

Review Article

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Role of connexin 26 (*GJB2*) & mitochondrial small ribosomal RNA (*mt 12S rRNA*) genes in sporadic & aminoglycoside-induced non syndromic hearing impairment

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Non syndromic hearing impairment is a common sensory disorder, which affects one in 600 newborns. Though more than 50 nuclear genes are involved in causing non syndromic hearing impairment, mutations in the connexin 26 (*GJB2*) gene explain a high proportion of congenital deafness in several populations worldwide. The diversity of genes and genetic loci implicated in hearing loss defines the complexity of the genetic basis of hearing. This review focuses on the role of connexin 26 and mitochondrial 12S rRNA genes in hearing which will be helpful for better understanding of genes in sporadic and aminoglycoside-induced non syndromic hearing impairment.

Key words Aminoglycosides - connexins - heterozygosity - homoplasmic - matrilineal

Hearing loss is the most common sensory deficit in humans. It affects approximately 10 per cent of the world population¹, which is significant enough to compromise the development of normal language skills and social development. It can appear at any age with varying degrees of severity. In India, one in every 600 children has hearing impairment². Hearing loss can be classified based on age at onset (pre or post-lingual), type of ear defect (conductive, sensorineural or mixed), degree of hearing loss (mild, moderate, severe and profound), and can be syndromic/non syndromic³. Congenital/pre-lingual forms of deafness are always of sensorineural type, of which half are due to environmental factors (ototoxic drugs like aminoglycosides, cisplatin; bacterial/viral

infections and acoustic trauma) and the remaining due to genetic causes⁴. Seventy per cent of genetic cases are classified as non syndromic and 30 per cent are syndromic. Among the non syndromic, autosomal dominant (DFNA) contributes 22 per cent, autosomal recessive (DFNB) – 77 per cent, X-linked (DFN) – 1 per cent and mitochondrial (<1-20%)⁵.

Over the past one decade, remarkable progress has been made in identifying and cloning the genes for hearing loss. More than 100 genes that are involved in syndromic hearing impairment, have been mapped. To date, about 132 hearing impairment loci have been mapped for non syndromic hearing impairment, of which 54 gene loci are associated with autosomal dominant mode of inheritance, 67 gene loci with

autosomal recessive mode of inheritance, eight are X chromosome linked, one is Y-linked, and 2 are mitochondrial gene loci⁶. Fifty nuclear genes [(22 genes for autosomal dominant (*DFNA*), 28 for autosomal recessive (*DFNB*)], one X-linked (*DFN*) gene and two mitochondrial genes for non syndromic deafness have been characterized⁶. In some instances, different mutations at the same locus have been found to cause both syndromic and non syndromic forms of deafness. For example, the *DFNB1* locus is shown to cause both syndromic (Palmoplantar Keratoderma - PPK, Keratitis Ichthyosis deafness - KID) as well as non syndromic hearing impairment. In autosomal dominant, *DFNA9* (*COCH* gene) locus is the most common one whereas in X-linked, *DFN3* locus (*POU3F4* gene), and in mitochondria, 12S rRNA gene are the most common ones involved in hearing impairment.

Anatomically, human ear is divided into three distinct compartments- the outer, middle and inner ear. The outer ear or pinna collects the sound waves and channels these down the ear canal to vibrate the ear drum. The vibrations are transmitted as mechanical waves by the three ossicles of middle ear and transfer into oval window of inner ear where these are converted to nerve impulses. Human ear consists of three major parts such as outer, middle and inner ear. Inner ear is composed of two fluid-filled labyrinths – membranous labyrinth and bony labyrinth. The membranous labyrinth is filled with a liquid known as endolymph. It lies in a similar shaped cavity of the temporal bone known as bony labyrinth filled with perilymph. The membranous labyrinth consists of a snail shaped cochlea-the auditory sensory organ and a vestibular organ. The vestibular organ is responsible for the balance and consists of the sacculle, the utricle, and three semicircular canals³.

