

# Genetics of Deafness

Guest Editors: Edi Lúcia Sartorato, Karen Friderici, and Ignacio Del Castillo



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Genetics Research International

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## Editorial

# Genetics of Deafness

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Hearing impairment is the most common sensorineural deficit worldwide. Deafness has a major genetic component, and understanding how genetic variation impacts hearing needs to be extensively studied. The importance of this work is reflected in the review by Lufkin: “the sense of hearing is one of the most crucial senses endowed to a living organism and its loss can have many ramifications.” This special issue about the Genetics of Deafness contributes to these studies by describing new mutations in genes important in hearing, by exploring the clinical implications of treatment based on genotype as well as reviewing of the literature.

Included in this issue are reviews of genetic syndromes where deafness is a significant component. Lassaletta and colleagues set out an important review of the molecular mechanism of vestibular schwannoma. Pillion and colleagues characterize hearing loss in osteogenesis imperfecta. Tomelleri’s group explores MELAS. All address molecular pathology of hearing loss and explore treatment options.

Several papers evaluate the distribution or effect of sequence variation in known hearing loss genes. Leal and colleagues describe novel mutations in two previously identified nonsyndromic hearing loss genes. Mateos et al. describe the effects of noncoding sequence variation in *GJB2*.

Vilarino and colleagues detail the distribution of mutations in pediatric patients in Portugal. Reports of this type improve our understanding of the mechanisms of hearing and the relevance of sequence variation in gene function.

Mutations in *GJB2* are the leading cause of congenital deafness in many countries. Malekpour and colleagues present a large study detailing the performance of cochlear implants in patients with and without *GJB2* mutation.

The complexity of genetic deafness and the tremendous progress associated with inherited hearing loss make it clear to us that the “silence genes” will have a lot more to tell us.

Edi Lúcia Sartorato  
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Ignacio Del Castillo

## Clinical Study

# Mitochondrial Sensorineural Hearing Loss: A Retrospective Study and a Description of Cochlear Implantation in a MELAS Patient

**Mauro Scarpelli,<sup>1</sup> Francesca Zappini,<sup>1</sup> Massimiliano Filosto,<sup>1,2</sup> Anna Russignan,<sup>1</sup> Paola Tonin,<sup>1</sup> and Giuliano Tomelleri<sup>1</sup>**

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Hearing impairment is common in patients with mitochondrial disorders, affecting over half of all cases at some time in the course of the disease. In some patients, deafness is only part of a multisystem disorder. By contrast, there are also a number of "pure" mitochondrial deafness disorders, the most common probably being maternally inherited. We retrospectively analyzed the last 60 genetically confirmed mitochondrial disorders diagnosed in our Department: 28 had bilateral sensorineural hearing loss, whereas 32 didn't present ear's abnormalities, without difference about sex and age of onset between each single group of diseases. We reported also a case of MELAS patient with sensorineural hearing loss, in which cochlear implantation greatly contributed to the patient's quality of life. Our study suggests that sensorineural hearing loss is an important feature in mitochondrial disorders and indicated that cochlear implantation can be recommended for patients with MELAS syndrome and others mitochondrial disorders.

## 1. Introduction

Mitochondrial diseases are disorders caused by impairment of the mitochondrial respiratory chain. The genetic error can affect both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) [1].

MtDNA mutations are classified as either large-scale rearrangements (partial deletions or duplications), usually sporadic, or point mutations, which are usually maternally inherited, and concern genes responsible for protein synthesis (rRNAs or tRNAs), or genes encoding subunits of the electron transport chain (ETC) [2]. The phenotypic expression of mtDNA mutations depends on the affected gene, its tissue distribution, and the different dependency of different organs and tissues on the mitochondrial energy supply. Visual and auditory pathways, heart, central nervous system (CNS), and skeletal muscle are the tissues mostly involved, because of their dependence on aerobic energy production [1].

Hearing impairment is common in patients with mitochondrial disorders, affecting over half of all cases at some time in the course of the disease [3]. Although the final common pathway for the hearing loss is thought to involve ATP deficiency secondary to a biochemical defect of the respiratory chain, the clinical presentation of mitochondrial deafness varies considerably, both in terms of associated clinical features and of natural history. In some patients, deafness is only part of a multisystem disorder, often involving the central nervous system, neuromuscular system, or endocrine organs; in other cases, deafness may represent a feature of an oligosyndromic disease [4].

By contrast, there are also a number of mitochondrial "pure" deafness disorders, the most common probably being maternally inherited deafness due to the A1555G mutation in the 12 s rRNA gene, MTRNR1 [5]. The use of streptomycin and to a lesser extent other aminoglycoside antibiotics can cause hearing loss in genetically susceptible individuals.

These drugs are known to exert their antibacterial effects at the level of the decoding site of the small ribosomal subunit, causing miscoding or premature termination of protein synthesis [6]. The hearing loss is primarily high frequency and may be unilateral. Risk factors for aminoglycoside ototoxicity include therapy lasting more than 7 days, elevated serum levels, prior exposure to aminoglycosides, noise exposure, high daily dose, use in neonates, and a background of predisposing mutations. Several mutations in the MTRNR1 gene encoding the 12S rRNA (961delT/insC, T1095C, C1494T, A1555G, and possibly A827G, T1005C and A1116G) and possibly also mutations (G7444A) in the COI/MTTS1 gene overlap can contribute to ototoxic hearing loss [7–11]. The MTRNR1 mutations probably alter the secondary structure of the 12S rRNA molecule, so that it resembles its bacterial counterpart, the 16S rRNA, more closely. As the bacterial 16S rRNA molecule is the target of aminoglycoside action, this might explain the cumulating effect of these MTRNR1 mutations and the use of aminoglycosides [12]. Mitochondrial nonsyndromic sensory neural hearing loss (SNHL) is also associated with the A7445G, 7472insC, T7510C, and T7511C mutations in the tRNASer (UCN) gene, MTTS1 [13].

The pathological examination of the inner ear is technically demanding, highly specialized, and only possible postmortem. There have, therefore, only been a few detailed pathological studies of the auditory system in patients with mitochondrial diseases. In the cochlea, the stria vascularis maintains the ionic gradient necessary for sound transduction and the complex interaction between inner and outer hair cells [14]. These components are highly metabolically active, and it is likely that a respiratory chain defect and the attendant relative deficiency of intracellular ATP would impair the function of both the stria and the air cells, ultimately leading to cell death, possibly through apoptosis [15].

Hearing loss is usually peripheral (due to cochlear or auditory nerve dysfunction), but in patients with a multi-system mitochondrial disorder, the auditory system may be affected at the brain stem, midbrain or at a higher level in the auditory cortex. The peripheral hearing loss typically affects high frequencies first, followed by intermediate frequencies, and finally involving low frequencies and causing the typical “flat” audiogram seen in a severely deaf individual. The preferential involvement of high frequencies may be related to the relatively high energy requirements of the basal cochlea [16]. The vast majority of patients with mitochondrial deafness have absent otoacoustic emission, providing strong evidence that the cochlea is the component most sensitive to mitochondrial dysfunction [17, 18].

In this review, we analyzed the results of a retrospective study about the presence of hearing loss in a cohort of patients with genetically confirmed mitochondrial disease and we described the results and follow-up of a MELAS patient who underwent cochlear implantation.

## 2. Patients and Methods

**2.1. Patients.** We retrospectively analyzed the last 60 genetically confirmed mitochondrial disorders diagnosed in our

Department in order to identify patients affected with hearing loss. Males and females were quite equally distributed (33 males and 27 females), with an age at diagnosis ranging from 8 to 73 years; the mean age of this group of patients was 45 years old. Three patients belonged to the same family.

**2.2. Methods.** Most of patients were referred to our centre for a suspected mitochondrial disease and underwent a muscle biopsy (quadriceps or deltoid muscle). In these cases, DNA was extracted from frozen muscle tissue and used for direct sequencing of mitochondrial and/or nuclear genes, with standard methods [19, 20]. In some other cases, family history was strongly suggestive for mitochondrial disorder so that patients were not submitted to muscle biopsy, and DNA to perform molecular analysis was extracted from their blood.

Presence of single or multiple deletions of mitochondrial DNA was revealed by long-range PCR or southern blot analysis, using standard methods [20]. *mtDNA* point mutations were searched by screening the whole *mtDNA* using the MitoScreen Assay Kit for the Transgenomic WAVE System and DHPLC (denaturing high-performance liquid chromatography) with single-stranded conformational polymorphism analysis, following supplier's indications or by using the ABI PRISM BigDye Terminators v3.0 Cycle Sequencing Kit.

All of the cases included in this study performed audiometric examination before to receive the genetic diagnosis. The cut-off of hearing loss was defined according to the mean hearing loss at frequencies of 250, 500, 1000, 2000, and 4000 Hz as follows: normal hearing  $\leq$  20 dB hearing loss.

## 3. Results

Out of the 60 cases, 28 had bilateral sensorineural hearing loss, whereas 32 did not present ear's abnormalities. Clinical findings and the frequency of SNHL are summarized in Table 1 and Figure 1. All of the three patients belonging to the same family had A3243G mitochondrial DNA mutation and hearing loss.

One of the 28 patients with hearing loss, a MELAS patient, was submitted to cochlear implantation. Following, we briefly reported his main clinical notes.

He was a 46-year-old man who started to complain mild bilateral hearing loss from his 20; he had normal prenatal and perinatal histories. His family history was also normal. Diabetes mellitus was noted at the age of 33, and insulin therapy was initiated. Serum biochemical studies showed a high level of lactic acidosis, which increased after exercise, and echocardiography disclosed the presence of hypertrophic cardiomyopathy. Neurological examination showed diffuse mild muscle weakness, more evident in distal lower limbs, with stepping gait. Computer tomography (CT) and magnetic resonance imaging (MRI) scans showed no abnormality in either inner ear. MRI demonstrated mild cerebellar atrophy.

