels of binding of aminoglycoside to the ribosome. Further work will need to be done to provide direct evidence of enhanced binding of one or all aminoglycosides to such mitochondrial ribosomes.

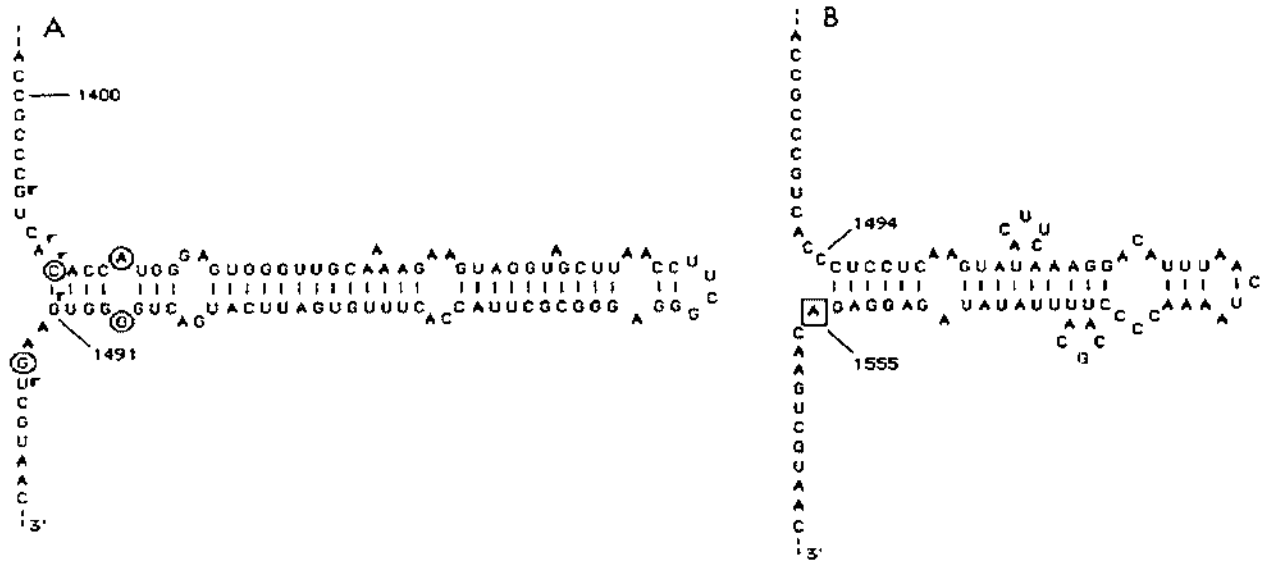

Fig 2.9 A1555G Mutation in the small rRNA

The figure shows the localization of 1555G mutation to aminoglycoside-binding sites in small rRNA. The secondary structures of the 3' ends of the Escherichia coli 16S rRNA (A) and human mitochondrial 12S rRNA (B) are shown. Bases protected by aminoglycosides are circled, bases conferring aminoglycoside resistance as a result of either mutation or methylation are marked r. Position 1555 on the mitochondrial 12S rRNA (boxed) is shown here as it exists in the wild type, i.e., an A residue, which when mutated to a G residue would be expected to pair to the C residue at position 1494.

cell death in individuals who carry the A1555G mutation. The cause of hair cell death in normal individuals who receive large doses of aminoglycosides may be the same. The aminoglycoside streptomycin is known to work by inducing the mistranslation of mRNAs at the ribosome, which has an effect similar to that of missense mutations in the DNA.

The mechanism that could bring about the mistranslation of mitochondrial mRNAs is the aminoglycoside treatment leading to a deficiency of mitochondrial complex I. Genes encoding proteins of mitochondrial complex I make up 56% of the protein-coding region of the mitochondrial DNA (mtDNA). Thus, mistranslation is more likely to affect complex I than any other mitochondrial protein. It has been shown by others in vitro that drugs which induce complex I deficiency are lethal to cells and induce the production of mitochondrial superoxide. It has been found that the lethality of a complex I inhibitor is suppressed by the mitochondrial superoxide dismutase (MnSOD). Thus, one possible result of aminoglycoside treatment is the mistranslation of complex I-encoding genes, leading to excess mitochondrial superoxide production and oxidative damage to mitochondria, which may trigger cell death. A prediction of this model is that animals which over express MnSOD should be resistant to aminoglycoside-induced hair cell death. (Tim Hutchin *et al.*, 1993)

2.7.6 Penetrance and Tissue Specificity

Study of the mitochondrial mutations has identified three factors that could modulate phenotypic expression. These factors all affect the level of oxidative-phosphorylation capacity in the cell. The first such factor is environmental agents, of which administration of aminoglycosides, as a triggering event in the case of the A1555G mutation, is the prime example. It is likely that other as-yet-unrecognized environmental factors could play similar, but perhaps less dramatic, roles. Both diet and drugs that affect oxygen-radical formation and breakdown should be taken into account. The second factor is the rest of the mitochondrial genome; as noted above, the A7445G mutation provides a dramatic example of that effect. The third factor is nuclear genes, of which the Arab Israeli pedigree is a good example. The entire family lives in the similar environmental surroundings of a small Arab village in Israel, and all maternal relatives share the same mitochondrial haplotype. for this hypothesis (Guan *et al.*, 1996). A genome wide search led to the conclusion that this effect is due to several or many The difference between family members with normal hearing and those with congenital profound hearing loss has been postulated to be due to nuclear genes, and biochemical differences between lymphoblastoid cell lines of hearing and of deaf family members provide support genetic loci

differences on either side of the threshold. (Nathan Fischel-Ghodsian, 1998)

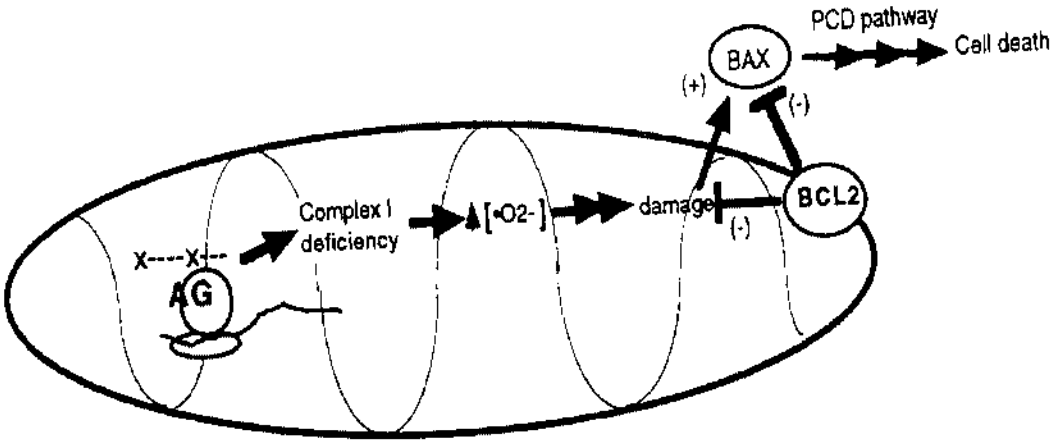

Fig 2.10 Superoxide induced hair cell death

Hypothetical model of mitochondrial superoxide-induced hair cell death. x----x---, complex I transcript misread by mitochondrial ribosomes in the presence of aminoglycoside (AG); 02-, superoxide anion; BCL2, B-cell lymphoma/leukemia locus-2 protein; BAX, B-cell accelerated cell death protein (20); PCD, programmed cell death.

Two different mechanisms where proposed in which the tissue specificity in these as well as in some heteroplasmic mitochondrial disorders can be explained.

First, it is possible that tissue-specific subunits of mitochondrial ribosomes or oxidative-phosphorylation complexes interact specifically with the mitochondrial defect only in tissues in which they are expressed, leading to insufficient oxidative phosphorylation. Tissue-specific subunits for general cellular processes, including oxidative phosphorylation, have been described .

Second, it cannot be excluded that human mitochondrial genes have functions in addition to their functions in oxidative phosphorylation. In this model the mitochondrial mutation would interfere with a tissue-specific secondary function of the mitochondrial gene, which also has to make the cell more sensitive to changes in oxidative- phosphorylation capacity. Precedent for part of this hypothesis can be found in studies of mice and of *Drosophila melanogaster* (Wong *et al.*. 1991).

Table 2.3 . PREVALENCE OF A1555G IN DIFFERENT ETHNIC GROUPS

OUNTRY(S)	AUTHORS	YEAR	SAMPLE SIZE	MUTATION STATE	MUTANT (%) FREQUENCY
rab–Israeli	Prezant *et al.*	1993	4 families	Homoplasmy	
pain and Cuba	Torroni *et al.*	1999	50-spain families 4-cuban families		
apan	Usami *et al.*	2000	319 unrelated HI 21 SNHL+AG 22 AG induced HI	Heteroplasmy	3.45 unrelated HI 33.3 AG exposed 59 AG induced HI
hina	Li *et al.*	2005	128 AG induced,NSHL	Homoplasmy	13- AG induced 2.9- NSHL
aucasia	R.Li *et al.*	2004	164 Sporadic	Homoplasmy	0.6
enmark	Ostergaard *et al.*	2002	85 sporadic	-	2.4
apan	Usami *et al.*	1997	5 families (32)	Homoplasmy	87.5
angolia	Pandya *et al.*	1997	480 Sporadic	Heteroplasmy	7.708
ew Zealand	Scrimshaw *et al.*	1999	206 Sporadic	Homoplasmy	0.48
urkey	Tekin *et al.*	2003	168 patients(families)	-	1.8
K, Italy, inland	Jacobs *et al.*	2005	80- UK (Postlingual) 128-Ity (Postlingual) 227-Fin (ARHI)	-	2/80 2/128 0/227
ermany, ungary,Poland	Kupka *et al.*	2002	139-German (Sporadic) 56-Hungary (Sporadic) 125-Polish (Sporadic)	-	0.7-Germany <1.8- Hungary 2.4- Polish

Mitochondrial DNA mutation at position 7445 involves the A-G transition in the ($tRNA^{ser(UCN)}$) gene. As a result,the rate of processing of the $tRNA^{Ser(UCN)}$ from its precursor is affected by the mutation at position 7445.Although some family members carried very low levels of wild-type mtDNA . there was no correlation between the level of heteroplasy and hearing loss.The mutation is homoplasmic,affecting almost all maternally related individuals,which is typically of childhood onset. progressive and affects the higher frequencies.

Reid *et al* (1994) reported a novel mitochondrial point mutation in a maternal pedigree with sensorineural deafness.The deafness was progressive,postlingual and involved high frequencies..The mutation was a T to C transition at nucleotide 7445 of the MTTS1 gene.Most researchers report mtDNA mutations by the standard sequence of Anderson *et al* (1981) , which is the sense strand for the majority of the open reading frames. Using this convention,the mutation in the MTTS1 gene described by Reid *et al* (1994) should be given as A7445G instead of the complementary T7445C. The pedigree studied by Reid *et al* (1994)was of Scottish maternal origin.

Fischel-Ghodsian *et al* (1995) suggested the high penetrance of hearing loss in one family was due to the presence of other, secondary mtDNA mutations, including T7416C and G13708A ,which have previously been reported as secondary mutations in Leber's hereditary optic Neuropathy.However ,these secondary mutations were not present in the other families.Members of Scottish,New Zealand,Japenese families presented with tne skin condition palmoplanar keratoderma(PPK).The coexistence of PPK and hearing loss may provide an understanding to the mechanism of mitochondrial defects in hearing.