The inner ear is lined with sensory and supporting cells and is covered by a cellular membrane. The sensory cells are also called hair cells. The hair cells at their apical surface have a bundle of stiff actin-filled microvilli, also called stereocilia. Thirty to 300 stereocilia form a hair bundle, which is the mechanoreceptive structure of the hair cell. Each stereocilia interconnected with the next tallest stereocilia. The hair bundle and the remaining surfaces of the hair cells are immersed in liquids of different ionic composition. The endolymph is rich in potassium but not in sodium and calcium. The perilymph has low K^+ and high Na^+ and Ca^{2+} ion concentration⁷.

The sensory patch of the cochlea is called the organ of Corti. It lies on the basilar membrane and has

two types of hair cells: a single row of inner hair cells (IHC) and triple rows of outer hair cells (OHC). Inner hair cells transmit the signals and the outer hair cells are responsible for frequency selectivity.

A variety of epithelial cells line the membranous labyrinth of the cochlea. The stria vascularis - a secretory structure in the lateral wall of the cochlea consists of two cell barriers formed by the marginal cells and basal cells. Each barrier consists of a continuous sheet of cells joined by tight junctional complexes. Between these barriers is the discontinuous layer of intermediate cells and the tight junctions connecting the marginal, basal and endothelial cells make the intrastrial space - a separate fluid filled compartment with a capillary bed. The basal cells are joined by gap junctions to intermediate cells and to fibrocytes of adjacent connective tissue, indicating exchanges between these three cell types. In contrast, strial marginal cells are not coupled to each other or to other cells by gap junctions⁸. The stria vascularis secretes K^+ into the cochlear endolymph and produces the endocochlear potential⁹.

Mechanism of hearing

During audition, external ear collects airwave pressure and transfers it to the tympanic membrane. From the tympanic membrane, the vibrations are picked up by the three ossicles of the middle ear and transmitted to the inner ear. In the inner ear, the vibrations cause a displacement of the acellular membranes relative to the neuroepithelia in the cochlear duct.

This displacement provokes a deflection of the sensory hair cell stereociliary bundles that in turn open the mechanotransduction channels. The minute deflection of less than 1nm causes the influx of potassium ions from the potassium rich endolymph. This endolymph bathes the hair cells, which results in a change in membrane potential that is proportional to the intensity of the acoustic stimulus³.

The influx of ions causes depolarization of the hair cells that activate calcium channels on the basolateral side of the cells, leading to calcium influx into the hair cells. This influx triggers the release of neurotransmitter to the brain. The hair cells are repolarized when potassium ions leave these cells through potassium channels and enter the epithelial supporting cells. The potassium ions then diffuse to the stria vascularis through gap junctions formed by connexins and are secreted back into the endolymph through potassium channel⁹. Hence, the mutations in

connexins or gap junctions lead to the disturbance in the potassium ion circulation, which in turn results in hearing impairment.

In autosomal recessive, the first locus *DFNB1* (*Cx26* gene) was identified in two Tunisian consanguineous families with bilateral profound prelingual hearing impairment¹⁰. Subsequently, several families of different ethnic origin were identified to this locus¹¹⁻¹³.

Connexins

Connexins are a family of transmembrane proteins that form intercellular gap junction channels to allow ions and small molecules between adjacent cells. They are found in all mammalian tissues except circulating blood cells and adult skeletal muscles. Twenty two connexins have been identified in humans and 19 in the mouse genome¹⁴.

Each connexon comprises six connexins which are assembled in the endoplasmic reticulum or in the Golgi apparatus to form a connexon hemi-channel. These hemi-channels are transported to the plasma membrane of the cell with the help of vesicles and form an intercellular connexon-connexon channel by disulphide bonding once these find their counter part in the adjacent cell¹⁵.