Muscle biopsy revealed strong reactive vessels on Succinate Dehydrogenase stain (SDH), without ragged red neither cytochrome C-oxidase negative fibers. Molecular analysis

TABLE 1: Clinical and molecular features of patients analyzed in our study.

Clinical syndrome	Molecular defect	Hearing loss	Non hearing loss	Total
MIDD	A3243G	4	0	4
MELAS	A3243G	11	10	21
MERRF	A8344G	6	1	7
PEO	Single deletion (4) Multiple deletions (20)	4	20	24
MNGIE	TYMP mutations	3	1	4

MIDD: Mitochondrial inherited diabetes and deafness; MELAS: mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MERRF: myoclonic epilepsy with ragged red fibers; PEO: progressive external ophthalmoplegia; MNGIE: mitochondrial neurogastrointestinal Encephalomyopathy.

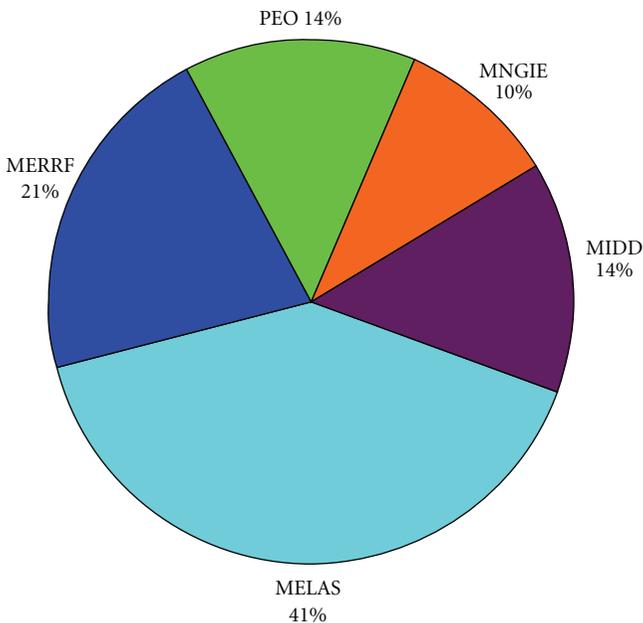


FIGURE 1: Graph of distribution of hearing loss into the clinical syndromes.

revealed the presence of the A3243G mitochondrial DNA heteroplasmic mutation.

During next years, hearing loss markedly worsened: pure-tone audiometry revealed bilateral profound hearing loss with an average of 130 dB in both ears. The hearing aid was no longer useful. In a promontory stimulation test, the patient responded to electrical stimulation in both ears at the intensity of 0.3 mA.

At 33 years old, he underwent cochlear implantation surgery using a 24-channel device (cochlear-contour) in the right ear. Twenty-five electrode rings were successfully inserted and all 22 channels and 2 extracochlear electrodes were found to be usable at the initial “switch on” of the cochlear implant.

Clinical followup was quite stable: he referred sometimes episodes of gastrointestinal pseudoobstruction and few

periods of scarce glycemic control; outcome of cochlear implantation was good: postoperative neural response telemetry (NRT), implant-evoked BAEP and middle latency response (MLR) showed good responses. Eight years after the surgery, the patient could use the telephone and was satisfied with the improvement in communication due to the cochlear implant.

#### 4. Discussion

Hearing impairment is a common feature of mitochondrial disease, either in isolation, or as a part of a complex multi-system disorder, with the cochlea bearing the brunt of the pathology. Ultimately, all forms of mitochondrial deafness arise through a respiratory chain defect causing ATP depletion, but it is not clear why hearing should be preferentially affected in some mitochondrial disorders and not in others, nor why cochlear pathology can vary between different disorders. There is clear evidence of a major environmental influence in some forms of mitochondrial deafness, and the interaction between nuclear and mitochondrial genes appears to be important [21].

The degree of hearing loss correlates well with the mutation load in skeletal muscle [3], and the progressive nature of the hearing loss may be related to the accumulation of mutated mtDNA within the cochlea. Although this appears to be the general trend, there are clear exceptions to the rule. In one patient with the A3243G mutation, severe hearing loss was associated with low levels of mutated mtDNA in skeletal muscle [22]. This may occur because of unequal segregation of mutated mtDNA among different tissues during early development, so that occasionally, by chance, high levels are present in the cochlear precursors, and lower levels in skeletal muscle precursor cells.

The percentage of mutated mtDNA undoubtedly contributes to the clinical variability seen among patients, but this does not provide the whole explanation. It is not currently known why certain maternal pedigrees transmitting A3243G tend to develop a pure deafness-diabetes phenotype, whereas others only show ptosis and external ophthalmoplegia, and yet others are affected by severe multisystem MELAS phenotype [23]. Additional genetic factors are likely to be important, but have yet to be identified [21].

Our study considers only the syndromic form of mitochondrial SNHL, showing that the frequency of hearing loss in our group of patients is the same of the most of studies reported in literature [3, 21]; moreover, it suggests how this form does not present difference about sex and age of onset into each single group of diseases (data not shown). One limit of our study is the retrospective analysis that does not allow to define in patients without hearing loss if this deficit will develop in the future and does not give the real severity of hearing loss within different categories.

However, the study offers important considerations, like the relative low frequency of hearing loss in patients with chronic progressive external ophthalmoplegia (CPEO) (16%), which could be considered in most of cases a “pure” myopathy, and, by contrast, the high frequency of hearing

loss in patients affected by mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (75%), in which the diagnosis may be difficult at the beginning and the presence of hearing loss could orient clinicians in considering a mitochondrial disease in the differential diagnosis [24].

The case presented in this study remarks the importance to consider cochlear implant in patients with mitochondrial SNHL. The patient suffered from hearing loss due to MELAS syndrome; after cochlear implantation his quality of life markedly improved and he could preserve his work.

Since the first recorded cochlear implant in a patient with Kearns-Sayre syndrome [25], many patients have successfully received implants [13]. In many ways, patients with mitochondrial disease are “ideal” recipients of a cochlear implant because the hearing loss develops well after speech development, and often in isolation (as in patients with diabetes and deafness due to A3243G, or non-syndromic deafness due to A1555G). A systematic review of literature (March 2003) identified 12 detailed descriptions of patients with mitochondrial sensorineural deafness who had cochlear implants [13]. All 12 cases had profound postlingual deafness. The age of onset of the deafness and the age at surgery varied, but 58% were able to converse on the telephone following the procedure, and the remainder had good open-set speech recognition. There were no reported complications. The procedure should, however, only be undertaken with caution, because the implantation procedure requires a general anesthetic and takes a number of hours, and because it is also important to consider the natural history of the disorder in the individual patient. This is the case of MNGIE, in which the mean age of death is 37 years [26], but the presence of new therapeutic options gives hopes for patients with this devastating neurodegenerative disorder and could modify the prognosis. Very recently, Li and colleagues described a successful multichannel cochlear implantation in a 28-years-old MNGIE woman [27], confirming the importance to consider and treat ear’s problems also in these types of disorders.

In conclusion, individuals who harbour mtDNA mutations may be at risk of developing severe hearing deficit, and these individuals should avoid ototoxic agents, such as aminoglycoside antibiotics, which may further compromise cochlear function [3].

Our study suggests that SNHL is an important feature in mitochondrial disorders and should be considered in the diagnostic workup and management of patients with suspected mitochondrial disease. We found that cochlear implantation in a patient with multisystem degenerative disease greatly contributed to the patient’s quality of life and made it possible for him to communicate with family members and caregivers better than previously.

The results indicated that cochlear implantation can be recommended for patients with MELAS syndrome and other mitochondrial disorders, if they have residual retrocochlear function.

## Conflicts of Interest

The authors declare that they have no conflict of interests.

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## Review Article

# The Molecular Biology of Vestibular Schwannomas and Its Association with Hearing Loss: A Review

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Hearing loss is the most common symptom in patients with vestibular schwannoma (VS). In the past, compressive mechanisms caused by the tumoral mass and its growth have been regarded as the most likely causes of the hearing loss associated with VS. Interestingly, new evidence proposes molecular mechanisms as an explanation for such hearing loss. Among the molecular mechanisms proposed are methylation of TP73, negative expression of cyclin D1, expression of B7-H1, increased expression of the platelet-derived growth factor A, underexpression of PEX5L, RAD54B, and PSMAL, and overexpression of CEA. Many molecular mechanisms are involved in vestibular schwannoma development; we review some of these mechanisms with special emphasis on hearing loss associated with vestibular schwannoma.

## 1. Introduction

Vestibular schwannomas (VSs) can be classified into two broad groups: unilateral sporadic vestibular schwannoma and those associated with neurofibromatosis type 2 (NF2). VSs constitute 8% of all benign intracranial tumors, and sporadic unilateral schwannomas represent up to 95% of all VSs [1]. As new population-based studies are performed, the true incidence of VS appears to be higher than expected [2–5]. A nationwide study performed in Denmark [2] revealed that the incidence of VS had been rising from 5 cases per million population per year in 1977–1981 to 10 cases in 1992–1995. In 2004, the same research group estimated an incidence of 11.5 cases per million inhabitants per year during a 25-year period (1976–2001) [3]. Data from a US national tumor registry (2010) reported a VS incidence rate of 1.1 cases per 100,000 people per year [4]. On the other hand, Evans et al. found an incidence of 1 case in 80,000 individuals for sporadic VS, and 1 in 70,000 if NF2-related

tumors were included [5]. These increasing numbers are probably due to the effect of newer and more sensitive diagnostic tests, especially magnetic resonance imaging (MRI). The age of presentation of VS is usually the fourth and fifth decades. Even though a benign tumor, if large enough, can cause neurological symptoms like hydrocephalus, brainstem compression, herniation, and ultimately death.