Sevior *et al* (1998) on clinical examination of the New Zealand family found that many relatives had palmoplanar keratoderma in addition to deafness.Review of this literature demonstrated three other large families with presumed autosomal dominant inheritance of palmoplanar keratoderma and hearing loss.One of these families, a Japanese pedigree reported by Hatamochi *et al* (1982) in which five members had deafness and palmoplanar keratoderma, had only maternal transmission.Analysis by Sevior *et al* (1998)documented the same A7445G mitochondrial mutation that had previously been identified in the New Zealand and Scottish pedigree.The mitochondrial sequence variants reported in the NewZealand and Scottish pedigrees were absent from the Japanese pedigree,which suggested that the A7445G mutation

Sevior *et al* (1998) noted that all affected persons in the Turkish family described by Bititci(1975), in which 10 of 11 persons in 5 generations with progressive perceptive hearing loss also had palmoplanar keratoderma,were maternally related. The A7445G mutation changes the stop codon AGA of the heavy strand (H-strand) encoded mRNA for subunit COI of cytochrome c oxidase to an equivalent AGG stop codon, and at the same time, changes a U to C transition in the light strand (L-strand) encoded tRNA $^{ser(UCN)}$ precursor.

Hutchin *et al* (2001) described a 3 generation family from Ukraine with nonsyndromic sensorineural progressive deafness due to the homoplasmic mtDNA A7445G mutation.The family members showed no signs of palmoplanar keratoderma .The authors stated that all 4 reported families with this mutation have been of different ethnic backgrounds,suggesting that the mutation arose on 4 independent genetic backgrounds.

Li *et al* (2005) identified a A7445G transition in the MTTS1 gene in 7 of 1542 Han Chinese individuals with aminoglycoside ototoxicity or nonsyndromic sensorineural hearing loss.All 7 probands had been administered aminoglycosides between 1 to 3 years of age and began suffering hearing loss within 3 months.Two of the probands had both 7444 G-A and a mutation in the MTRNR1 gene (A1555G).Family history suggested very low penetrance for the A7445G mutation alone.In contrast,there were several members of the 2 families with both A7444G and A1555G who had sensorineural hearing loss without aminoglycoside exposure,indicating a higher penetrance of hearing loss in those with the two mutations.

2.9 T7510C and T7511C Mutation

T7510C base change disrupts a hydrogen bond in the acceptor stem of the tRNA$^{ser\ (UCN)}$, which may affect tRNA levels or function that results in insufficient mitochondrial protein synthesis and respiration defects. The 7510 residue is highly conserved in a wide range of species forming an A-U base pair in all except bovine.

Hutchin *et al.*, (2000) described a white family with nonsyndromic sensorineural hearing impairment transmitted in a manner consistent with maternal inheritance. The proband was formally diagnosed as having sensorineural hearing impairment aged 15 months.

illness in the family. DNA was extracted from the proband, his sister, and both parents. Analysis by PCR and restriction enzyme digestion showed the absence of the A1555G, A7445G, T7511C, and 7472insC mutations. Further analysis, however, showed the gain of a *Hinf*I site around base pair (bp) 7510 in the $tRNA^{Ser(UCN)}$ gene of the proband, his sister, and mother. Sequencing showed this to be a T to C transition at bp 7510. The T7510C mutation was heteroplasmic in all three affected family members tested, that is, >95% mutant in the two sibs and 90% in the mother. The following points provide further support for the T7510C mutation as the most probable cause of the hearing impairment in this family. (1) The *Hinf*I site gain at bp 7510 is extremely rare. This was not found in 141 white controls, nor was it reported in 520 other controls in published reports. The mechanism behind the mutation is base change disruption a hydrogen bond in the acceptor stem of the $tRNA^{Ser(UCN)}$, which may affect tRNA levels or function. Similar mutations in this acceptor stem at bp 7511 and 7512 have previously been shown to cause hearing loss.

Hutchin *et al.*, (2001) analyzed total of 202 subjects with non syndromic SNHL. They all had sensorineural hearing impairment which was congenital childhood onset. One subject had a history of aminoglycoside antibiotic exposure. There was no family history of hearing impairment in 110 of the subjects (that is, sporadic) and 75 had one or more affected sibs but no other family history of hearing impairment. The remaining 17 subjects all had a family history of hearing impairment in subjects from at least two generations, with 10 families showing a pattern of transmission only through the maternal lineage. DNA was screened for mtDNA mutations using Restriction Fragment Length Polymorphism analysis (RFLP) for each of the following mutations associated with non-syndromal hearing impairment: A1555G, A3243G, A7445G, 7472insC, T7510C, T7511C, and T7512C.

Three subjects with a family history of hearing impairment were found to carry mtDNA mutations A7445G, T7510C, and A3243G. In all the cases the hearing impairment was inherited only through the maternal lineage, that is, 30% of such families. Typical of mitochondrial hearing impairment, the age of onset and severity of hearing impairment varied widely within each family, though it was always sensorineural and progressive. The proband with the T7510C mutation had a profound hearing impairment from 15 months of age. His sister had a severe, progressive hearing impairment from the age of 5 years, whereas their mother had only a slight hearing loss. In all three subjects, the mutation was heteroplasmic and present at similar levels (about 95%).

the pathogenic mutation (Hutchin *et al.*,2000). The same change was also identified in a Spanish pedigree (Castillo *et al.*, 2002). This mutation is predicted to disrupt base pairing in the acceptor stem of the tRNA.

Castillo *et al.*, (2002) analyzed 148 unrelated Spanish families with non-syndromic sensorineural hearing loss. In all of these families, the pattern of inheritance of the hearing loss was consistent with maternal transmission. At least one patient from each of these 148 families was tested for the presence of the mitochondrial A1555G mutation, the result being positive in 66 families. In the remaining 82 cases, detection of the T1095TC and T7510C mutations was performed by tests that are based on PCR amplification of a DNA fragment containing the mutation, followed by digestion with a specific restriction endonuclease (Hutchin *et al.*, 2000). Mutation T1095C was not found in any of these 82 families, but mutation T7510C was detected that was also confirmed by DNA sequencing.

Subsequently, the presence of the mutation was shown in a total of 26 subjects from this family. In all of them, the mutation was homoplasmic, considering detection limits (>95% mutant copies). No additional mutation was found in the $tRNA^{Ser(UCN)}$ gene in these patients. They also investigated the A4336G mutation in the tRNA gene, since it had been described in the only pedigree previously reported with the T7510C mutation [Hutchin *et al.*, 2000]. No patient in this family S258 carried the A4336G mutation.

The finding of the T7510C mutation in a second Spanish family with maternally inherited hearing impairment lends further support to its pathogenic role. The comparison of the clinical data of affected subjects from the two families so far reported shows wide phenotypic variation, both intrafamilial and interfamilial, concerning age of onset, progression, symmetry, severity, and shape of the audiogram. This suggests that other factors, environmental and/or genetic, contribute to modulate the phenotype of hearing loss. It is intriguing that several females in the Spanish family associated pregnancy with an increase in their hearing loss, which suggests that the complex hormonal changes that take place during this period may play a role in accelerating the progression of the auditory impairment. On the other hand, the hypothesis of the existence of nuclear genes acting as modifiers of mitochondrial hearing impairment has recently received strong support from different experimental approaches *(Li et al, 2005).*

Hospital Medical Center, participated in the investigation conducted by Greinwald *et al.*, (2004). The 226 controls DNA used for screening for the presence of mtDNA mutations were obtained from a panel of unaffected individuals from comparable ethnic backgrounds. All DNA fragments spanning the entire mitochondrial 12S rRNA gene or $tRNA^{Ser(UCN)}$ gene were amplified by PCR and sequenced corresponding to the mitochondrial genome at positions 618–635 and 1988–2007, 9 and 7151–7170 and 8504–8623.

In this study, they performed a systematic and extended mutational screening of the mitochondrial 12S rRNA and $tRNA^{Ser(UCN)}$ genes in the clinical population of CHDR at the Cincinnati Hospital Medical Center. They failed to detect the presence of the A7445G, T7510C, 7472insC, T7511C, or T7512C mutations in the $tRNA^{Ser(UCN)}$ gene in 164 affected and 226 control subjects. However, the C7476T variant in the $tRNA^{Ser(UCN)}$ gene, which was previously described in a control population, was found in two affected and two control subjects. These data suggest that the deafness-associated mutations in the $tRNA^{Ser(UCN)}$ gene are not common in this Caucasian population (Greinwald *et al.*, 2004).

MATERIALS AND METHODS

This work studies two subsets of hearing impaired which is available as the Deafness genetic resource.belonging to an ongoing research programme at the Department of genetics.PGIBMS.University of Madras.

3.1 Subgroup 1

1.19 samples have been included in this study, who belong to a multigenerational family with a history of progressive postlingal deafness expressed across nearly four generations.

2.This family belongs to a caste known as Tulava Vellalar. Blood has been collected from 19 individuals belonging to these five clusters after informed consent.

3.A pedigree has been drawn using several elders from the second generation as informants

4.According to this information 21 members have postlingual and progressive deafness with variable age at onset.Some have mild phonological and severe speech articulation defects.

5.The transmission pattern appears to be matrilineal spread across nearly four generations.

6.Among the 19 subjects included in this study 12 are affected with hearing loss of variable severity ,age at onset and progression,8 of them are females and four are males.Seven of the individuals reported normal hearing.

7 Normal hearing + 8 Affected females + 4 Affected males= 19 Subjects included in the study

This large extended family has been discussed into five clusters named as TRIP1,TRIP2,TRIP3,TRIP4,TRIP5 (Appendix 1– Pedigrees) who all share a set of common grand parents.

vere analyzed . They all had 'Sensory Neural Hearing impairment'.

2.The history of aminoglycoside antibiotic exposure could not be established definitely as the cause for their deafness, through the interview made during the collection of samples.

3.This sub group has an important feature which makes them unique .All 15 of them had 1 or more siblings affected with Hearing loss but no other family history of Hearing Impairment.

4.The probands hail from Salem,Chennai ,Erode and Madurai districts.

5.Pedigrees were drawn (Appendix 2) after interviewing them.Most of the time , parents of the proband and the elders of the family have been the informants. All the subjects were younger than 27 yrs.

3.3 DNA EXTRACTION FROM 10 ml BLOOD BY AMMONIUM ACETATE METHOD*

Reagents

1) WBC lysis buffer: 200 ml
   40 ml of 1M Tris, pH 8.5
   40 ml of 0.5M EDTA, pH 8
   20ml of 10% SDS
2) RBC lysis buffer: 200ml
   140 mg of ammonium bicarbonate
   14 g of ammonium chloride
   Dissolve in 1000ml of water. Make it up to 2 liters and store at 4° C.
3) 5M ammonium acetate (192.7g/500ml)

Procedure

- 3ml RBC lysis buffer was taken in 15ml centrifuge tubes.
- Buffy coat (WBC) was taken from blood, mixed well and made up to 14ml.
- Then incubated in ice for 30 min, mixed well and centrifuged at 3500 rpm (10 min).

  * developed by Molecular Otolaryngology laboratories, University of Iowa, USA.
- The supernatant was discarded and the white pellet in the tube was broken.
- 3ml of WBC lysis and 2.5ml of 5M ammonium acetate was added.

- The DNA sample stored in 70% ethanol was vortexed and was spun at 5000rpm for 7 min.
- The supernatant was discarded and 1ml of 70% ethanol was added,vortexed and spun at 1100rpm.
- The supernatant was discarded and the pellet was air dried for 1 to 2 and 1/2 hours.
- 750ml of 1X buffer (pH8) was added to the dry pellet.
- The dissolved DNA (5µl) was diluted in 95µl 1X TE buffer and used.

ble 3.1. PRIMERS AND RESTRICTION ENZYMES USED FOR THE MUTATION STUDIES

Mutations	Gene Amplified	Primer sequence	Amplicon size	Restriction enzyme used	Restriction site status in the mutant
A1555G	12SrRNA	**Forward:** 5'-AGA AAT GGG CTA CAT TTT CTA CCC-3' **Reverse:** 5'-GTT CGT CCA AGT GCA CTT TCC A-3'	1354-1601 bp	BsmA1	Abolishes the restriction site single 248bp band
A7445G	tRNAser(UCN)	**Forward:** 5'-GGA TGC CCC CCA CCC TAC C-3' **Reverse:** 5'-CCT ACT TGC GCT GCA TGT GCC-3'	7392-7608 bp	Xba 1	Abolishes the restriction site single 248bp band
T7510C	tRNAser(UCN)	**Forward:** 5'-GGA TGC CCC CCA CCC TAC C-3' **Reverse:** 5'-CCT ACT TGC GCT GCA TGT GCC-3'	7392-7608 bp	Hinf I	Creates the restriction site triple102+48+67bp bands
T7511C	tRNAser(UCN)	**Forward:** 5'-GGA TGC CCC CCA CCC TAC C-3' **Reverse:** 5'-CCT ACT TGC GCT GCA TGT GCC-3'	7392-7608 bp	Mbo II	Creates the restriction site triple106+71+40bp bands

PrimerReference: Kupka *et al.*,Human mutation.2002, Hutchin *et al.*, J.Med.Genet.2002

The primers used in the reaction, amplicon length and the number of cycles involved are given in Table below.