Once the channel is formed, permeation and gating processes will occur in which permeation depends upon the number of small molecules, inorganic ions like Na⁺, K⁺, Ca²⁺, *etc.* and second messengers like cyclic AMP, inositol 1,4,5 triphosphate (IP3)¹⁶.

Gating is a reversible transition between connexon channel due to the result of non covalent and covalent modifications of the channel structure. Several extracellular and intracellular messengers like voltage, calcium and cell adhesion molecules can modify the gating property¹⁶. Connexons could be either homotypic or heterotypic and homomeric or heteromeric. Connexins have common structural organization with four hydrophobic transmembrane domains, two extracellular loops and one cytoplasmic loop with N and C termini¹⁷.

The N-terminus plays a role in voltage gating by non covalent or covalent modifications in the surrounding aminoacids. The transmembrane domains form the pore of the gap junction channel and decide channel permeability. Domain TM1 forms a charge complex and acts as a voltage sensor. The TM2 domain is involved in oligomerisation of connexins. TM3 is amphipathic and involved in the

lining of the channel¹⁸. Extracellular loops are highly conserved which consists of three cysteine residues mainly responsible for proper alignment of connexons between adjacent cells¹⁹.

Certain *Cx26* gene mutations are ethnic-specific such as, 35delG mutation prevalent in Caucasians, R143W in Africans, 167delT in Ashkenazi Jews, 235delC in Orientals and W24X in Indians²⁰. The high frequency of W24X in certain Caucasian populations like Slovak Romany, Spanish Romany where Romans also known as “Gypsies” implies their origin from the Indian subcontinent about a thousand year ago²¹. Worldwide, *Cx26* gene mutations accounts for about two to 69 per cent of the autosomal recessive non syndromic hearing impairment (ARNSHI) and it varies between different populations (Table I). Among the Caucasians, populations of Slovak Romany, Italy and Northeastern Hungary show very high rate of *Cx26* mutations. On the contrary, relatively low frequency rate was observed in a few African populations *viz.*, Kenya (2%) and Sudan (7%) while high frequencies in Tunisia (17%) and Egypt (19.8%) among other African countries. In India, *Cx26* gene frequency varies between 10-20 per cent.

In all these populations, *Cx26* gene mutations were highly observed in heterozygous condition, which proves the heterogeneity of non syndromic hearing impairment. Apart from *Cx26* gene in NSHI, certain mitochondrial genes may also be involved in NSHI.

At least five per cent of non syndromic hearing impairment (NSHI) is caused by known mitochondrial DNA (mt DNA) mutations which represent the second common mutation next to 35delG in Caucasian populations⁶⁰. This frequency may increase in the Oriental and Indian population. Recently, many novel mitochondrial mutations in NSHI have been reported⁶⁰.

Mitochondrial genetics

Mitochondria play an important role in the life and death of cells. There are hundreds of mitochondria in each cell with a variety of metabolic functions, the most important being the synthesis of ATP (chemical energy) by oxidative phosphorylation (OXPHOS). These also play an important role in the regulation of cell death and protection against reactive oxygen species⁶¹.

Each mitochondrion consists of 2-10 mitochondrial chromosomes, so, each cell contains thousands of mitochondrial chromosomes. The human

Table I. Frequency of Cx26 mutations among childhood hearing impaired in populations of different ethnic origin