NF2 is an autosomal dominant disease representing 5% of all VSs. Patients with NF2 are characterized by having bilateral vestibular schwannomas. Half of these patients do not have a family history of the disease [1] and therefore represent new germline mutations. The Manchester criteria for the diagnosis of NF2 have been described elsewhere [6, 7]. These patients can also present other intracranial benign tumors. There are three types of NF2, distinguished according to clinical presentation and severity: Wishart type, Gardner type, and mosaic NF2. The Wishart type appears in childhood or late adolescence and consists of bilateral vestibular schwannomas associated with spinal tumors. The

Gardner type appears later in life and is less debilitating, with patients developing bilateral vestibular schwannomas but few meningiomas. Mosaic NF2 occurs when a postzygotic mutation takes place and only a portion of the cells carry this mutation. Around 25% of NF2 patients with apparently healthy parents have a mosaicism [8].

Schwannomatosis has been recently defined as a new form of neurofibromatosis. It consists of multiple schwannomas without associated vestibular schwannomas [1]. The main symptom of affected patients is pain. The SMARCB1 gene has been found to be mutated in schwannomatosis patients [9–12]. SMARCB1, located in chromosome band 22q11.2 [9–12], is a tumor suppressor gene that encodes the INI1 protein. Germline mutations have been described in both familial [10–12] and sporadic [9, 11, 12] cases of schwannomatosis. Immunohistochemical analysis of VS samples [12] detected a mosaic pattern of INI1 expression in 93% of familial schwannomatosis patients, 55% of sporadic schwannomatosis cases, 83% of NF2-associated tumors, and 5% of solitary sporadic schwannomas. These findings suggest that the SMARCB1 gene might also play a role in NF2 tumors [12].

It is estimated that 80% of patients with VS initially complain of hearing loss or tinnitus. Such hearing loss could be the result of various mechanisms [13]. Among them are direct compression of the cochlear nerve by the tumor; occlusion or vascular compression of the internal auditory artery; intratumoral bleeding; biochemical changes in the inner ear, caused by vascular compression or internal auditory canal occlusion. Another cause that has emerged as an interesting possible explanation of this symptom relates to molecular data. Some authors [13, 14] suggest that a degeneration of the inner ear may be caused by a toxic substance produced by the tumor, or by a deficiency in a factor that is crucial for proper inner ear function. Dissociation between tumor size and hearing loss has been described elsewhere [13, 15] and could be an objection to the theory that compression is the sole cause of hearing loss. New ways to explain deterioration of hearing in patients with VS are needed.

The molecular biology of VS has been explained by several pathogenic mechanisms including NF2 gene mutation [1, 16, 17], chromosome 22 loss [16–20], NF2 gene mitotic recombination [18, 20], DNA methylation [21, 22], deregulation of genes [23], immunogenic factors [24, 25], cytokines, and growth factors [26–30]. Early data on the genetic alterations of VS were provided by cytogenetic studies [31–37]. Monosomy 22 was identified in various types of schwannomas, including VS. The incidence of total loss of chromosome 22 varies among VS studies and can reach 50% [31–37]. Other chromosome changes have been observed rarely, and, although these variations do not show a consistent pattern, losses of chromosomes 14, 16, 17, 18, and Y have been observed in at least one sample [31–37]. Warren et al. [38] studied 76 vestibular schwannoma samples, finding that 10% of the tumors showed copy gains in chromosome 9q34. Three tumors had gains in 17q, and, in three or fewer tumors, copy gains and copy losses were identified in chromosomes 10, 11, 13, 16, 19, X, and

TABLE 1: Molecular mechanisms described in VS growth.

NF2 gene mutation
Loss of chromosome 22
NF2 gene mitotic recombination
DNA methylation
Deregulation of genes
Immune response alteration
Growth factors and cytokines

Y. The relevance of copy gains in chromosome 9 is still under investigation. In parallel, loss of heterozygosity (LOH) studies have demonstrated that deletions of chromosome 22 have occurred in up to 80% of schwannomas, including VS cases. These findings suggest that genes located on this chromosome play an essential role in VS development [39–41]. Allelic losses at 1p have also been described in a few cases [42]. Mitotic recombination consists of deletion followed by reduplication. This mutational mechanism can generate two identical copies of a mutated gene in the absence of a wild-type copy [17]. This mechanism is responsible for LOH in a proportion of schwannoma cases [18, 20] (see Table 1).

## 2. NF2 Gene

The molecular study of VS began in 1993 with the identification of the NF2 gene which contains 17 exons and is located in chromosome 22q12.2 [43, 44]. The coding region of messenger RNA is 1785 base pairs in length and encodes 595 amino acids, producing a protein named merlin (for “moesin-ezrin-radixin-like protein”) or schwannomin (derived from schwannoma). This family of proteins presents an N-terminal globular domain (FERM domain), an  $\alpha$ -helical stretch, and a charged C terminus at the end [1, 16]. It is believed that this protein acts by linking the actin cytoskeleton to the plasma membrane. Merlin has the ability to change its conformation status. It can fold into itself (closed conformation) or be unfolded (open conformation). This can be achieved by folding its alpha helical portion and c-terminal portion [45]. P21-activated kinase 1 (PAK), a downstream effector of Rac1, promotes the phosphorylation of merlin at S518 with conversion to an open conformation, initiating its degradation [46]. The folded form of merlin is known for its tumor-suppressing properties [47]. The folded version of merlin binds to DCAF1 and suppresses cell proliferation by inhibiting E3 ubiquitin ligase CRL4 [48]. CRL4<sup>DCAF1</sup> plays a role in DNA replication and, if inactivated by merlin, favors the upregulation of genes related to apoptosis and cell-cycle arrest [48].

Rac1 is a member of the Rho GTPase family and regulates signaling pathways such as MAPK, JNK/SAPK, NF- $\kappa$ B, and PI-3K [49, 50]. Rac1 is associated with tumorigenesis [50]. Merlin downregulates Rac1-mediated canonical Wnt signaling, becoming a negative feedback loop preventing Rac1 activation and therefore phosphorylation by PAK [51–53].

There is an association between Merlin, CD44, and  $\beta$ 1-integrin. CD44 is a transmembrane hyaluronic acid receptor

implicated in cell-cell adhesion, cell matrix adhesion, cell motility, and metastasis [1]. Herrlich et al. [54] demonstrated that at high cell density, hypophosphorylation of merlin occurs, which inhibits cell growth. On the other hand, at low cell density, phosphorylation of merlin occurs, becoming growth permissive. In both scenarios, merlin interacts with CD44, promoting cellular contact-dependent inhibition.

The NF2 gene has been shown to be a tumor suppressor gene. This was evident in mouse models, where overexpression of the NF2 gene (merlin) has been proven to limit cell growth in mouse fibroblast and rat schwannoma cells [55, 56].

In various studies, epidermal growth factor (EGF) and epidermal growth factor receptor B (ErbB2) are upregulated in VS cells [57, 58]. Merlin's proliferative activity depends on the regulation of these factors. Neuroglin induces proliferation of VS cells by binding to ERBB2 and ERBB3 [58], as subsequent activation of PI3K and MAPK (mitogen-activated protein kinase) pathways occurs. Both pathways are associated with cellular invasion [27]. MAPK is regulated by mitogen stimuli mixed lineage kinase 3 (MLK3). Finally, merlin has been shown to inhibit MLK3 and epidermal growth factors, demonstrating its tumor suppressive properties [59].

These molecular and signaling pathways can help us to understand new therapies for VS treatment, some of which will be discussed further below.

### **3. Mutations of the NF2 Gene, DNA Methylation, and Hearing Loss**

Mutations of the NF2 gene have been found in both NF2 and unilateral sporadic schwannoma patients. More than 200 mutations have been identified to date, including single-base substitutions, insertions, missense, and deletions [60]. NF2 gene inactivation is necessary for VS to grow. According to Knudsen's two-hit hypothesis, in NF2 patients, the germline NF2 allele is inactivated and tumors occur when a wild-type allele is inactivated by allelic loss, silencing, or mutation [17]. On the other hand, sporadic unilateral VS formation is explained by somatic biallelic NF2 inactivation [17]. Regarding the effect of age on unilateral sporadic VS formation, Evans et al. [61] have hypothesized that new somatic mutations are added to the NF2 gene due to impaired DNA repair mechanisms. They also found an increased ratio of somatic frameshift to nonsense mutations with increased age at diagnosis.

Hadfield et al. [20] identified germline mutations in 89% of a sample of 97 patients with an NF2 diagnosis and a second mutational event in 79% of the sample. LOH was the most common form of second hit. Mitotic recombination was the cause of LOH in 14 out of 72 total evaluable NF2 tumours. On the other hand, in a sample of 104 patients with unilateral sporadic VS, 66% had at least one somatic point mutation identified by MLPA (Multiplex ligation dependent probe amplification), loss of heterozygosity (LOH) was found in 56%, and mitotic recombination was a cause of LOH in 6%.

The genotype-phenotype correlation in NF2 patients has been studied. While some authors deny any correlation [1, 62], others affirm it [63, 64]. Recent evidence has detected genotype-phenotype correlation in NF2 patients [65–68]. The Manchester group [65] studied 411 NF2 patients and correlated the presence of meningiomas with gene mutations. Interestingly, they did a genotype-phenotype correlation based not only on the type of mutation but also on the position effect of the mutation itself. Patients with mutations in exons 14 or 15 develop meningiomas less frequently; in other words, patients with mutations located in exons 1 to 13 had a higher risk of developing meningiomas. Regarding the type of mutation, it was observed that individuals with truncating mutations (nonsense or frameshift) had a higher risk of meningiomas than patients with missense or splicing mutations (58% versus 26% versus 35%, resp.). This finding had already been described by Selvanathan et al. [66] and Evans et al. [67], where nonsense and frameshift mutations were associated with more severe NF2 symptoms.