Table 3.2 PRIMERS USED IN PCR REACTION

Primer Type	Primer Sequence	Amplification Length	PCR Cycles
Forward	5'-AGA AAT GGG CTA CAT TTT CTA CCC-3'	248bp	30
Reverse	5'-GTT CGT CCA AGT GCA CTT TCC A-3'		

SEQUENCE OF 12SrRNA OF mtDNA

■ - Sequence of 12SrRNA ■ - Sequence of Primer ■ - Mutated bp

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                                                    648-650          660
                                                      AAT AGGTTTGGTC
                                                      TTA TCCAAACCAG
670             680             690             700             710             720
  CTAGCCTTTC TATTAGCTCT TAGTAAGATT ACACATGCAA GCATCCCCGT TCCAGTGAGT
  GATCGGAAAG ATAATCGAGA ATCATTCTAA TGTGTACGTT CGTAGGGGCA AGGTCACTCA
730             740             750             760             770             780
  TCACCCTCTA AATCACCACG ATCAAAAGGA ACAAGCATCA AGCACGCAGC AATGCAGCTC
  AGTGGGAGAT TTAGTGGTGC TAGTTTTCCT TGTTCGTAGT TCGTGCGTCG TTACGTCGAG
790             800             810             820             830             840
  AAAACGCTTA GCCTAGCCAC ACCCCCACGG GAAACAGCAG TGATTAACCT TTAGCAATAA
  TTTTGCGAAT CGGATCGGTG TGGGGGTGCC CTTTGTCGTC ACTAATTGGA AATCGTTATT
850             860             870             880             890             900
  ACGAAAGTTT AACTAAGCTA TACTAACCCC AGGGTTGGTC AATTTCGTGC CAGCCACCGC
  TGCTTTCAAA TTGATTCGAT ATGATTGGGG TCCCAACCAG TTAAAGCACG GTCGGTGGCG
910             920             930             940             950             960
  GGTCACACGA TTAACCCAAG TCAATAGAAG CCGGCGTAAA GAGTGTTTTA GATCACCCCC
  CCAGTGTGCT AATTGGGTTC AGTTATCTTC GGCCGCATTT CTCACAAAAT CTAGTGGGGG
970             980             990             1000            1010            1020
  TCCCCAATAA AGCTAAAACT CACCTGAGTT GTAAAAAACT CCAGTTGACA CAAAATAGAC
  AGGCGTTATT TCGATTTTGA GTGGACTCAA CATTTTTTGA GGTCAACTGT GTTTTATCTG
1030            1040            1050            1060            1070            1080
  TACGAAAGTG GCTTTAACAT ATCTGAACAC ACAATAGCTA AGACCCAAAC TGGGATTAGA
  ATGCTTTCAC CGAAATTGTA TAGACTTGTG TGTTATCGAT TCTGGGTTTG ACCCTAATCT
1090            1100            1110            1120            1130            1140
  TACCCCACTA TGCTTAGCCC TAAACCTCAA CAGTTAAATC AACAAAACTG CTCGCCAGAA
  ATGGGGTGAT ACGAATCGGG ATTTGGAGTT GTCAATTTAG TTGTTTTGAC GAGCGGTCTT
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   AGCCTGTTCT GTAATCGATA AACCCCGATC AACCTCACCA CCTCTTGCTC AGCCTATATA
   TCGGACAAGA CATTAGCTAT TTGGGGCTAG TTGGAGTGGT GGAGAACGAG TCGGATATAT
1270          1280          1290          1300          1310          1320
   CCGCCACTCT CAGCAAACCC TGATGAAGCC TACAAAGTAA GCGCAAGTAC CCACGTAAAG
   GGCGGTAGAA GTCGTTTGGG ACTACTTCCG ATGTTTCATT CGCGTTCATG GGTGCATTTC
1330          1340          1350          1360          1370          1380
   ACGTTAGGTC AAGGTGTAGC CCATGAGGTG GCAAGAAATG GGCTACATTT TCTACCCCAG
   TGCAATCCAG TTCCACATCG GGTACTCCAC CGTTCTTTAC CCGATGTAAA AGATGGGGTC
1390          1400          1410          1420          1430          1440
   AAAACTACGA TAGCCCTTAT GAAACTTAAG GGTCGAAGGT GGATTTAGCA GTAAACTAAG
   TTTTGATGCT ATCGGGAATA CTTTGAATTC CCAGCTTCCA CCTAAATCGT CATTTGATTC
1450          1460          1470          1480          1490          1500
   AGTAGAGTGC TTAGTTGAAC AGCGCCCTGA AGCGCGTACA CACCGCCCGT CACCCTCCTC
   TCATCTCACG AATCAACTTG TCCCGGGACT TCGCGCATGT GTGGCGGGCA GTGGGAGGAG
1510          1520          1530          1540          1550          1560
   AAGTATACTT CAAAGGACAT TTAACTAAAA CCCCTACGCA TTTATATAGA GGAGACAAGT
   TTCATATGAA GTTTCCTGTA AATTGATTTT GGGGATGCGT AAATATATCT CCTCTGTTCA
1570          1580          1590          1600     1601
   CGTAACATGG TAAGTGTACT GGAAAGTGCA CTTGGACGAA   C
   GCATTGTACC ATTCACATGA CCTTTCACGT GAACCTGCTT   G
```

PCR REACTION MIXTURE

The polymerase chain reaction was carried out in 10µl volume consisting.

10 x buffer	- 1 x PCR buffer
[160 mM $(NH_4)_2$ SO_4, 670 mM Tais - HCl,	
PH - 8.8 (at 25°C), 0.1% Tween 20]	
50 mM $Mgcl_2$	- 0.28µl
10mM dNTP^s (dATP, dTTP, dGTP and dCTP)	- 0.1µl
Forward Primer	- 5 Pmol/µl
Reverse Primer	- 5 Pmol/µl
Taq DNA polymerase	- 0.3u
Template DNA	- 50-100 ng

Table 3.3 PCR REACTION CONDITION

REACTION	TEMPERATURE	DURATION
Initial denaturation	95^0C	5 minutes
Denaturation	95^0C	30 seconds
Annealing	56^0C	30 seconds
Extension	72^0C	45 seconds
Final extension	72^0C	5 minutes
Cycles	30	

Agarose Gel Electrophoresis

The PCR products were diluted with gel loading buffer (6x: 0.25% bromophenol blue,30% glycerol) and run on 2% agarose gel containing ethidium bromide (EtBr- 10mg /ml).The electrophoresis was carried out in 0.5x TBE buffer (5.4g tris base, 2.75g boric acid, 0.372 mg EDTA / lit. pH -8.3) for 20 minutes at 170V using 50 bp ladder for reference. The DNA bands were visualized under gel documentation system (Vilber Lourmat).

3.4.2 BsmA I RFLP ANALYSIS – A1555G

The primers used in this analysis will generate 248bp amplicon. The A1555G mutation will lead to loss of BsmA 1 restriction site.

5'...G T C T C $(N)_1\downarrow$...3'

3'...C A G A G $(N)_5\uparrow$...5'