Continent / Country	N	Frequency (%)	Reference
<i>Asia:</i>			
British Asian	51	23.5	22
Japan	53	26.4	23
	1227	12.0	24
China	60 (sporadics)	38.3	25
	58 (familial)	39.7	
India	215	50.2	26
	45 (families)	13.33	27
Pakistan	196	28.6	28
Indonesia	120	20.0	29
<i>Middle East Asia:</i>			
Israeli Arab	75	38.7	30
Lebanon	48	33.3	31
Palestine	48	23.0	32
Iran	168	11.0	33
	(83 families)		
Turkey	60	31.7	34
	14 (families)	21.4	35
(Anatolia)	371	27.8	36
	93	31.2	37
<i>Europe:</i>			
France	65 (families)	60.0	38
	140	41.3	39
	(104 families)		
	(159 families)	33.0	40
Italy	53	53.0	41
Poland	102	40.0	42
Greece	210	36.2	43
	(45 familial)		
	(165 sporadics)		
	173	35.2	44
Austria	46 (familial)	30.4	45
	40 (sporadic)	27.5	
	204	15.2	46
Jordan	68 (families)	16.2	47
Germany	342	11.11	48
	228	16.7	49
Slovak Romany	54	68.5	50
Czech	156	48.1	51
North Eastern	102	46.4	52
Hungary	(28 familial)		
	(92 sporadics)	64.1	
Spanish Romany	34 (families)	52.9	53
Altai	76	23.7	54
<i>Asia:</i>			
Tunisia	70 (families)	17.0	55
Kenya	406	2.2	56
Sudan	183	6.6	
Egypt	111 (families)	19.8	31
<i>Australia:</i>	243	21.0	57
<i>America:</i>	209	35.0	58
	737	22.2	59
N, no. of individuals			

mitochondrial DNA is a 16,569 nucleotide pair (np) closed, double-stranded circular molecule located within the cytoplasmic mitochondria. It transcribes polycistronic RNA transcripts that is subsequently cleaved to produce tRNAs, rRNAs and mRNAs. It consists of a promoter region called D-loop, which accounts for 7 per cent of mitochondrial genome length. Mitochondrial DNA contains 37 genes (heavy strand-28, light strand-9 genes) all of which are essential for normal mitochondrial function. These consists of 13mRNA (involved in oxidative phosphorylation), 2rRNAs and 22 organelle specific tRNAs which are required for assembling a functional mitochondrial protein-synthesizing system. The 13 mitochondrial proteins together with approximately 60 nuclear encoded proteins form the five enzyme complexes of the respiratory complex that are required for OXPHOS: complex I reduced nicotinamide adenine dinucleotide dehydrogenase, complex II, complex III cytochrome c oxidoreductase, complex IV cytochrome c oxidase and complex V ATP synthetase^{62,63}.

Eight of the nine genes on the light strand code for mitochondrial tRNA molecules. The entire molecule is regulated by only one regulatory region which contains the origins of replication of both heavy and light strands. The total mtDNA accounts only for about 0.5 per cent of the DNA in a nucleated somatic cell⁶⁴. But the rate of mutation in mtDNA is calculated to be about ten times greater than that of nuclear DNA, possibly due to a paucity of DNA repair mechanisms. This high mutation rate leads to a high variation between mitochondria, not only among different species but even within the same species^{60,64,65}.

Mitochondrial diseases

Mutations in mitochondrial genes encoding mitochondrial proteins or in the nuclear genes encoding mitochondrial proteins may lead to dysfunction of the OXPHOS system, apoptosis and oxidative stress control leading to a variety of multi-system disorders with pleiotropic effects. Mitochondrial DNA mutations have been implicated in a great variety of diseases ranging from rare neuromuscular syndromes, such as Kearns-Sayre syndrome (KSS), mitochondrial encephalomyopathy, lactic acidosis, and stroke like episodes (MELAS), myoclonic epilepsy and ragged red fiber (MERRF) and neuropathy, ataxia and retinitis pigmentosa (NARP) to common disorders such as hearing impairment, diabetes, Parkinson disease, Leber's hereditary optic neuropathy (LHON) and Alzheimer disease⁶⁶. Among the broad spectrum