Abo-Dalo et al. [68] demonstrated that clinical features in individuals with large intragenic deletions were similar to those in individuals with mutations affecting single or multiple nucleotides. Milder phenotype was seen in deletions affecting 3' exons 15 and 16 of the NF2 gene, corroborating the same finding of Smith et al., where mutation position was fundamental in phenotype expression.

Currently, there is a new understanding of the role played by the type and position of NF2 mutations in the phenotype of the disease. Nevertheless, it has been reported that families and identical twins with NF2 carrying the same mutation can develop different phenotypes [69, 70].

Besides the type and position of the mutation [71], there are other possible mechanisms that can explain NF2 inactivation [1]. Presence of a modifier gene [72], methylation of the regulatory region [21, 22, 73], posttranscriptional alternative splicing, and differential polyadenylation of the NF2 gene [74] are proposed as possible causes. On the other hand, the NF2 gene promoter area is a region of DNA that facilitates transcription. Welling et al. [75] described how methylation of the regulatory region of the NF2 gene occurs. Both positive and negative cis-acting regulatory elements required for transcription of the NF2 gene have been found on the 5' flanking region of the promoter. This region is rich in G/C and susceptible to inactivation and methylation.

Robertson [76] covered the DNA methylation process extensively, defining it as a crucial epigenetic modification of the genome that is involved in regulating many cellular processes, including transcription. This epigenetic modification of DNA consists of methylation of cytosine at position C5 in CpG dinucleotides. CpG islands are generally unmethylated. It has been claimed that DNA methylation represses transcription directly, by inhibiting the binding of transcription factors, or indirectly, by recruiting methyl-CpG-binding proteins. Hypermethylation of the promoter-associated CpG islands leads to transcriptional silencing and finally to epigenetic inactivation of tumor suppressor genes [21]. The DNA methylation of VS has not been fully studied. Data on the methylation status of the promoter region of RASSF1A in brain tumors have been reported

[77]. Only 10% of schwannoma cases displayed this DNA modification. Kino et al. [73] found methylation of 3 CpG islands in 14 of 23 VS patients. Gonzalez-Gomez et al. [22] and Bello et al. [78] identified the 5 tumor-related genes most frequently methylated in VS cases (THBS1, TP73, MGMT, NF2, and TIMP3). These tumor-related genes were chosen on the basis of their critical cancer-related functions, since they are frequently hypermethylated and silenced in other neoplasms. Among their known functions are tumor suppression, angiogenesis and invasion inhibition, DNA repair, and detoxification. NF2 gene promoter elements showed hypermethylation in 18% of cases, which suggests an alternative mechanism of NF2 gene inactivation. Aberrant methylation of the NF2 gene could be considered a relatively early event, whereas hypermethylation of other tumor-related genes might represent secondary changes [22]. These results were demonstrated to be specifically due to negativity on control tissues (nonneoplastic nerve sheath and brain samples).

To understand the role of TP73 (located at 1p36.3), we have to be aware of its homology, in terms of structure and conformation, with TP53 (located at 17p13.1). Both genes are involved in apoptosis and inhibition of cell growth. It has been proposed that p53 haploinsufficiency has a role in the development of VS [79]. TP73 null mice show specific developmental defects but no spontaneous tumors, giving rise to multiple protein isoforms with opposite biological properties [80]. Ahmad et al. [79] stated that p73 can produce multiple protein isoforms generated by alternative promoters and alternative splicing. Overexpression of p73 in various carcinomas correlates with poor prognosis [80]. Allart et al. [81] suggested that TP73 plays a major role in cellular differentiation and apoptosis in neuronal tissues. Ahmad et al. [79] confirmed this in their study of 34 VS tissues, which found expression of p73 in 41% of the specimens. Furthermore, after transfecting experimental vestibular schwann cells with p73 plasmid and exposing them to ionizing radiation, an increase in early apoptosis, late apoptosis, and necrosis was observed, as compared to a control group.

Lassaletta et al. [21] explored the methylation status of 16 tumor-related genes in 22 unilateral VSs. DNA methylation values of 9 to 27% were found in 12 of the genes tested: RASSF1A, VHL, PTEN, TP16, CASP8, TIMP3, MGMT, DAPK, THBS1, HMLH1, TP73, and GSTP1. The association discovered between TP73 aberrant methylation and hearing loss was remarkable. The corrected hearing thresholds for patients with methylated and unmethylated TP73 genes were 43 dB and 17 dB, respectively ( $P = 0.04$ ). The frequency most affected was 1000 Hz.

Additionally, Lassaletta et al. [21] found that methylation of TP73 had no association with age, clinical growth index, or tumor size. Other clinical findings concerning tumor-related genes included an association of methylation of CASP8 with age and tumor size and an inverse correlation between RASSF1A methylation and clinical growth index.

The scientific relevance of methylation to hearing loss needs further study, as, at present, methylation of TP73 is the only mechanism implied in hearing loss pathogenesis [6].

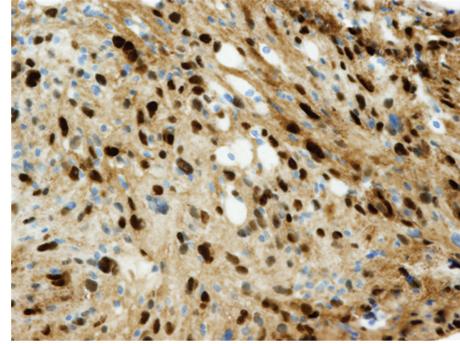


FIGURE 1: Positive immunostaining (+++) for cyclin D1.

#### 4. Deregulated Genes in Vestibular Schwannoma

Molecular studies are based on the gene expression of tumors. According to new data [1, 19, 82, 83], it is now possible to differentiate one tumor from another in ways unavailable to histopathology. Molecular investigation also suggests that mutations alone cannot explain the diverse behavior of VS. Welling et al. [82] studied 7 vestibular schwannomas by microarray DNA analysis, concluding that 42 genes were significantly upregulated in six of seven tumors studied. Among the upregulated genes were mediators of angiogenesis like endoglin (an endothelial marker of angiogenesis) and osteonectin (a promoter of cell migration). Downregulated genes included an apoptosis-related putative tumor suppressor gene, LUCA-15. The retinoblastoma protein (pRb) is encoded by the RB1 gene. Lasak et al. [84] examined a deregulated signaling pathway, the retinoblastoma protein (pRb)-cyclin-dependent kinase (CDK) pathway, which was downregulated in 7 of 8 tumors. This signaling pathway is involved in the G1 to S cell cycle progression, promoting cell proliferation. As already stated by Cayé-Thomasen et al. [23], the available data for the CDK pathway is conflicting, since one group found downregulation [84] while the other found upregulation [23]. Gonzalez-Gomez et al. [22] had previously suggested an inverse link between the methylation of RB1 and p16<sup>INK4a</sup> (found in 15% of samples) and cell cycle regulation, with the latter being altered through epigenetic changes. This in turn may explain the RB1-CDK pathway deregulation [84].

Deregulated expression of growth regulatory genes might play a role in VS progression. Cyclin D1 is a cell-cycle regulatory protein for the mammalian G1-S phase transition and is implicated in cell proliferation and differentiation. Lassaletta et al. [85] found, using immunohistochemistry, cyclin D1 expression in 52% of their cases. This deregulated gene was more frequently present in tumors with nuclear degenerative changes (Figure 1). Patients with negative cyclin D1 expression had a longer duration of deafness ( $P = 0.02$ ) and higher 2,000 Hz hearing thresholds ( $P = 0.04$ ) than cyclin positive patients. In spite of the need for more research to fully understand these results, this is the only study that has made a correlation between clinical symptoms and cyclin deregulation.

By contrast, in a 2006 study, Neff et al. [86] found no staining of cyclin D1 in 15 VS specimens. Stankovic et al. [14] collected VS surgical specimens from 13 patients and classified them into two groups, one with good hearing (word recognition >70% and pure tone average  $\leq 30$  dB) and another with poor hearing. The entire genome expression was tested by microarray technology. The expression of selected genes was validated using real-time quantitative reverse transcription-polymerase chain reaction and immunohistochemistry. A chromosomal region (3q27) was found to be expressed differently in the two groups of patients. The peroxisomal biogenic factor 5-like gene (PEX5L), a gene within this chromosomal region with a recognized role in hearing, was found to have underexpression in VS patients with poor hearing. Another 3 genes were found to be underexpressed in VS patients with poor hearing: RAD54, homolog B (RAD54B), and the prostate-specific membrane antigen-like gene. In contrast, carcinoembryonic antigen was highly expressed. The negative results were also of interest: no correlation was found between the presence of axons and preservation of hearing, nor between vessel density and hearing loss. Finally, no significant differences in platelet-derived factor 4 expression were found between the groups.

Stankovic et al. [14] also explained each of their positive results and their possible roles in hearing loss. PEX5L, located in the chromosomal region 3q27, is generally expressed in brain tissue, and it is linked to the regulation of peroxisomal protein import. It is believed that peroxisomal dysfunction could aggravate hearing loss in patients with VS, due to underexpression of PEX5L. The authors suggest pathologic accumulation of fat and/or demyelination and neurodegeneration of the acoustic nerve as possible mechanisms of such hearing loss. They argue that hereditary demyelinating disease or peroxisomal disorders manifest with sensorineural hearing loss similar to that of VS. Moreover, histopathology has also shown demyelinating changes in the nearby vestibular nerve. All of these factors offer support for the theory of peroxisomal dysfunction.