Two fragments of the size 192bp and 56bp will be generated upon digestion of the 248bp amplicon for normal sequence, whereas the whole 248bp will be present for mutant sample. The source of BsmA I restriction enzyme used for digestion is from *Bacillus steprothermophilus.*

Restriction Enzyme Digestion/ BsmA I

2µl of PCR product was digested with 1.5U of BSm AI restriction enzyme for 16 hours at 37^0C as per the protocol provided by the manufacturer (Fermentas). Individuals for the wild type sequence shows 192bp and 56bp fragments whereas the individuals for the mutant allele shows 248bp fragment following BsmA 1 restriction enzyme digestion and on separation by agarose gel electrophoresis (UNIQUIEP).

The digested PCR products were run on 3% agarose gel electrophoresis. The electrophoresis was carried out in 0.5X TBE buffer (5.4g tris base, 2.75g boric acid, 0.372mg EDTA / L, pH-8.3) at 170V for 25-minutes using 50bp ladder for reference. The separated DNA bands were visualized and documented using a gel documentation system (Vilber Lourmat).

3.5 A7445G T7510C T7511C Mutation Detection

3.5.1 Polymerase Chain Reaction

The polymerase chain reaction was performed for the purpose of amplification of the 7392-7608 fragments. This is achieved by selecting a suitable primer and an appropriate reaction set up. The primers used in the reaction, amplicon length and the number of cycles involved are given in Table 3.4

Table-3.4 : PRIMERS USED IN THE PCR REACTION

Primer Type	Primer Sequence	Amplification Length	PCR Cycles
Forward	5′-GGA TGC CCC CCA CCC TAC C-3′	217 bp	25
Reverse	5′-CCT ACT TGC GCT GCA TGT GCC-3′		

SEQUENCE OF tRNAser(UCN) OF mtDNA

7321 gagaagcctt cgcttcgaag cgaaaagtcc taatagtaga agaaccctcc ataaacctgg

7381 agtgactata **tggatgcccc ccaccctacc** acacattcga agaacccgta tacataaaat

7441 ctagacaaaa aaggaaggaa tcgaacccct caaagctggt ttcaagccaa ccccatggcc

7501 tccatgactt tttcaaaaag gtattagaaa aaccatttca taactttgtc aaagttaaat

7561 tataggctaa atcctatata tcttaat**ggc acatgcagcg caagtaggt**c tacaagacgc

7621 tacttccccc atcatagaag agcttatcac ctttcatgat cacgccctca taatcatttt

■ – Primer sequence ■ - amplified region ■ - mutated bp

PCR reaction mixture

The polymerase chain reaction was carried out in 10µl volume consisting

10 x buffer [160 mM $(NH_4)_2 SO_4$, 670 mM Tris - HCl, PH - 8.8 (at 25°C), 0.1% Tween 20]	- 1 x PCR buffer
50 mM $Mgcl_2$	- 0.47µl
10mM dNTP^s (dATP, dTTP, dGTP and dCTP)	- 0.08µl
Forward Primer	- 5 Pmol/µl
Reverse Primer:	- 5 Pmol/µl
Taq DNA polymerase	- 0.3u
Template DNA	- 50-100 ng

Table 3.5 PCR REACTION SET UP

Reaction	Temperature	Duration
Initial Denaturation	95°C	5 minutes
Denaturation	95°C	30 seconds
Annealing	60°C	30 seconds
Extension	72°C	45 seconds
Final Extension	72°C	5 minutes
Hold	4°C	
Cycles	25	

Agarose Gel Electrophoresis

The PCR products was diluted with 6X gel loading buffer (0.25% Bromophenol Blue, 30% glycerol) and run on 2% Agarose gel containing Ethidium Bromide (EtBr) (10mg/ml). Electrophoresis was carried out in 0.5X TBE buffer (5.4g Tris Base, 2.75g Boric acid, 0.372 mg EDTA/ liter .pH 8.3) for 20 minutes at 170 V using 50bp ladder for reference. The separated DNA bands were visualized using a Gel documentation system (Vilber Lourmat).

The primers used in this analysis will generate 217bp amplicon. The A7445G mutation introduced Xba I restriction site.

5`.....T CTAGA.....3`

3`.....AGAGC T.....5`

Two fragments of size 49bp and 168bp will be generated upon digestion of the 217bp for normal sequence whereas the whole 248bp will be present for mutant sample. DNA samples from the database were subjected to RFLP analysis to confirm the A7445G mutation. The source of Xba I restriction enzyme used for digestion is from *Xanthomonas badrii.*

Restriction Enzyme Digestion/ Xba I

1.7µl PCR product was digested with 1.8U of Xba I restriction enzyme by incubation it at 37°C for 16 hours as per the protocol provided by the manufacturer (Medox Biotech India Pvt.Ltd). Individuals for the wild type sequence shows 49bp and 168bp fragments where as the individuals with the mutant allele shows 217bp for Xba I restriction enzyme digestion on separation by agarose gel electrophoresis (UNIQUIEP).

Agarose Gel Electrophoresis

The digested PCR products were run on 3% Agarose gel. The electrophoresis was carried out in 0.5 x TBE buffer (5.4 g Tris base, 2.75g Boric acid, 0.372 mg EDTA / litre, pH 8.3) at 160v for 30 minutes using 50bp ladder for reference. The separated DNA bands were visualized and documented using a gel documentation system (Vilber Lourmat)

The primers used in this analysis will generate 217bp amplicon. The T7510C mutation introduced Hinf I restriction site.

5`.....GANTC .....3`

3`.....CTNAG.....5`

Two fragments of size 150 bp and 67bp will be generated upon digestion of the 217bp for normal sequence whereas 101bp, 49bp, 67bp will be generated for mutant sample by Hinf I restriction enzyme. DNA samples from the database were subjected to RFLP analysis to confirm the T7510C mutation. The source of Hinf I restriction enzyme used for digestion is from *Hemophilus influenzae*.

Restriction Enzyme Digestion/ Hinf I