of mitochondrial depletion syndromes, progressive external ophthalmoplegia (PEO), mitochondrial neuro gastrointestinal encephalomyopathy (MNGIE), sensory ataxia neuropathy, dysarthria, and ophthalmoparesis (SANDO), spino cerebellar ataxia with epilepsy (SCAE), and alpers⁶⁰, are a few conditions resulting due to mutations in the nuclear genes which control the integrity and replication of mitochondrial DNA, OXPHOS and apoptosis. The progressive breakdown of mitochondrial function with age might result in presbycusis-an age related hearing loss. It is the most frequent form of non syndromic deafness, affecting over 50 per cent of elderly. Fischel-Ghodsian *et al*⁶⁷ reported mutations in the mitochondrially encoded cytochrome oxidase II gene in the auditory system of five patients with presbycusis with great individual variability in both quantity and cellular location of these mutations.

Mitochondrial DNA mutations involve point mutations or large rearrangements. Large rearrangements usually cause syndromes involving various organ systems such as KSS, Pearson syndrome and progressive external ophthalmoplegia. Point mutations such as deletions, insertions, duplications or inversions can sometimes be limited to a few base pairs thereby unaffacting other organ systems⁶⁸.

Ototoxicity

Aminoglycoside antibiotics such as gentamycin, streptomycin, kanamycin and tobramycin, are commonly clinically used in the treatment of patients with aerobic Gram negative bacterial infections, especially in patients with chronic infections such as cystic fibrosis or tuberculosis^{69,70}. Aminoglycoside antibiotics are composed of amino sugars linked to a 2-deoxystreptamine ring. These drugs are highly polar cations, which are not easily metabolized⁷¹. The use of aminoglycosides can often lead to renal, vestibular and/ or auditory toxicity^{69,70,72}. The renal impairment is usually reversible, but ototoxicity is usually irreversible. While streptomycin and gentamycin cause vestibular damage, neomycin and kanamycin are responsible mainly for cochlear damage. Tobramycin affects both equally⁷⁰. Aminoglycosides are known to exert their antibacterial effects by directly binding to 16S ribosomal RNA in the 30S subunit of the bacterial ribosome, causing mistranslation or premature termination of protein synthesis^{73,74}. Decline in ATP production might hence cause an increase in the production of reactive oxygen species (ROS), thereby damaging mitochondrial and cellular proteins, lipids and nuclear acids. This in turn

would lead to the neuronal dysfunction or death of cochlear and vestibular cells in the auditory system, thereby causing hearing impairment or irreversible deafness⁷⁵.

12S rRNA gene mutations

The most common mutations in 12S rRNA (*MTRNR1*) gene causing non syndromic hearing impairment are A1555G, T961deT/insC, T961G, T1005C, T1095C, A1116G, T1243C, T1291C, C1494T and A827G. These gene mutations have also been involved in aminoglycoside induced non syndromic hearing impairment⁷⁶⁻⁷⁸.

The first homoplasmic mutation, A1555G in mitochondrial 12S rRNA was identified in Arab-Israeli family with NSHI with matrilineal mode of inheritance⁷⁹. Most of these individuals had congenital/prelingual sensorineural hearing impairment. The A1555G mutation is localized in a highly conserved region which is involved in decoding small ribosomal subunit⁸⁰. The new G-C pair in 12S rRNA created by the A1555G transition facilitates the binding of aminoglycosides^{81,82}, and causes hearing loss in individuals with this mutation when exposed to aminoglycosides^{79,83}. Studies from Asian, Caucasian and African populations worldwide showed that, the A1555G mutation has been found in many families with aminoglycoside-induced and NSHI^{81,84-86}. Nonsyndromic hearing loss with the history of aminoglycoside exposure in Japanese population showed 33 per cent of A1555G mutation⁸⁷, whereas only 13 per cent in Chinese population⁸⁸. However, children with non syndromic deafness without any exposure to aminoglycoside antibiotics were observed to be only 2.9 per cent in China and 3 per cent in Japan (Table II). Aminoglycosides appeared as a major modifier factor for the phenotypic expression of the A1555G mutation in the families with non syndromic hearing loss in studies carried out from China⁸⁸, as the penetrance on hearing loss was shown to increase with aminoglycosides⁹⁴. The prevalence of the A1555G mutation was shown to be between 20-30 per cent in deaf individuals in Spain and Asia of which 15 per cent of them had the history of aminoglycoside ototoxicity⁸⁵.