RAD54B is another gene that is underexpressed in patients with VS and poor hearing [14]. This gene has been associated with the recombinational repair of DNA damage. Stankovic et al. found a nuclear distribution of RAD54B in VS patients with poor hearing and cytoplasmic distribution of this same gene in the good hearing group. The authors suggest this may be due to different ways of responding to DNA damage. Nonetheless, a question still remains: how does an impaired ability to repair DNA in VS patients contribute to hearing loss? Mutations in RAD54B have also been identified in non-Hodgkin lymphoma and colon cancer [87], and their relationship to VS development must be fully determined.

PSMAL (glutamate carboxypeptidase III) also showed lower levels of expression in the group with VS and poor hearing [14]. This gene has been reported to have a high expression in brain tissue. Unfortunately, its biological implications for VS remain unknown. On the other hand, the CEA-CAM7 gene and CEA protein had high expressions in VS patients with poor hearing. This differs from the overall

results, in which underexpression was the rule. Likewise, high levels of CEA in the cerebrospinal fluid (CSF) have been associated with benign and malignant tumors of the central nervous system (CNS), and it has been proposed that they are also associated with hearing loss [88]. Recently, a new mammalian protein, a member of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM), has been identified [89]. CEACAM16mRNA is expressed in outer hair cells, and its product localizes to the tips of the tallest stereocilia and the tectorial membrane. According to Zheng et al. [89], this localization might imply a role in maintaining the integrity of the tectorial membrane as well as the connection between the outer hair cell stereocilia and the tectorial membrane, which is essential for mechanical sound amplification. Furthermore, a mutation in CEACAM16 leads to autosomal dominant nonsyndromic deafness. These data could clarify the relationship between CEA and hearing loss. Again, more studies in this area are needed.

Stankovic et al. [14] also found a possible link between CEA levels and peroxisomal dysfunction. The authors explained that the activation of the nuclear hormone peroxisome proliferator-activated receptor gamma induces CEA. RAD54B and PSMAL could play an indirect role in the degeneration of the inner ear. According to these authors, this role might be a decrease in the production or responsiveness required for normal auditory nerve and inner ear function.

Using microarray gene expression technology, Lassaletta et al. [90] studied tumor samples surgically removed from 11 patients with unilateral vestibular schwannomas. The expression of platelet-derived growth factor A (PDGFA) was inversely correlated with hearing loss ( $r_s = -0.942$ ,  $P < 0.001$ ). Mean PDGFA expression for tumor patients with <40 dB and  $\geq 40$  dB pure tone threshold was 0.73 and 0.56, respectively. PDGFs are mitogenic factors for smooth muscle cells and also act as paracrine growth factors that mediate epithelial-mesenchymal interactions in various tissues. Increasing evidence suggests they may have a role in signaling pathways with a tumor angiogenesis effect. There are 4 members of PDGF (A, B, C, and D), and these bind to two tyrosine kinase receptors that are specific to each member. PDGF A, B, and C bind to PDGFR $\alpha$ , and PDGFB and D bind to PDGFR $\beta$ . Ultimately, activation of these pathways leads to cellular responses such as proliferation and migration. The authors had no explanation for the relation between hearing loss and PDGF.

## 5. Immune Response, Vestibular Schwannomas, and Hearing Loss

The literature contains studies of the immunogenic potential of tumors and recently that of the vestibular schwannomas. Rossi et al. [91] described the presence of macrophages, CD8 and CD4 lymphocytes, in VS. Leukocyte migration inhibition has been measured in serum, CSF, and perilymph in VS patients [92]. Archibald et al. [24] proposed that B7 homolog 1 (B7-H1) was a protein aberrantly expressed in malignant tumors (renal, breast, lung, and head and neck

cancer) and also in VS. It is reported that B7-H1 acts as a ligand that interacts with its counterreceptor, programmed death-1 (PD-1) on activated T cells, inducing apoptosis and inhibiting their proliferation and cytokine production. As a consequence, there is a diminished immune response to tumor cells and unrestricted growth. Archibald et al. [24] studied 48 VS samples and correlated them with clinical data and with immunohistochemical staining of B7-H1. Patients with failure of tumor control after stereotactic radiation therapy were significantly more positive ( $P = 0.029$ ) in B7-H1 staining. This finding suggests a role for B7-H1 in immune evasion and might explain the continued growth of VS despite radiotherapy. Additionally, patients with worse hearing at the moment of surgery tended to stain more strongly for B7-H1 than the better hearing patients, although no significant difference between both groups was found.

## 6. Cytokines and Growth Factors

Identification of growth factors implied in VS progression can provide new treatment options. Different authors have studied Ki67, proliferation cell nuclear antigens, nerve growth factor receptors, transforming growth factors, fibroblast growth factors, interleukin 6, and hormones [25–30]. Nonetheless, these tumor growth factors have not been identified as independent causes of hearing loss.

Vascular endothelial growth factors (VEGFs), on the other hand, are associated with hearing loss and are highly expressed in vestibular schwannoma [93–95]. Cayé-Thomasen et al. [94] demonstrated a correlation between the concentration of vascular endothelial growth factors in VS samples and rate of tumor growth. Plotkin et al. [95] found VEGF expressed in 100% of 21 NF2-related schwannomas and 22 sporadic schwannoma samples. Moreover, they found hearing improvement in 4 of 7 patients under treatment with Bevacizumab. Bevacizumab is a VEGF neutralizing antibody approved by the FDA for the treatment of cancers. This finding supports the idea that VEGF plays a role in tumor growth and also suggests a possible treatment for hearing loss in VS patients [96].

## 7. Novel Therapies in VS Treatment

Bevacizumab is the most studied agent of the new therapies for vestibular schwannoma [16]. It consists of a humanized monoclonal IgG1 antibody against VEGF. As previously stated, vascular endothelial growth factors are associated with tumor growth and therefore represent a new target in VS therapy.

Recently, there has been increased attention to the auditory benefit registered in patients treated with Bevacizumab [95, 97]. Mautner et al. [97] treated 2 patients, one for 3 months and one for 6 months. Improved hearing could be registered in the patient treated for 6 months. The only side effect mentioned was high blood pressure in one of the patients.

Plotkin et al. [95] reported the best auditory benefit described in the literature. They administered Bevacizumab to 7 patients, of whom 4 (57%) reported hearing

improvement. The results were evaluated on the basis of the word recognition score (WRS). Patient number 2 had the best result, with a WRS of 8% prior to therapy and 98% after therapy. Hearing improvement was sustained for up to 16 months. The authors explained the improvement in hearing as due to reduction of intraneural edema and reduction in tumoral size. This conclusion was based on the evolution of hearing loss, the correlation between the mean apparent diffusion coefficient (a measurement of the magnitude of diffusion of water molecules within tissue and a marker of edema on imaging MRI), tumor shrinkage, and measurements (on a dynamic contrast MRI) of the changes in intratumoral vascular permeability.

Both of these previous studies corroborated a reduction in tumoral burden. In another study published in 2010 [98], the authors demonstrated that anti-VEGF therapy normalizes vasculature in schwannoma xenografts in nude mice and controls tumor growth. Vascular normalization in benign tumors is an important issue when considering this treatment. New anti-VEGF agents are being developed (e.g., PTC299), which have mitigation of hearing loss as a main clinical outcome [16, 99].

Erlotinib is an oral EGFR tyrosine kinase inhibitor. Its main target is the arresting of the proliferative properties of the tumor. Plotkin et al. [100] evaluated 10 patients who underwent treatment with Erlotinib. In three patients, the disease was stable, as measured by radiographic evaluation. Regarding their hearing outcomes, one patient presented a transient hearing response, 2 experienced minor hearing response (lasting 19 and 24 months), 3 patients were stabilized, and 2 presented progressive hearing loss. The authors commented that Erlotinib may have more cytostatic properties as well as being less effective in progressive VS. More studies are needed with emphasis on time to progression as an important outcome, rather than tumoral volume or hearing status.

An in vitro study by Ammoun et al. [101] included VS samples and identified overexpression and activation of EGFR family receptors. They found that lapatinib inhibited ErbB2 phosphorylation and downstream ERK1/2 and AKT activation, resulting in decreased proliferation. Phase II studies are pending.

Other pathways such as PAK inhibitors are under investigation [99].

## 8. Conclusions

In this paper we describe some of the molecular mechanisms involved in vestibular schwannoma development. The NF2 gene mutation, chromosome 22 loss, NF2 gene mitotic recombination, DNA methylation, deregulation of genes, immunogenic factors, cytokines, and growth factors are the key to understand the molecular pathophysiology of VS.

Also, many theories have been advanced to explain hearing loss associated with VS patients, but there is growing evidence concerning molecular-based data. The methylation of TP73, the negative expression of cyclin D1, the positive expression of B7-H1, increased expression of platelet-derived growth factor A, underexpression of PEX5L, RAD54B, and

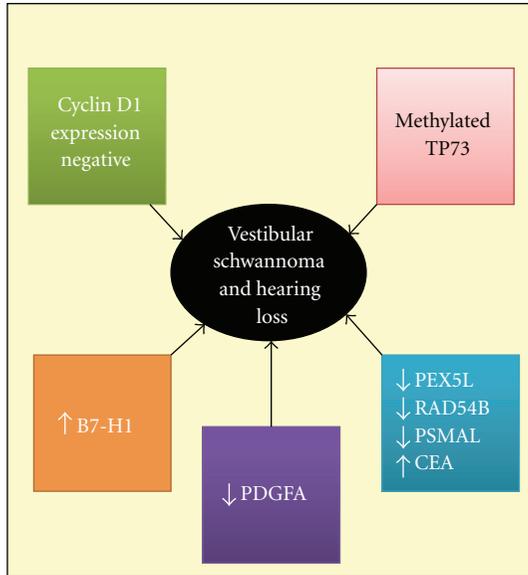


FIGURE 2: Available evidence of molecular mechanisms implicated in VS patients with hearing loss PEX5L (peroxisomal biogenesis factor 5-like gene), RAD54B (RAD54 homolog B), PSMAL (prostate-specific membrane antigen-like), CEA (carcinoembryonic antigen), PDGFA (platelet-derived growth factor A, B7-H1 (B7 homolog 1).