2µl PCR product was digested with 0.5U of Hinf I restriction enzyme by incubation it at 37°C for 16 hours as per the protocol provided by the manufacturer (New England Biolabs). Individuals for the wild type sequence shows 150bp and 67bp fragments where as the individuals for the mutant allele shows 101bp, 49bp and 67bp fragment following Hinf I restriction enzyme digestion on separation by agarose gel electrophoresis (UNIQUIEP).

Agarose Gel Electrophoresis

The Digested PCR products were run on 3% Agarose gel. The electrophoresis was carried out in 0.5 x TBE buffer (5.4 g Tris base, 2.75g Boric acid, 0.372 mg EDTA / litre, pH 8.3) at 170v for 25 minutes using 50bp ladder for reference. The separated DNA bands were visualized and documented using a gel documentation system (Vilber Lourmat)

PCR / Hinf I RFLP ANALYSIS – T7511C

The primers used in this analysis will generate 217bp amplicon. The T7511C mutation introduced Mbo II restriction site.

5`.....G A A G A (N)8.....3`

3`.....C T T C T (N)7.....5`

Two fragments of size 177 bp and 40bp will be generated upon digestion of the 217bp for normal sequence whereas 107bp, 70bp, 40bp fragments will be generated for mutant sample by Mbo II restriction enzyme. DNA samples from the database were subjected to RFLP analysis to confirm the T7511C mutation. .

Restriction Enzyme Digestion/ Hinf I

2µl PCR product was digested with 2U of MboII restriction enzyme for 16 hours at 37°C as per the protocol provided by the manufacturer (Fermentas). Individuals for the wild type sequence shows 177bp and 40bp fragments whereas the individuals for the mutant allele shows 107bp, 70bp and 40bp fragment following Mbo II restriction enzyme digestion on separation by agarose gel electrophoresis

Agarose Gel Electrophoresis

The Digested PCR products were run on 3% Agarose gel. The electrophoresis was carried out in 0.5 x TBE buffer (5.4 g Tris base, 2.75g Boric acid, 0.372 mg EDTA / litre, pH 8.3) at 170v for 25 minutes using 50bp ladder for reference. The separated DNA bands were visualized and documented using a gel documentation system (Vilber Lourmat)

RESULTS AND DISCUSSION

enetic resource.belonging to an ongoing research programme at the Department of enetics,PGIBMS,University of Madras.As a trainee I had the oppurtunity to participate in is programme for a short period.

UB GROUP 1

19 subjects have been included in this study who belong to a multigenerational family vith a history of progressive postlingual deafness expressed across nearly 4 generations.

Among the 19 subjects included in the study , 12 are affected with hearing loss of ariable severity , age at onset, and variable progression; 8 of them are females and 4 are nales. 7 of the individuals reported normal hearing.

7 Normal hearing + 8 Affected females + 4 Affected males= 19 Subjects included in the tudy

(TRIP)		(Years)				MUTATION
1-1	F	53	Mother of TRIP 1-2	Post lingual; progressive	Progression of hearing impairment enhanced after each delivery	Mutant
1-2	M	27	Son	Post lingual	Educated in a deaf school; language well developed	Mutant
3-1	M	45	Grandson of the presumed founder (Grandmother) of this mutation	Moderate hearing loss; Can speaak on phone	Spouse related	Mutant
4-1	F	58	Mother	Post-lingual; Onset 13th year; mild	Underwent stapedectomy ;enhanced progression of hearing impairment with each delivery;history of epileptic seizures	Mutant
4-2	M	38	Son of TRIP 4-1	Mild	educated in a normal school -10th standard	Mutant
4-3	M	36	Son of TRIP 4-1	Severe bilateral congenital	Educated till 5th standard .speech not well developed ;Phonological deficits;speech articulation deficit	Mutant
4-4	F	30	Daughter of TRIP 4-1	Severe bilateral;left ear worse; onset 5th year	Increased hearing loss with each delivery .10th standard normal school	Mutant
4-5	F	8	Daughter of TRIP 4-3	Normal	Molecular testing positive for A1555G Mutation. Highly predisposed to hearing loss Aminoglycoside to be used with caution .needs to be monitored regularly for hearing loss	Mutant
4-6	M	6	Son of TRIP 4-3	Normal	-	Normal
4-7	F	13	Daughter of TRIP 4-4	Normal	Molecular testing positive for A1555G mutation. Highly predisposed to hearing loss Aminoglycoside to be used with caution.	Mutant

O	ID NO (TRIP)	SEX	AGE (Years)	RELATIONSHIP	HEARING STATUS	REMARKS	A1555G MUTATION
	4-8	M	10	Son of 4-4	Normal	Molecular testing positive for A1555G mutation.Risk of being affected	Mutant
.	5-1	F	50	Mother	Tinnitus (Right ear);very mild hearing loss ;onset 48th year	All her daughters have hearing loss and are positive for A155G mutation while she has is found negetive for A1555G mutation.	Normal
.	5-2	F	34	Daughter of TRIP 5-1	Post lingual hearing loss;moderate	-	Partial digestion
.	5-3	F	31	Daughter of TRIP 5-1	Mild; post-lingual ; late onsetat 29 yrs.	Respiratory congestion,allergic to paracetamol;skin allergy and eruptions to sweets.	Mutant
5.	5-4	F	29	Daughter of TRIP 5-1	Severe ;post-lingual HL	Highly progressed after delivery	Mutant
6.	5-5	F	17	Daughter of TRIP 5-2	Mild hearing loss	Tested positive for A1555G mutation .Needs careful monitoring while administering aminoglycosides.	Mutant