Recent studies indicate that the phenotypic expression of mtDNA mutations is highly variable which indicates that these mutations are not sufficient to produce the clinical phenotype⁹⁵. Some differences in either the nuclear gene content or activity appears to contribute significantly to the biochemical defect

Table II. Frequency of mt. A1555G mutation in NSHI with/without aminoglycoside exposure among the different ethnic origin

Population	Frequency (%)		Reference
	Amino-Glycoside Induced	Amino-Glycoside Non Induced	
<i>Asian</i>			
China	13	2.9	88
Japan	33	3	87
Indonesia	--	5.3	89
<i>Middle East Asian</i>			
Turkey	--	1.8	90
<i>Caucasian</i>			
UK	--	2.5	5
USA	--	0.6	91
Germany	--	0.7	92
Hungary	--	1.8	92
Danish	--	2.4	93

responsible for the penetrance of non syndromic deafness associated with this mutation.

A genome-wide scan carried out by Bykhovskaya *et al*⁹⁶ using parametric analysis of the Arab-Israeli family failed to identify a single major nuclear modifier gene. However, they reported a candidate locus for a nuclear modifier gene associated with the mtDNA A1555G mutation in a same family using nonparametric analysis.

In the presence of additional 12S rRNA gene mutations or nuclear mutations involved in transfer RNA modification (*TRMU-MTO2* and *GTPBP3* genes) or rRNA modification (*TFBIM* gene), the penetrance is higher⁷⁵. Some studies reported a high prevalence of *GJB2* heterozygous mutations in patients bearing the 1555A→G mitochondrial mutation suggesting that *GJB2* mutations may aggravate the phenotypic expression of 1555A-G 12S rRNA gene mutation⁹⁷. Thus, nuclear modifier genes or other modifier factors may modulate the phenotypic manifestation of the 12S rRNA gene mutations by interacting with the gene either suppressing/enhancing the effect of the mutation^{75,98}.

To facilitate the identification of additional mutations causing drug susceptibility in the 12S rRNA gene, an additional mutational analysis of the mitochondrial 12S rRNA gene was initiated in two hearing impaired Chinese populations which revealed a homoplasmic C-to-T transition at position 1494 (C1494T) in a large Chinese family with maternally transmitted aminoglycoside-induced and nonsyndromic deafness^{77,88,99}. Further sequencing of the complete

mitochondrial genomes in four Chinese subjects with aminoglycoside induced and non syndromic hearing impairment identified homoplasmic T1095C mutation in 12S rRNA gene^{77,88}. This mutation was also identified in an Italian family with neuropathy, Parkinsonism and ototoxicity¹⁰⁰ and another Italian family with maternally inherited non syndromic hearing loss¹⁰¹ and a Chinese subject with auditory neuropathy¹⁰². The genetically unrelated cases with aminoglycoside induced and non syndromic deafness showed the mtDNA mutations at position 961 in 12S rRNA gene. These include ET961C mutation in Caucasian and Asian subjects^{76,103}, 961-C insertion in Caucasian and Asian subjects¹⁰⁴, T961G mutation in Caucasian subjects⁹¹ and T961C mutation in Chinese subjects⁸⁸.

Although there have been significant advances in the knowledge of the molecular basis of hereditary deafness, the magnitude of the problem in India remains largely undefined. Extrapolating the worldwide incidence to one billion population, compounded by the large prevalence of consanguineous marriages, prevalence of deafness in our country is likely to be of significant health concern for India. Studies are warranted to understand the implication of both nuclear and mitochondrial mutations in the causation of non syndromic hearing impairment.

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