PSMAL, and overexpression of CEA are factors associated with hearing loss and VS (Figure 2).

Novel therapies also confirm that molecular investigation could be a promising alternative for the treatment of VS. More studies are needed to corroborate these results and, more broadly, to establish links between molecular and clinical data.

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## Review Article

# Hearing Loss in Osteogenesis Imperfecta: Characteristics and Treatment Considerations

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Osteogenesis imperfecta (OI) is the most common heritable disorder of connective tissue. It is associated with fractures following relatively minor injury, blue sclerae, dentinogenesis imperfecta, increased joint mobility, short stature, and hearing loss. Structures in the otic capsule and inner ear share in the histologic features common to other skeletal tissues. OI is due to mutations involving several genes, the most commonly involved are the COL1A1 or COL1A2 genes which are responsible for the synthesis of the proalpha-1 and proalpha-2 polypeptide chains that form the type I collagen triple helix. A genotype/phenotype relationship to hearing loss has not been established in OI. Hearing loss is commonly found in OI with prevalence rates ranging from 50 to 92% in some studies. Hearing loss in OI may be conductive, mixed, or sensorineural and is more common by the second or third decade. Treatment options such as hearing aids, stapes surgery, and cochlear implants are discussed.

## 1. Introduction

Osteogenesis imperfecta (OI) is the most common of the heritable disorders of bone as first defined by McKusick in 1956 [1]. Approximately 25,000 individuals with this condition live in the US. However, the real incidence may be twice that number because of persons with mild OI who experience a small number of fractures and in whom no diagnosis is made. Hearing loss is a significant complication of OI.

The hallmark of OI is the occurrence of fractures with relatively minor injury. Blue sclerae, dentinogenesis imperfecta, increased joint mobility, and short stature are common to the 8 phenotypes that are currently recognized (Table 1). As shown in Table 1, these vary considerably in their relative incidence and clinical severity. It is not commonly appreciated, however, that OI is a systemic disorder of connective tissue due to the wide distribution of type I collagen in multiple organs.

Type I collagen is the most abundant protein in the body. OI is the result of mutations involving several genes

responsible for the synthesis or intracellular processing of type I collagen (COL1). To date, 7 genes have been implicated as causative in OI [2, 3]. Ninety-five percent of OI cases are due to dominantly transmitted mutations involving COL1A1 or COL1A2 genes which are responsible for the synthesis of the proalpha-1 and proalpha-2 polypeptide chains that form the type I collagen triple helix. Approximately 3–5% of cases involve mutations which are transmitted recessively and are associated with severe or lethal OI. These genes modify the intracellular processing of type I collagen: CRTAP (collagen-related protein), P3H1/LEPRE1 (prolyl 3-hydroxylase 1), and PPIB (Cytophyllin B protein) and several chaperone proteins such as HSP-47 in the endoplasmic reticulum [4–6]. CRTAP and P3H1 form a complex with cyclophilin B (CyPB) in the endoplasmic reticulum (ER) which 3-hydroxylates the Pro986 residue of collagen proalpha1 (1) and proalpha (2I) chains [7]. Mutations in these genes lead to severe or lethal skeletal disease (OI types VII and VIII) [8]. No correlation has been established to date between mutations affecting the COL1A1 and COL1A2 genes and hearing loss in OI [9].

TABLE 1: Types of osteogenesis imperfecta (adapted from [18]).

Type	Inheritance**	Clinical	Incidence**	Mutations
I	AD	Mild, blue sclerae fractures with little or no deformity hearing loss, DI	60%	Type I collagen COL1A1, COL1A2
II	AD, AR	Lethal, pulmonary insufficiency, beaded ribs, rhizomelic hearing loss	10%	Type I collagen COL1A1, COL1A2
III	AD, AR	Progressive deforming intrauterine fractures, deformed limbs, scoliosis white or blue sclerae hearing loss, DI	10%	Type I collagen COL1A1, COL1A2
IV	AD	Moderately severe, limb deformity, sclerae blue early and lighten with age scoliosis	15%	Type I collagen COL1A1, COL1A2
V	AD	Variable phenotype like IV hyperplastic callus, dislocated radial head calcified interosseous membrane	5%	Unknown
VI	unknown	More fractures than IV mineralization defect on biopsy, vertebral fractures, no DI	Unknown	Type I collagen SERPINF1 (chaperone protein)
VII	AR	First nations family, Quebec congenital fractures white sclerae, severe rhizomelia	++	CRTAP, LEPRE1, PPIB (prolyl-3 hydroxylation)
VIII	AR	Severe or lethal similar to OI type II (Sillence)	++	CRTAP, LEPRE1 SERPINH1

\*\* AD = autosomal dominant.

\*\* AR = autosomal recessive.

++: the incidence of OI types I–IV is reasonably established. However, for the less common types, OI types VI, VII, and VIII, the incidence is not clearly defined. However, it is estimated that the recessively inherited forms (VII and VIII) constitute approximately 3–5% of the total OI population.

As noted above, disordered type I collagen synthesis affects multiple organs in addition to bone. Blue sclerae result from altered light reflectance in the presence of abnormal scleral collagen [10]. Corneal defects occur because collagen is the dominant corneal protein. Tendon and ligament involvement leads to hyperextensible joints. Dentinogenesis imperfecta is the result of abnormal collagen in dental pulp which leads to enamel breakage. Type I collagen in vascular structures leads to mitral and aortic valve involvement as well as aortic dilatation in some cases. Finally, disordered type I collagen in the ear involves each of the auditory structures, both hard and soft tissues, leading to early-onset hearing loss.

There is limited information about the histopathology of the temporal bone in OI. Berger et al. examined the histopathologic findings in 8 temporal bones from 5 patients with type III osteogenesis imperfecta [11]. Evidence of both deficient and abnormal ossification was found in the bony walls of middle ear and ossicles. Microfractures were found in the otic capsule and in the anterior process and handle the malleus and at the crura of the stapes. The cochlear and vestibular end organs appeared normal. It is of interest that the OI auditory system did not show changes compatible with otosclerosis. Zajtchuk and Lindsay [12], reporting on three OI cases (Type III), observed sclerotic fixation of the footplate, lack of deposition of the skein-like bone in the endochondral layer, sparse bony septae in marrow spaces, and a deficiency of the periosteal layer. The stapedial crura were thin, deformed, and fractured in 2 cases [12]. Examination with the scanning electron microscope using the Ca/P ratio as the criterion based on characteristic X-ray fluorescence showed that the stapes in OI had a higher Ca/P ratio (2.6:1) as compared to the normal stapes 2:1 ratio. The stapes in OI were poorly mineralized, with low calcium

salt and apparent increase of phosphates. This finding suggested a possible change from hydroxyapatite (or apatite) to brushite, which implied an acidification of bone [13].

Computerized tomography (CT) of the temporal bone in an OI type I patient demonstrated otic capsule demineralization that appeared to progress as hearing diminished [8]. Band-like areas of lucency were seen surrounding the cochlea. Similarly, magnetic resonance imaging (MRI) examination of the otic capsule in type I OI demonstrated demineralized pericochlear lesions with soft tissue signal intensity and contrast enhancement [14, 15].

## 2. Hearing Loss in Osteogenesis Imperfecta

Hearing loss is a significant clinical feature in many patients with OI. In national surveys of hearing loss in OI, prevalence rates of hearing loss of 46% [16] to 57.9% [17] have been reported. In a recent study, hearing loss was found in 62% of ears with the hearing loss ranging from mild to profound [18]. Slightly lower prevalence of hearing loss (50%) [19, 20] or even a higher prevalence of hearing loss (65–72%) [16, 21] has been reported. Methodological or definitional differences may account for the varying results across studies [22]. Some investigators have excluded cases of conductive hearing loss believed to be secondary to otitis media with effusion (OME) in younger patients with OI [16, 23] which would lead to underestimation of the prevalence of hearing loss. Subject selection issues are also a consideration. Hearing loss varies with the type of OI and is reported to be most common in type I OI and rarely reported in type IV OI [24]. Studies including only type I OI patients [23] have reported a higher prevalence

of hearing loss in OI than studies with more inclusive selection criteria [18]. For example, when only patients with type I OI over 30 years of age are considered, a 95% rate of hearing loss has been reported [23]. No relationship has been reported between the degree, nature of hearing loss, and mutated gene or mutation type [25]. Sykes et al. reported that 13/16 patients with early onset of hearing loss had a mutation in the COL1A1 gene [26]. OI may be associated with neurological complications such as skull fractures, seizure disorders, and brainstem compression secondary to basilar invagination [27]. Some of these complications can result in hearing loss. Some studies have excluded patients with these or other complications believed to be unrelated directly to OI [17], whereas others have not [18, 22].

It is frequently reported that conductive hearing loss is found in younger patients with OI, whereas sensorineural hearing loss is found more in older patients with OI [17, 19, 21, 23, 28]. This view is not uniform as one study has reported that hearing loss in OI is most frequently sensorineural in origin [29] in the initial presentation. However, it should be noted that there are definitional differences across studies in terms of how conductive and sensorineural hearing loss are defined. For example, Riedner et al. [19] defined as conductive any hearing loss in which the air-bone gap was 5 dB or greater at two or more octave frequencies from 500 to 4000 Hz. On the other hand, Shapiro et al. [29] required a 15 dB or greater air-bone gap at one or more frequencies to classify a loss as conductive. Other studies have not explained how conductive versus sensorineural hearing loss was defined [28].