7.	5-6	F	13	Daughter of TRIP 5-2	Not known	-	Mutant
8.	5-7	F	6	Daughter of TRIP 5-4	Not known	Positive for A1555G mutation .To be monitored on aminoglycoside medication	Mutant
9.	5-8	F	$3^{1/2}$	Daughter of TRIP 5-4	Normal	To be monitored for A1555G mutation	Partial digestion

Phenotypically ,the deafness is of post-lingual and progressive with variable age at onset.Some have mild phonological and severe speech articulation defects.The transmission pattern appears to be matrilineal spread across nearly 4 generations.This large extended family can be well visualised as 5 clusters for convenience named as TRIP 1, TRIP 2, TRIP 3, TRIP 4 ,TRIP 5 .All the clusters share a set of common grandparents.

subjects were tested negative (or) normal for A1555G mutation .The hearing status for those diagnosed as normal is also reported to be normal.However TRIP 5-1 who is a 50 year old mother reports tinnitus in her right ear and a mild hearing loss with recent (48^{th} year)onset. TRIP 4-6 who is tested normal for the A1555G mutation phenotypically also shows a normal hearing.

Individuals who showed partial digestion are TRIP 5-2 and TRIP 5-8. Both are females aged 34 years and $3^{½}$ years respectively.As far as the hearing status is concerned,TRIP 5-2 has a moderated post-lingual hearing loss but TRIP 5-8 is recorded according to the informant's knowledge as normal hearing .Coming to the A1555G positive mutants,there are 15 individuals tested positive,among them 5 individuals phenotypically show no hearing loss .Interestingly , all of them belong to a young age group between 6 – 13 years age range.So they are highly predisposed to hearing loss which can be aggrevated with the use of aminoglycoside antibiotics.So as an immediate application to this research outcome we are informing the parents of all those five youngsters to be very cautious about administering the aminoglcoside antibiotics to the individuals.As far as the remaining 10 mutants are concerned,the hearing loss is post- lingual with variability in terms of onset, severity, laterality and progression.In general the women have shown enhanced progression with each child birth. Except TRIP 1-2 all the individuals have been educated in a normal school. TRIP 4-3 shows severe phonological and speech articulation deficits.

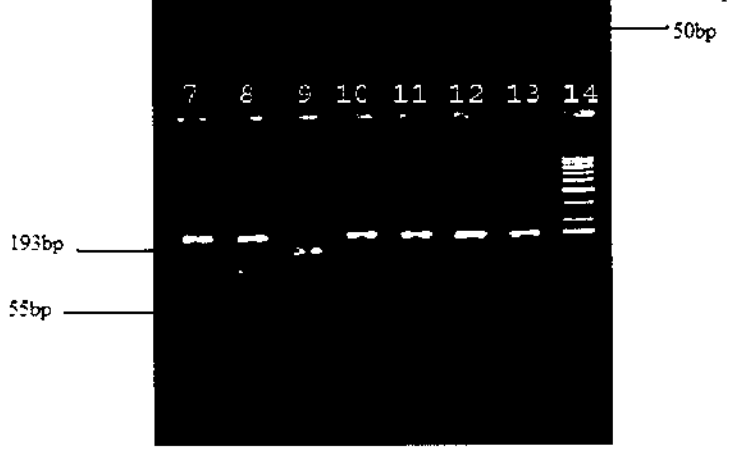


Fig 4.1

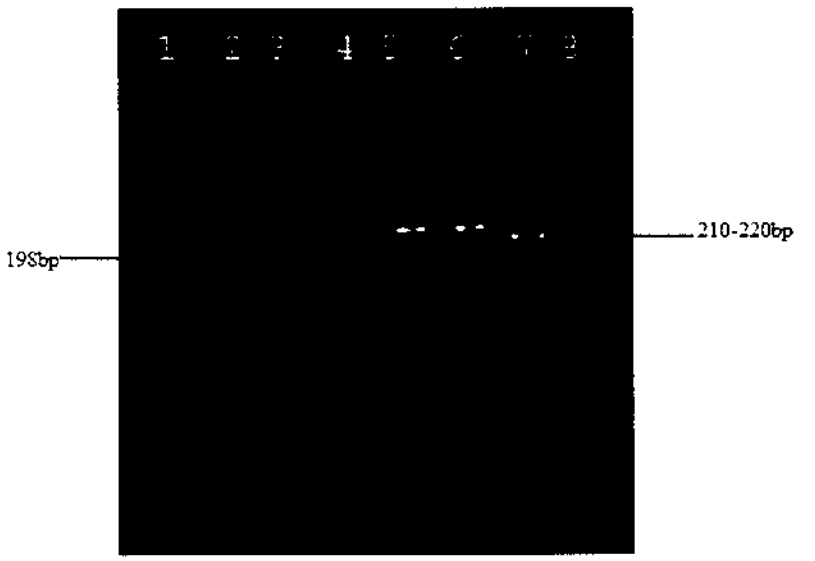


Fig 4.2

Fig 4.1 and Fig 4.2 Gel image of Restriction enzyme(BsmA1) digested samples.

The figures show the RFLP analysis of A1555G mutation in 12SrRNA gene of mtDNA. The PCR amplified segment of the mitochondrial 12SrRNA gene was digested with restriction enzyme BsmA1. In Fig 4.1, all lanes except lane 9 and 10 show a single band of length 248 bp and show the presence of A1555G mutation.Lane 9 shows two fragments of 193bp and 55bp and hence the absence of the A1555G mutation .Lane 10 has a band of about 210-220 bp,showing partial digestion. In fig 4.2 all the lanes except 2 and 7 show a single band(248bp) and the presence of A1555 mutation.Lane 2 shows the absence of A1555G mutation and lane 7 shows the presence of partial digestion.

FIG 4.1 - LANE NUMBER	ID NUMBER (TRIP)
1	1-1
2	1-2
3	4-1
4	4-4
5	5-4
6	LADDER(50bp)
7	3-1
8	4-3
9	5-1
10	5-2
11	5-3
12	5-5
13	4-2
14	LADDER(50bp)
FIG 4.2 - LANE NUMBER	**ID NUMBER (TRIP)**
1	4-5
2	4-6
3	4-7
4	4-8
5	5-6
6	5-7
7	5-8
8	LADDER(50bp)

It should be mentioned that as far as the TRIP family is concerned,the hearing loss can be genetically attributed to A1555G mutation.There is a need to complete the molecular testing on all the 21 members reported to be affected with post-lingual progressive deafness with variable age of onset.This family also requires a thorouh clinical documentation to arrive at the correct phenotype.Such multiplexed families with matrilineal transmission are very important owing to the rarity of this mutation , as there are no published reports as of

conformation.

SUB GROUP 2

The study consists of 15 subjects where they have 2 or more affected siblings in the sibship .8 families show both affected and normal hearing in the sibship; and 6 families have exclusively affected in their sibship; the maximum sibship size of the 'only affected' is 3.

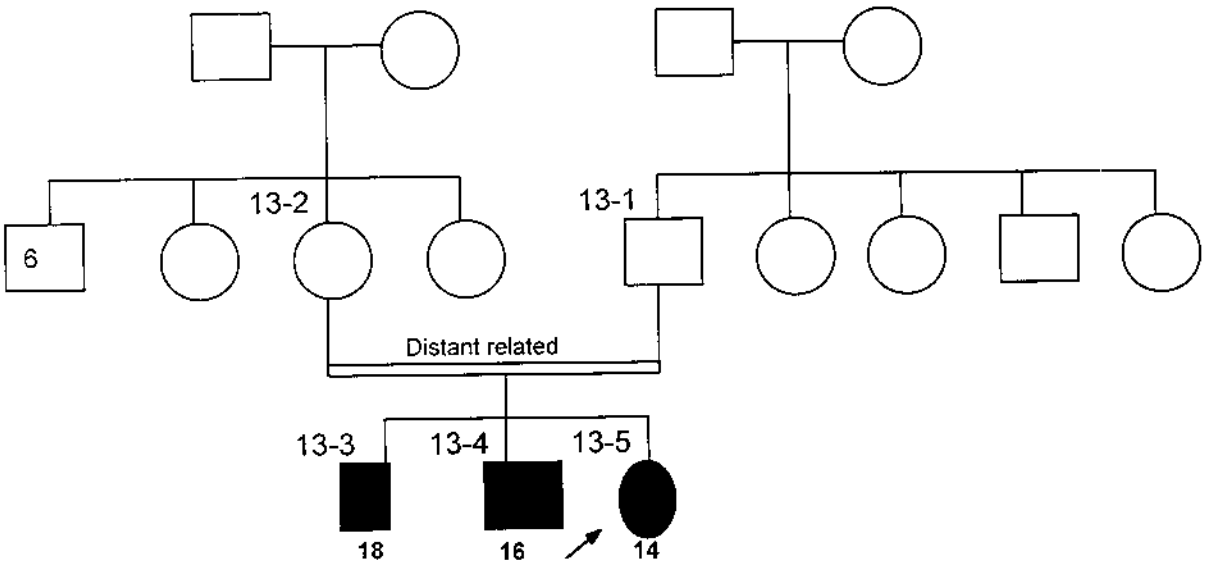


Fig.4.3 ZSIV 13-5

The figure shows the pedigree of this subset.Among the 15 subjects 10 were males and 5 females, who were tested for the mtDNA mutations.In other words 1 affected from each of the 15 families have been tested for the mtDNA mutations A1555G , A7445G, T7510C, T7511C causing non – syndromic deafness.

O	FAMILY ID AND PROBAND TESTED FOR MUTATION	SIBSHIP SIZE (ALIVE) INCLUDING PROBAND		NO. OF AFFECTED IN THE SIBSHIP (INCLUDING PROBAND)		GJB2 TESTED	GENDER (PROBAND)	REMARKS
		M	F	M	F			
	ZDE 11-4	-	2	-	2	NOT TESTED	F	-
	ZSIV 13-5	2	1	2	1	NOT TESTED	F	-
	ZRAM 16-4	5	-	2	-	NOT TESTED	M	-
	ZVTD 62-4	2	-	2	-	TESTED	M	-
	ZSL 64-5	2	1	1	1	TESTED	M	PROBAND TESTED POSITIVE FOR W24X
	ZMDU104-4	3	-	2	-	NOT TESTED	M	PROBAND HAS A NORMAL HEARING MALE TWIN SIB
	ZSAL 141-3	1	1	1	1	TESTED	M	-