Several investigators have reported that hearing loss is infrequent in OI until the 2nd or 3rd decade of life, increasing with advancing age [19, 21, 28]. However, hearing loss in younger patients with OI is not uncommon [17, 22], and mixed or sensorineural hearing loss may be observed at any age [17]. When sensorineural hearing loss is found in children, it is typically found in type I OI [17, 18]. Other investigators have reported that hearing loss in OI is typically sensorineural in origin [29] and begins with a characteristic loss in the 6000–8000 Hz frequency region. One recent study reported a prevalence of sensorineural hearing loss in 12% of ears, conductive hearing loss in 21% of ears, and mixed hearing loss in 29% of ears [18] in patients with OI. As has been reported in other investigations [17, 22], sensorineural hearing loss was present in a younger age group consisting of children (mean age = 9.87) and was found only in association with type I OI [18].

The etiology of sensorineural hearing loss in OI has not been definitively determined but may be a consequence of atrophy of the cochlear hair cells and the stria vascularis as well as from abnormal bone formation in the cochlea and surrounding structures [25]. Patients with OI are at greater risk for skull fractures which are associated with hearing loss. The cause of conductive impairments in OI is often associated with footplate fixation [25] although fractures of the crura do occur [30]. Atrophy of the stapes crura and hypervascularized mucosa have also been reported [25]. An association between conductive hearing loss due to stapes fixation and a mutation in the COL1A1 gene has been

suggested [25]. On the other hand, in a much larger study, no relationship was found between the mutation types and the presence, type, severity, or age of onset of hearing loss [9]. Autopsy findings have shown hair cell loss, abnormalities of the tectorial membrane, perilymph hemorrhage, atrophy, and calcification of the stria vascularis in OI [31]. In newborns with severe OI, evidence of incomplete ossification of the otic capsule has been reported as well as microfractures in the otic capsule, deficient ossification of the ossicles, and microfractures in the anterior process and handle of the malleus [11]. In older patients with OI who had been seen surgically for stapedia fixation or discontinuity, histological examination of the recovered stapes revealed evidence of an otospongiotic-like lesion in the footplate of the stapes [32]. CT scans in OI have revealed undermineralization of the otic capsule [33] as well as involvement of the cochlea, semi-circular canals, the distal internal auditory canal, and oval window in an individual with hearing loss of mixed origin [33]. It has been noted that in some respects the features of hearing loss in OI such as ossicular involvement and hearing loss progression mimic those found in otosclerosis [21]. However, OI and otosclerosis are biochemically divergent [34]. Examination of histopathological data has shown that OI is a generalized disorder of the bone and connective tissue, whereas otosclerosis is a localized disease of the temporal bone [32]. Another difference between OI and otosclerosis is that sensorineural hearing loss is more commonly reported in OI than in otosclerosis and at earlier ages [17].

Hearing loss is very common in older patients with OI and in one recent study in older patients (Mean age = 44 years) with OI, 87% of ears had some degree of hearing loss [18]. In younger patients with OI, hearing loss was not as common but was still present in 38% of ears [18]. Other studies with pediatric patients with OI have reported prevalence rates ranging from 9.1% [35] to 77.3% [22]. When patients with a range of medical conditions including otitis media with effusion, skull fracture, and neonatal meningitis are included in the sample studied, higher prevalence rates are reported [22]. Some investigators [18, 22] have not made assumptions as to whether factors present in the medical history were related or not to the complex of symptoms experienced by patients with OI. For example, the presence of OME, history of head trauma, and bacterial meningitis were not exclusion criteria in recent investigations as long as the patients presented with the diagnosis of OI [18, 22]. This practice has been challenged by some investigators [36].

Conductive hearing loss is typically reported as the initial manifestation of hearing loss in OI [16, 17, 19]. Clinically, pediatric patients with OI may initially present with conductive hearing loss associated with otitis media. This clinical observation has recently been supported in two investigations [18, 22], which have suggested the possibility of increased susceptibility in pediatric patients with OI to otitis media with effusion. In pediatric patients with OI, tympanometric abnormalities consistent with the presence of middle ear effusion were common in that 43% of the sample failed tympanometry [18]. In contrast, failure rates for tympanometry in preschool and school age children have

been reported to be in the range of to 8.6%–13.5% [37, 38], which suggests a higher rate of failure for tympanometry in OI than in the general preschool and school age population. As noted recently [18], the high incidence of otitis media with effusion in younger patients with OI may be attributable to the presence of craniofacial dysmorphism in OI [22]. A high frequency of OME is also commonly observed in other syndromes characterized by morphological abnormalities of the temporal bone [39] or as a consequence of cranial molding or deformation [22].

As noted recently, measurement of hearing loss and the determination of etiology of the hearing loss presents some challenges in OI [18]. The use of bone conduction thresholds in determination of hearing loss etiology is potentially confounded by the presence of middle ear pathology. Immobility of the stapes footplate with consequent mechanical restriction of the perilymph may alter cochlear function sufficiently to elevate bone conduction thresholds [40]. Middle ear pathologies contributing to increased stiffness in the middle ear system impact on the ossicular inertial component of bone conduction hearing [41]. This results in poorer bone conduction thresholds. For example, bone conduction thresholds are known to be elevated in otosclerosis in the 2000 Hz region. Tympanometric abnormalities may not support the presence of conductive involvement in OI as it was recently reported that only 65% of ears with conductive hearing loss and 48% of ears with mixed hearing loss were found to have abnormal tympanograms [18]. Other investigators have reported normal tympanograms in individuals with conductive and mixed hearing loss [19]. It has been noted that it is possible that the presence of multiple otologic pathologies in OI may confuse the interpretation of tympanometric findings [18]. This is the case because pathology contributing to high admittance may be masked by pathology resulting in reduced admittance of the tympanic membrane/middle ear system [42, 43]. It has been reported that the most prevalent abnormal tympanometric types observed in young patients with OI are consistent with the presence of middle ear effusion (A<sub>S</sub>, B, and C) [18]. In contrast, for older patients with OI, the Type A<sub>D</sub> pattern is more commonly found [18]. The type A<sub>D</sub> pattern is often associated with ossicular discontinuity although it can also be consistent with atrophic scarring of the tympanic membrane. The use of only a low-frequency probe tone in these studies may explain the poor test performance of tympanometry clinically [18, 19]. The use of a two-component higher frequency (678 Hz) tympanogram has been shown to be more accurate in identification of ossicular chain disruptions than when only a 226 Hz probe tone is utilized [44].

### 3. Management of Hearing Loss in OI

Treatment of hearing loss in OI can be divided into categories based on the severity of the hearing loss and the etiology. As noted above, the loss can be conductive (involving the ossicles, ear drum, and middle ear), sensorineural (involving the cochlea, auditory nerve, and brain), or a combination of both. The degree of hearing loss helps determine if any

TABLE 2: Results of stapedectomy in people with and without OI. Findings depict the number of cases, percent of patients with postoperation air-bone gap of less than 10 dB HL, and percent of patients with postoperation hearing loss.

	Cases	Post-op air bone gap <10 dB	Post-op sen- sorineural hearing loss
OI series			
Garretsen and Cremers [49]	40	78	1.7
Shea and Postma [50]	51	75	8
Ferekidis et al.[51]	9	75	0
Vincent et al.[52]	21	85.7	0
Non-OI series			
Vincent et al. [53]	2525	94.2	0.7

intervention is necessary, and if so, what that intervention should be.

Conductive hearing loss theoretically can be fully corrected. In people without OI, that is unfortunately not always possible. In people with OI, the percentages that are not correctable are higher.

*Serous Otitis Media.* Fluid in the middle ear causes loss of hearing by limiting ear drum and ossicle motion. This occurs commonly in young children but can occur at any age. Common causes include Eustachian tube dysfunction, colds, ear infections, and allergies. When treatment of the underlying cause does not resolve the fluid, ventilation tubes can be placed. The incidence of serous otitis may be higher in OI. But the treatment outcomes are the same.

*Ossicular Problems.* OI can cause deformity of the ossicles described earlier. These deformities can lead to a conductive hearing loss from ossicular fracture and stapes footplate fixation. Surgical correction is usually possible, but results of such surgery are significantly worse than similar surgery in non-OI series reports (Table 2). Stapedectomy results in type I OI show closure of the air-bone gap to within 10 db in 75–85% compared to published results in normal patients of 90–95%. Postoperative hearing loss is also higher in OI patients with up to 8% losing hearing instead of the 1% seen in the non-OI group. The reasons for the failure are easily understood. In stapes surgery, the footplate may be very thick and highly vascular. This limits visibility and may narrow the distance between the footplate and the inner ear balance organs (utricle and saccule) to less than 0.5 mm. Also in all reconstructions including stapedectomy, the supporting bone being used is weaker than and thus not as stable as non-OI bone.

Treatment for sensorineural hearing loss in people with OI is similar to people without OI. Hearing aids can help manage sensorineural, conductive, and mixed (both sensorineural and conductive) hearing loss. Since hearing aids amplify the sound going into the ear, the amount of the loss is more important than the cause of the loss. However, it should be noted that individuals with sensorineural hearing

loss typically have greater problems with speech recognition and loudness processing than is the case with conductive hearing loss. Fitting a person with OI with a hearing aid is the same as fitting someone without OI.