S.NO	FAMILY ID AND PROBAND TESTED FOR MUTATION	SIBSHIP SIZE (ALIVE) INCLUDING PROBAND		NO. OF AFFECTED IN THE SIBSHIP (INCLUDING PROBAND)		GJB2 TESTED	GENDER	REMARKS
		M	F	M	F			
8	ZSAL 142-3	1	1	1	1	TESTED	M	-
9	ZSAL145-3	2	1	2	-	TESTED	M	PROBAND HAS A DEAF MALE TWIN SIB
10	ZSAL 146-4	1	2	1	1	TESTED	F	-
11	ZSAL 148-5	4	1	3	0	TESTED	M	-
12	ZSAL 151-5	3	-	2	-	TESTED	M	-
13	ZSAL154-3	1	1	1	1	TESTED	M	-
14	ZERD 183-5	3	-	2	-	NOT TESTED	M	PROBAND HAS A PAIR OF YOUNGER TWIN BROTHERS : ONE IS A DEAF
15	ZERD 191-4	1	1	1	1	NOT TESTED	F	-

Table 4.2 shows the distribution of affected among the sibs of the probands. In this type of sample looking at the distribution , one cannot exclude non- syndromic deafness causing mtDNA mutation for certain.

Table 4.3 shows the distribution of probands by parental mating type.The frequency of consanguinity in this subset is 60%. Due to the high frequency of blood related marriages,we cannot completely rule out the role of autosomal recessive (DFNB) genes in the causation of deafness . So it should be borne in mind that there is a need to screen for common DFNB loci (in addtion to CONNEXIN 26) reported in South India.

FAMILY ID AND PROBAND TESTED FOR MUTATION	UNRELATED	CONSANGUINEOUS		DISTANT RELATED
		UN	FC	
ZDE 11-4	–	✓	–	–
ZSIV 13-5	–	–	–	✓
ZRAM 16-4	✓	–	–	–
ZVTD 62-4	–	✓	–	–
ZSL 64-5	–	–	–	✓
ZMDU104-4	–	–	–	✓
ZSAL 141-3	✓	–	–	–
ZSAL 142-3	✓	–	–	–
ZSAL145-3	✓	–	–	–
ZSAL 146-4	–	–	✓	–
ZSAL 148-5	✓	–	–	–
ZSAL 151-5	✓	–	–	–
ZSAL154-3	–	✓	–	–
ZERD 183-5	–	–	✓	–
ZERD 191-4	–	–	✓	–
TOTAL	6 (40%)	3(20%)	3(20%)	3(20%)

UN - UNCLE NIECE
FC - FIRST COUSIN

60 %

AND PROBAND TESTED FOR MUTATION	MITOCHONDRIAL MUTATION				CONNEXIN 26			
	A1555G	T7445C	T7510C	T7511C	W24X	W77X	35delG	Q124X
ZDE 11-4	NORMAL	NORMAL	NORMAL	NORMAL	NOT TESTED	NOT TESTED	NOT TESTED	NOT TESTED
ZSIV 13-5	NORMAL	NORMAL	NORMAL	NORMAL	NOT TESTED	NOT TESTED	NOT TESTED	NOT TESTED
ZRAM 16-4	NORMAL	NORMAL	NORMAL	NORMAL	NOT TESTED	NOT TESTED	NOT TESTED	NOT TESTED
ZVTD 62-4	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
ZSL 64-5	NORMAL	NORMAL	NORMAL	NORMAL	MUTANT	NORMAL	NORMAL	NORMAL
ZMDU104-4	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
ZSAL 141-3	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
ZSAL 142-3	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
ZSAL145-3	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
ZSAL 146-4	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
ZSAL 148-5	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
ZSAL 151-5	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
ZSAL154-3	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL	NORMAL
ZERD 183-5	NORMAL	NORMAL	NORMAL	NORMAL	NOT TESTED	NOT TESTED	NOT TESTED	NOT TESTED
ZERD 191-4	NORMAL	NORMAL	NORMAL	NORMAL	NOT TESTED	NOT TESTED	NOT TESTED	NOT TESTED

Table 4.4 shows the genotype correlation of the proband tested.This table also shows the results of screening for GJB2 (Connexin 26) in the nuclear genome for some of the probands.Some cases were tested for connexin26 mutations of the gene encoding for gap junction protein known as CX26(belonging to the nuclear genome) and have been found negative except in one subject(ZSL 64-5).These results were made available to us.

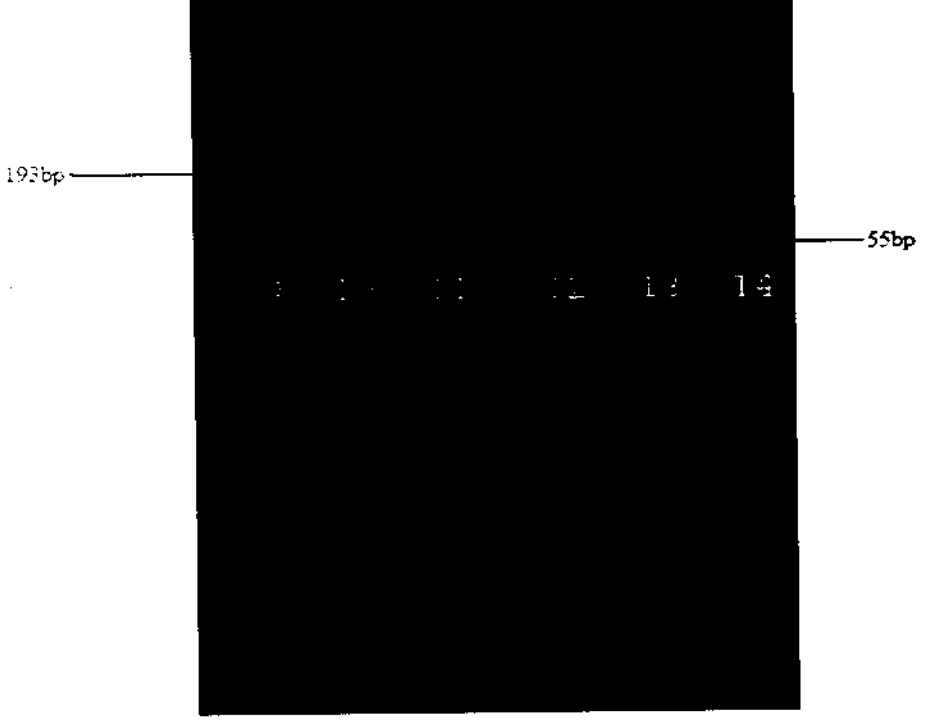


Fig 4.4. RFLP Analysis – A1555G

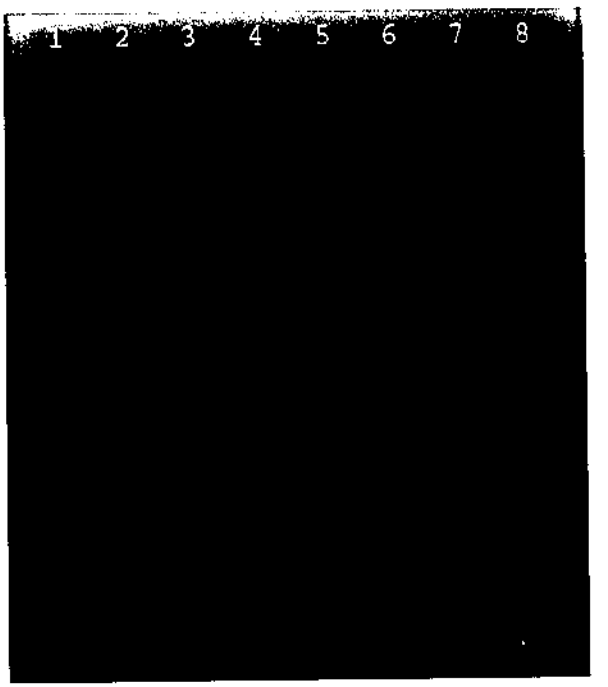


Fig 4.5 RFLP Analysis – A1555G

Fig 4.4 ; 4.5 shows the RFLP analysis of A1555G mutation in 12 SrRNA gene of mtDNA .The PCR amplified segment of the mitochondrial 12SrRNA gene was digested with restriction enzyme BsmA 1 .All lanes show two fragments of 193 bp and 55 bp and hence absence of the A1555G mutation.

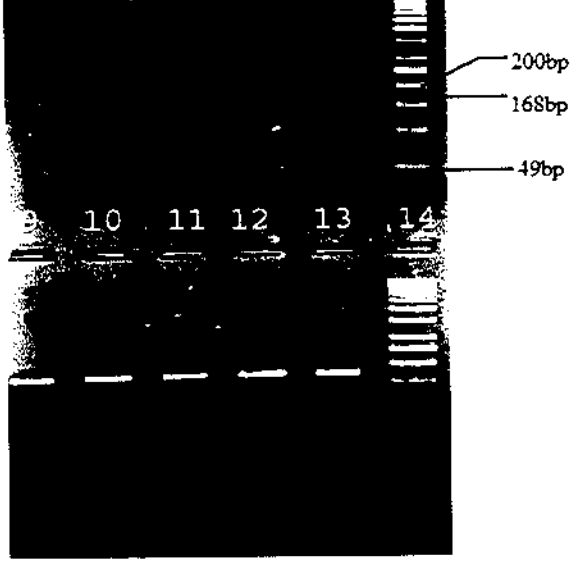


Fig 4.6. RFLP Analysis – A7445G

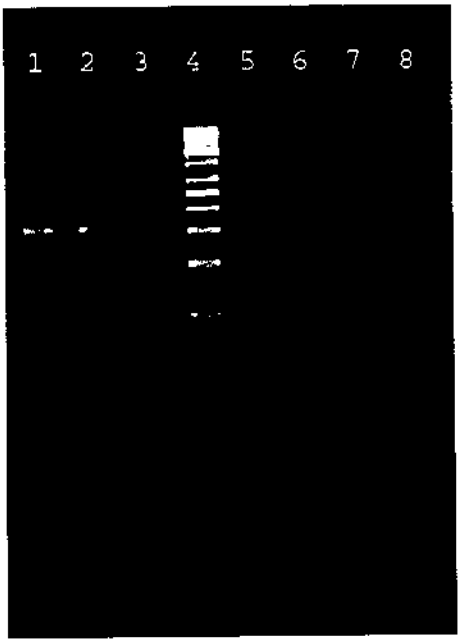


Fig 4.7. RFLP Analysis – A7445G

Figure 4.6 , 4.7 show the gel photographs of PCR amplicons digested with the restriction enzyme Xba I. In the above figures all lanes shows 169bp and 48bp and hence absence of the A7445G mutation. Lane 8 (Fig 4.6) and Lane 4(Fig.4.7) corresponds to 50bp ladder DNA.

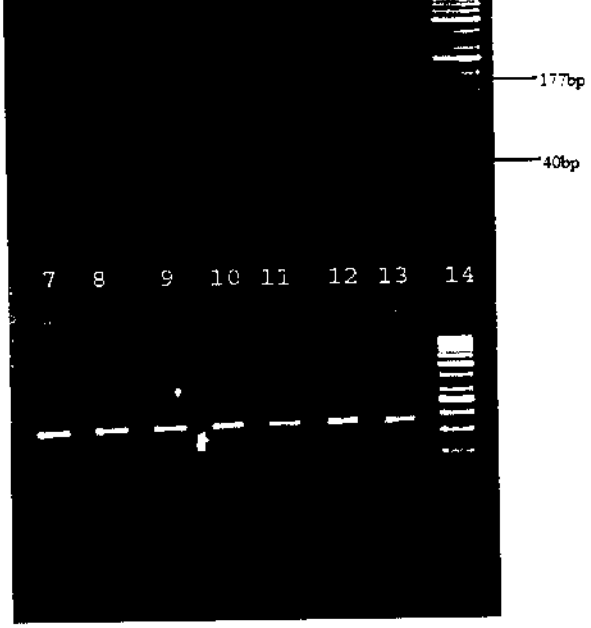


Fig 4.8. RFLP Analysis – T7511C

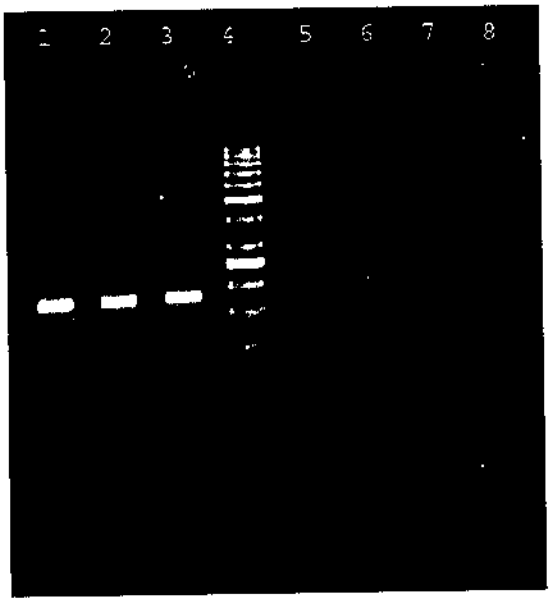


Fig 4.9. RFLP Analysis – T7511C

Figure 4.8; 4.9 show the gel photographs of PCR amplicons digested with the restriction enzyme Mob II. In the above figures all lanes shows 177bp and 40bp and hence absence of the T7511C mutation. Lane 8 (Fig 4.8) and Lane 4(Fig.4.9) corresponds to 50bp ladder DNA.

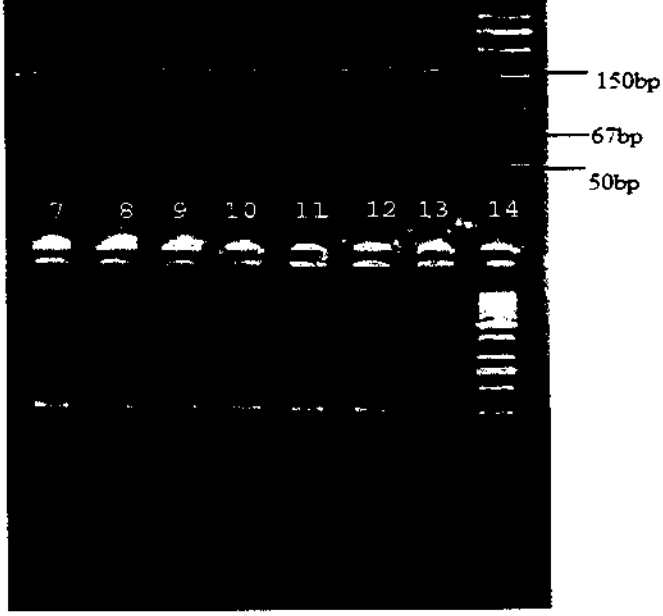


Fig 4.10. RFLP Analysis – T7510C

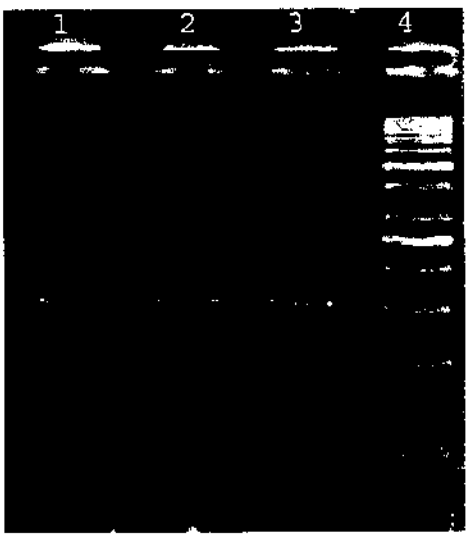


Fig 4.11. RFLP Analysis – T7510C

Figure 4.11:4.11 show the gel photographs of PCR amplicons digested with the restriction enzyme Hinf I. In the above figures all lanes shows 150bp and 67bp and hence absence of the T7510C mutation. Lane 8 (Fig 4.10) and Lane 4(Fig.4.11) corresponds to 50bp ladder DNA.

Fig 4.4 ,Fig 4.6, Fig 4.8, Fig 4.10 (LANE NUMBER)	ID NUMBER
1	ZDE 11-4
2	ZSIV 13-5
3	ZRAM 16-4
4	ZVTD 62-4
5	ZSL 64-5
6	LADDER (50bp)
7	ZMDU104-4
8	ZSAL 141-3
9	ZSAL 142-3
10	ZSAL145-3
11	ZSAL 146-4
12	ZSAL 148-5
13	ZSAL 151-5
14	ZSAL154-3

Fig 4.5,Fig 4.7, Fig 4.9 , Fig 4.11 LANE NUMBER	ID NUMBER
1	ZSAL154-3
2	ZERD 183-5
3	ZERD 191-4
4	LADDER (50bp)

Therefore on testing for A1555G,A7445G,T7510C,T7511C mutation in all the 15 subjects ,none were found to be having these mutations implying that this is not the cause for their hearing impairment.This is the 1st report that has attempted to screen for these mtDNA mutations among a group of 15 subjects with affected sibs in the sibship.Mitochondrial inheritance cannot be certainly ruled out without a systematic screening for each mutation in the type of sib ships tested here.This is the 1st report that has attempted in screening for this mutation in South Indian population.The A1555G mutation is associated with the susceptibility to aminoglycoside antibiotics.It has been found in recent studies,several

in patients from high risk population.

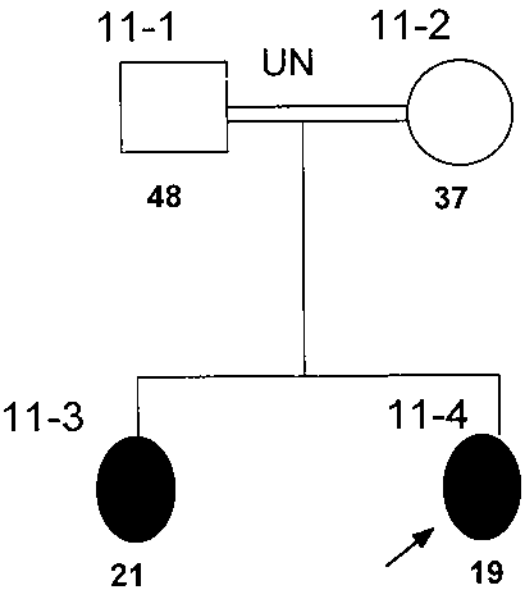
CONCLUSION

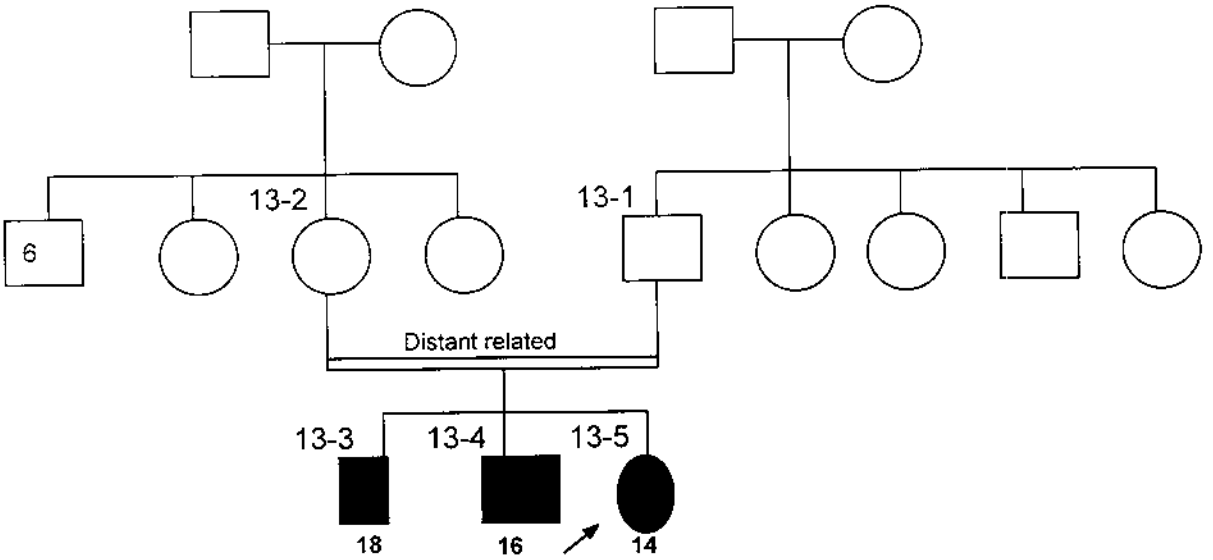
The present work is a part of an ongoing study at the host institute (Dept.of Genetics.PGIBMS.University of Madras)networking for deafness in INDIA and Tamilnadu in particular. The work studies two subgroups of hearing impaired which is available as the deafness genetic resource.Subgroup1 consists of members of a multigenerational family with a history of progressive post-lingual deafness expressed across nearly 4 generations.The demographic ,genotypic and phenotypic description of the relatives of the 5 clusters screened , showed hearing loss of variable severity , age at onset, and variable progression among them ,with matrilineal pattern of transmission .There is a high frequency of A1555G mutation in this family.Some subjects phenotypically found to be normal tested postive for A1555G mutation ,hence being highly predisposed to hearing loss which can be aggrevated with the use of aminoglycoside antibiotics.

The role of Autosomal recessive genes cannot be ruled out as the cause of mutation in this family because there is a considerable frequency of consanguinity within the familial clusters.Subgroup 2 consists of 15 subjects where they have 2 or more affected siblings in the sibship .The results of the distribution of the affected among the sibs show the existence of random mitotic segregation of the primary oocyte to the daughters cells.explaining heteroplasmy and the threshold effect.The results of the distribution of probands based on parental mating type shows the frequency of consanguinity in this subset to be 60%. Due to the high frequency of blood related marriages,we cannot completely rule out the role of autosomal recessive (DFNB) genes in the causation of deafness .The genotype correlation of the proband tested shows the results of screening for GJB2 (Connexin 26) in the nuclear genome for most of the probands.Almost all the subjects showing negative results for the A1555G, A7445G , T7510C, T7511C mutations ,showed negative results for Connexin 26 mutations also .This is the first report that has attempted in screening for these mitochondrial mutations in South Indian population.The A1555G mutation is associated with the susceptibility to aminoglycoside antibiotics.It has been found in recent studies,several hearing impaired patients bearing the A1555G mutation have no history of aminoglycoside injection.A rapid screening as well as careful counseling is only possible when such studies are made.Genetic background should be adequately checked before aminoglycoside are used in patients from high risk population.

Pedigrees of the 'siblings affected' subjects. The subgroup consists of 15 subjects.

ZDE 11-4

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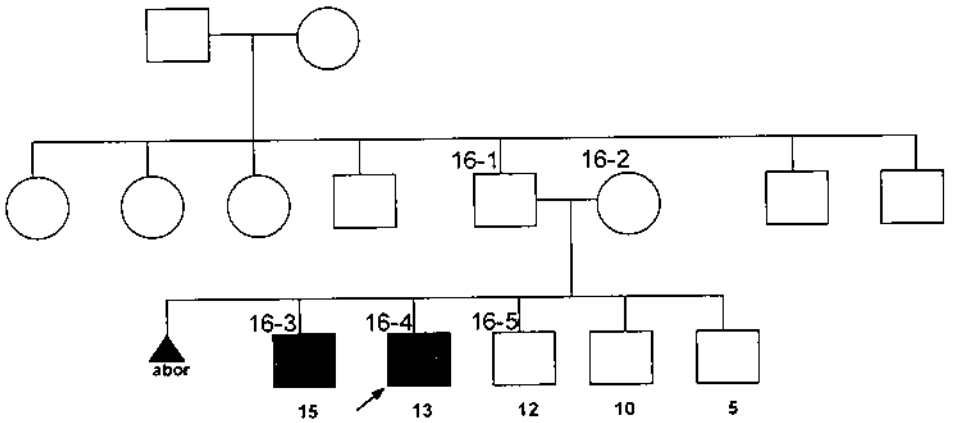
13-1
6
Distant related
13-3
13-4
13-5
18
16
14

ZRAM 16-4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16-4
16-5
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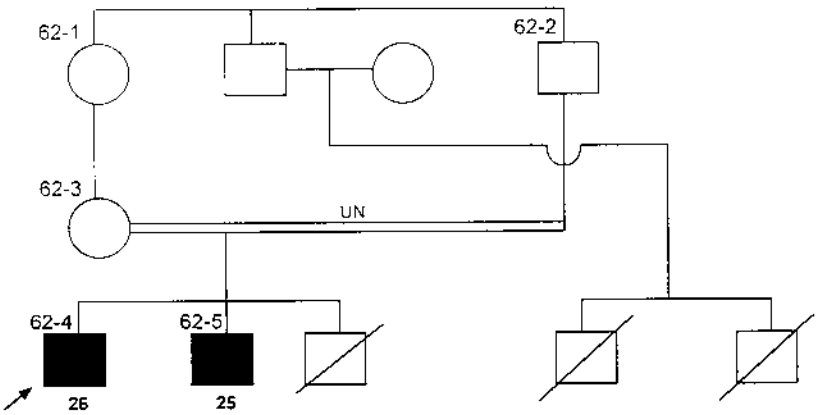
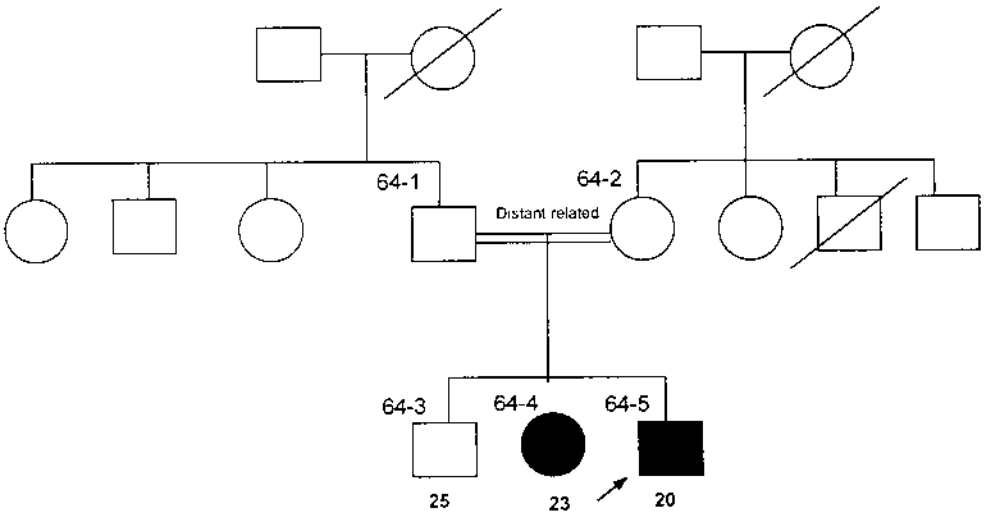
12
10
5


62-1
62-2
62-3
UN
62-4
62-5
26
25

ZSL 64-5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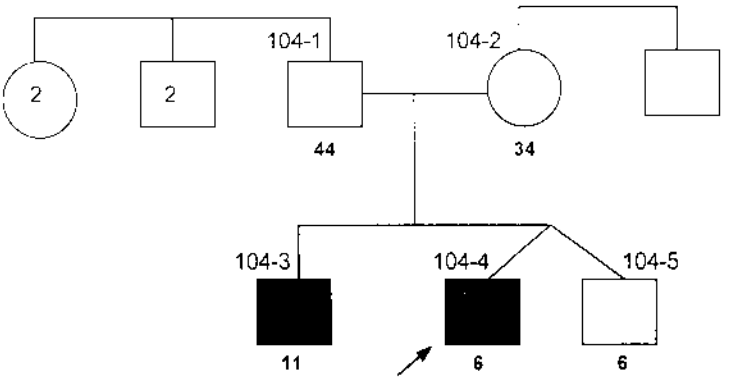
64-2
Distant related
64-3
64-4
64-5
25
23
20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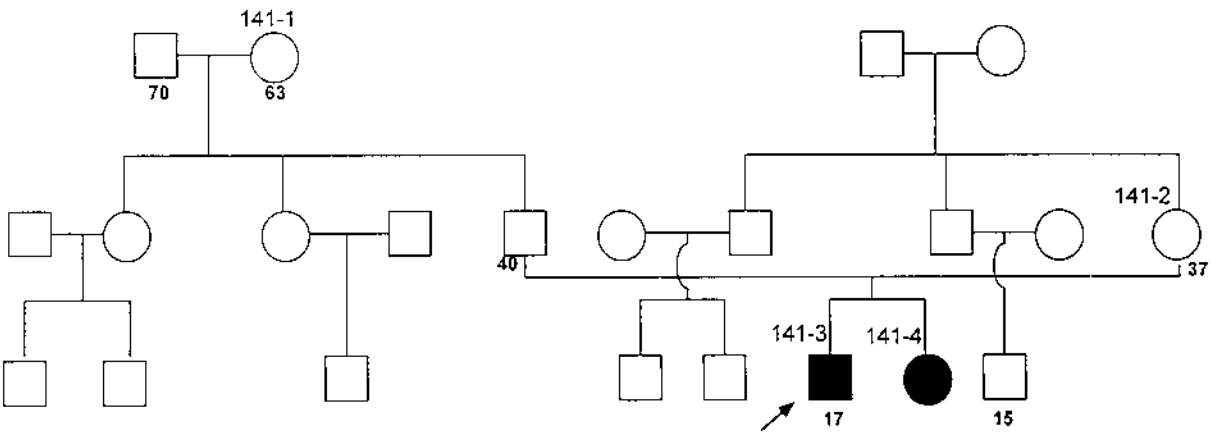
104-3
104-4
104-5
11
6
6

ZSAL 141-3


141-1
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63
141-2
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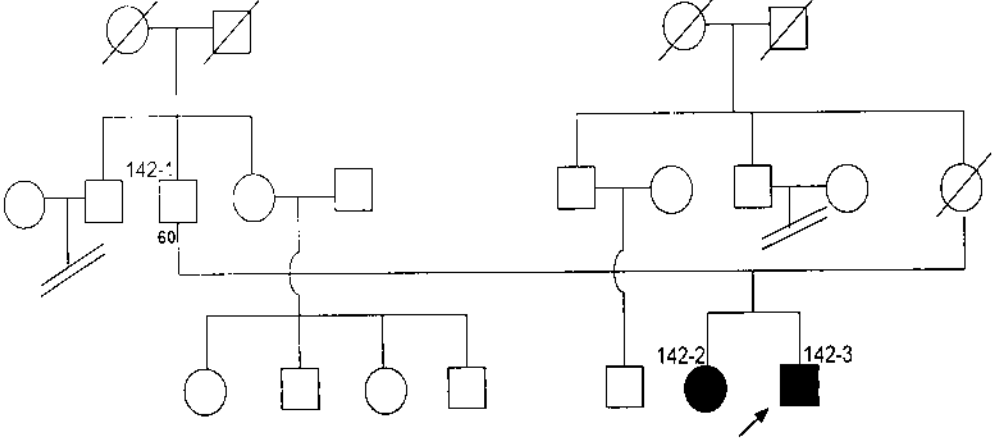
37
141-3
141-4
17
15



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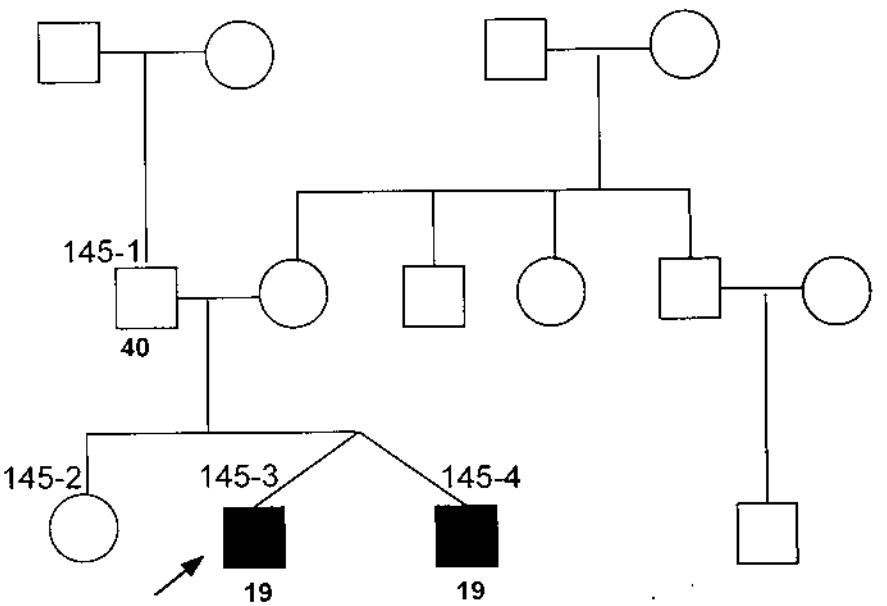
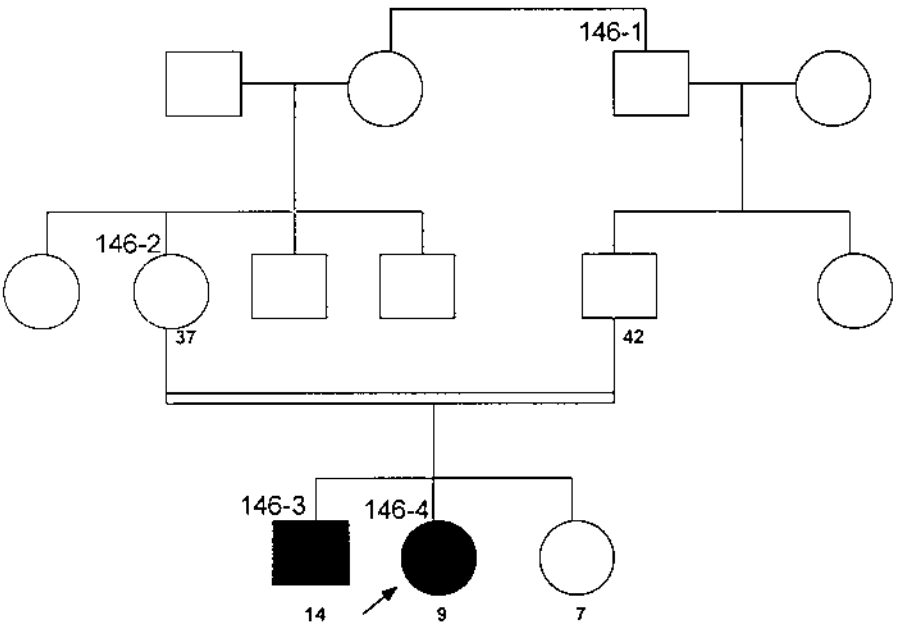
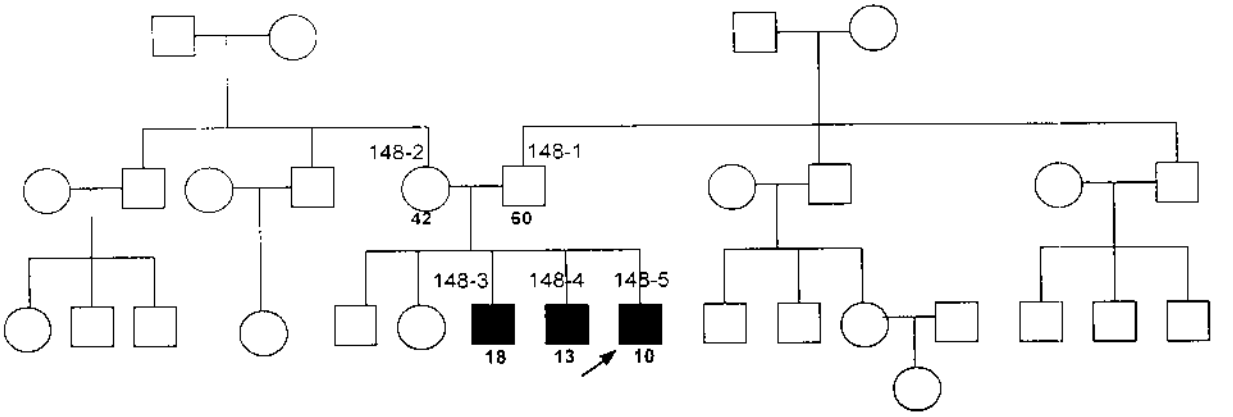


ZSAL 146-4





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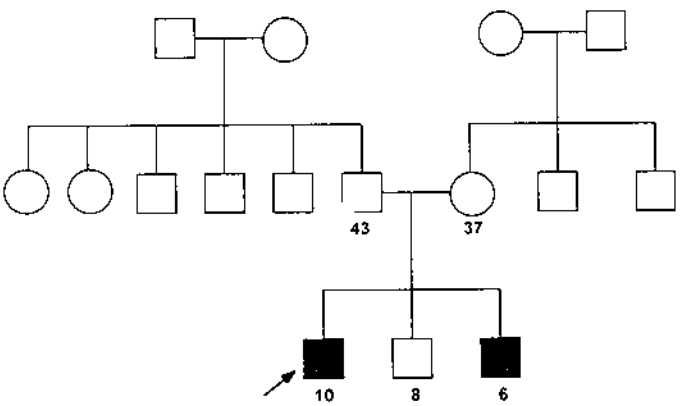

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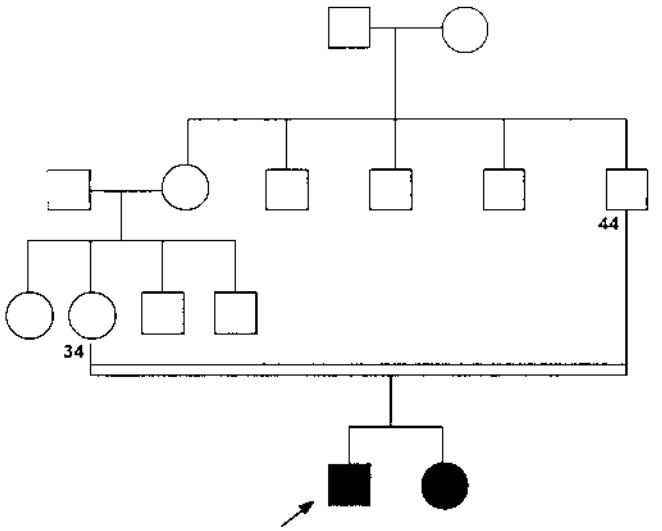
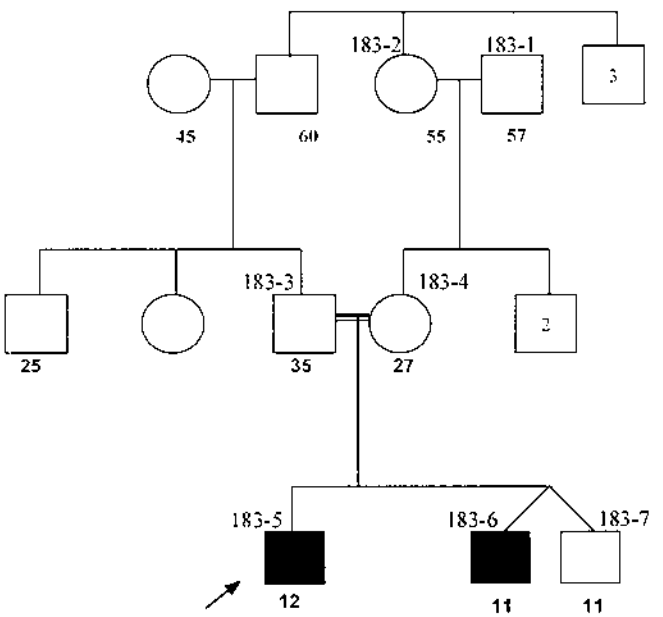


ZERD 183-5



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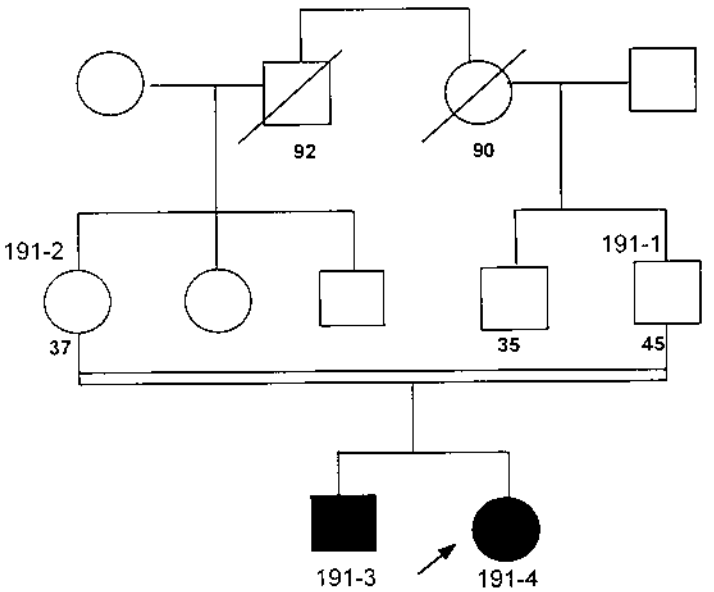

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