**Cochlear Implants.** ~11% of patients with OI will have hearing deterioration such that hearing aids will no longer be useful. Cochlear implants provide an option for restoring hearing [45–47]. They are surgically implanted into the inner ear and electrically stimulate the auditory nerve directly. Some problems have been encountered that are specific to people with OI. The operation to implant the device can be more difficult because of the hypervascularity of the bone and the possible narrowing of the inner dimensions of the cochlear channel. Also, once the device is placed, the bone does not shield the electrical stimulating current as well because of the lower bone density. This can sometimes lead to stimulation of the facial nerve and require some of the electrodes to be turned off. Despite these limitations, cochlear implants provide great benefit in deaf individuals with OI.

**Bone-anchored hearing aids (BAHA).** This is an alternative treatment for conductive hearing loss and single-sided deafness. It requires one normal functioning inner ear. It also requires that the titanium implant osseointegrates into the bone. A hearing aid is then attached to the implant to directly stimulate the bone. This vibration is sent to the inner ear to stimulate hearing by bypassing the ossicles. This is the same integration technology used with some dental implants. Since many patients with OI develop bilateral sensorineural hearing loss, they may not be a candidate for the BAHA anyway. With the high percentage of people with sensorineural hearing loss, the number of qualified BAHA candidates will be low. To date, no data on the use of this implant in OI is available.

Implantable hearing aids are starting to make their way into the option list for treating hearing loss. These devices drive the ossicular chain directly rather than amplifying the sound through air. They are attached to the ossicles surgically. Given the fragility of the ossicles in OI, they may not be a good option. However, beneficial results of Vibrant Sound-Bridge implantation in combination with stapedectomy have recently been reported in a small sample of three patients with OI [48].

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## Research Article

# Clinical Application of Screening for *GJB2* Mutations before Cochlear Implantation in a Heterogeneous Population with High Rate of Autosomal Recessive Nonsyndromic Hearing Loss

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Clinical application of mutation screening and its effect on the outcome of cochlear implantation is widely debated. We investigated the effect of mutations in *GJB2* gene on the outcome of cochlear implantation in a population with a high rate of consanguineous marriage and autosomal recessive nonsyndromic hearing loss. Two hundred and one children with profound prelingual sensorineural hearing loss were included. Forty-six patients had 35delG in *GJB2*. Speech awareness thresholds (SATs) and speech recognition thresholds (SRTs) improved following implantation, but there was no difference in performance between patients with *GJB2*-related deafness versus control (all  $P > 0.10$ ). Both groups had produced their first comprehensible words within the same period of time following implantation (2.27 months in *GJB2*-related deaf versus 2.62 months in controls,  $P = 0.22$ ). Although our findings demonstrate the need to uncover unidentified genetic causes of hereditary deafness, they do not support the current policy for genetic screening before cochlear implantation, nor prove a prognostic value.

## 1. Introduction

Hereditary sensorineural hearing impairment (SNHI) affects 2–4 children per 1000 in the developing countries [1]. Burden of hearing impairment is described *in extenso* and is felt by the clinician and families taking care of the deaf [2]. Early identification of hearing loss and initiation of rehabilitation measures are the cornerstones of language development [3].

For patients with profound SNHI, cochlear implant (CI) is considered to be an effective intervention bypassing the inner ear organs and activating the auditory nerve directly.

Outcome of CI differs significantly between the implantees. Since the etiology and demographic data of the implantees is diverse, different factors could be attributed to the difference of the CI success [4]. The operation is expensive and requires advanced training, therefore, identification of the most important factors affecting the outcome of CI becomes necessary especially in settings with limited resources and a need for judicious patient selection [5].

In the last two decades, molecular genetics had shed light on many aspects of hereditary deafness and elucidated the pathophysiology of deafness in some cases of genetic hearing loss. A number of studies had argued that *GJB2*

mutations demonstrate a better language performance following cochlear implantation, but the justifications are not persuasive and authors frequently suggested further studies [6–9]. Since genetic testing is expensive and not covered by most healthcare insurances, identification of the role that genetics plays in the outcome of CI is far beyond necessary. In this study we investigated the role of commonly identified genetic mutations and their association with subjective and objective outcomes in a population of pediatric patients with profound hearing impairment that received CI. This population has a high rate of consanguineous marriage and therefore high rate of autosomal recessive diseases, which facilitated sampling nonsyndromic cases of congenital hearing loss [10, 11].

## 2. Methods

**2.1. Subject Recruitment and Preimplantation Evaluation.** Two hundred and one consecutive and unrelated children with profound SNHI (108 boys and 93 girls) who underwent CI from 2003 to 2006 were enrolled in this study. All children were native Persian speakers and had been identified to have hearing impairment before the age of 3, also known as prelingual hearing impairment. All of these children were found to be suffering from nonsyndromic hearing impairment, which means no additional physical problem had been identified accompanying their hearing loss. Comprehensive clinical evaluations were fulfilled for each child encompassing history, physical examination, audiological tests, radiological studies of the temporal bone, and genetic testing. These evaluations were done free of charge for all the candidates.

All the implantees had gone through 60 sessions of mandatory oral and language rehabilitation classes. These classes take 2 hours per session, and provide the minimum of the required time for sufficient tonal information of Persian language. All the parents and/or guardians of the implantees had previously signed a written consent for their implantation procedures and had exclusively signed a second consent for this study. The procedure and studies had been approved by the Institutional Review Board of Tehran University of Medical Sciences.

**2.2. Genetic Examination.** Consenting subjects provided 7–10 cc of whole blood from which DNA was extracted using established techniques. Samples were screened for the 35delG allele of *GJB2* using a previously described ARMS-PCR method [12]. All 35delG homozygous samples were excluded from further testing. In the remaining samples, *GJB2* and *GJB6* mutation screening was completed using a previously described method [12].

**2.3. Evaluation of Speech Perception.** The date on which the CI recipient produced the first comprehensible two-syllable word was reported and confirmed by an audiologist. Data regarding the shape of audiogram and level of hearing was collected before the CI surgery for both ears and at 1, 3, 6, 12, and after 12 months from the date of CI for the implanted ear. None of the study subjects received bilateral

cochlear implants. After implantation, speech awareness thresholds (SATs) were measured at 1 and 3 months and speech recognition thresholds (SRTs) were measured at 6 and 12 months. Most comfortable level (MCL) was also determined at 6 and 12 months. In some CI recipients, additional testing was done within the first 3 years following surgery. Although newer tests for assessment of auditory function exist, including the open-set and close-set tests, in the lack of standardized equivalent tests in Persian, we used level of hearing, SAT, SRT, and MCL to assess the outcome of CI and compare it between the two arms of the study. We should highlight that results from nonstandardized tests that are equivalent to tests in English language were all reproducing the same results but are excluded from the analysis for the sake of robust standard comparisons to other studies.

**2.4. Statistical Analysis.** SAT, SRT, and MCL were expressed in dB referring to the hearing level; speech recognition and word generation were compared to the genetic diagnosis. Continuous variables were compared using the Student's *t*-test, ANOVA, and repeated measures analysis; chi-square was used to compare dichotomous variables. All tests were two tailed. Differences were reported as significant if the *P* value was less than 0.05. Because age at the time of implantation could potentially confound the association between the genetic status and some of the primary outcomes (e.g., time of first vocalization), we used linear regression models to adjust the analyses for age.

## 3. Results

Genetic analysis identified 62 patients with mutations in *GJB2*, and 35delG was the most frequent type of mutation. Forty-six patients were found to have 35delG mutation in *GJB2*: thirty (14.9%) were found to be homozygote for 35delG mutations and 13 (6.5%) were heterozygote; the second mutation was not found in the remaining three heterozygotes. Table 1 lists the mutations found in the 16 patients without 35delG. No *GJB6* mutation was found in our population.

Ethnicity of our patients and the ethnicity of the Iranian population are shown in Table 2. In 124 families (61.7%) parents had been consanguineous relatives of each other. The mean age of implantation was 2.5 years (ranging from 8 months to 5.5 years). They were followed for an average of 3.72 years (standard deviation = 2.95 years), and their age at last data point ranged between 2 and 14 years (mean  $\pm$  standard deviation = 6.38  $\pm$  3.37 years). Duration of followup was not statistically different among patients with and without mutations (4.1 years versus 3.6 years, *P* = 0.234, 95% CI = -1.43 to 0.35 years, power > 99%).

Following implantation, audiograms improved from severe-to-profound or profound levels before the surgery to moderate-to-severe or moderate levels by 3 months after surgery, all acquiring a flat shape. By 6 months after surgery, all audiograms were at moderate level or better. Nearly half of implant recipients improved to mild hearing loss after one year following implantation (Table 3). Comparison of

TABLE 1: *GJB2* genotypes in patients carrying no 35delG mutations. Homozygote mutations are marked with an asterisk.

Genotype	Frequency	Percent
V153I	4	25%
R184P*	2	12.5%
167DelT*	2	12.5%
V27I SNP*	2	12.5%
G130V	1	6.3%
235delC/R184P	1	6.3%
R127H	1	6.3%
SNP N26N*	1	6.3%
R32C*	1	6.3%
R151P*	1	6.3%
Total	16	100%

TABLE 2: Ethnicity of the studied population compared to overall composition in Iran.

Ethnicity	Number	Percentage	Percentage in Iran
Fars	68	33.8	51
Turk	44	21.9	24
Gilak/Mazandarani	34	16.9	8
Kurd	16	8.0	7
Lur	13	6.5	2
Arab	9	4.5	3
Pakistani	1	0.5	<1
Afghan	1	0.5	<1
Mixed	14	7.0	5 (other)
Not known	1	0.5	—
Total	201	100	100

level of hearing among carriers of *GJB2* mutations versus control yielded no significant association ( $P > 0.10$  in all comparisons).

At baseline, level of hearing had not been significantly different between the *GJB2* mutation carriers and the rest of implantees ( $P = 0.053$ , power = 96.65%, alpha level 0.05). In addition, there was no correlation between *GJB2*-related deafness and other demographic factors including race of patient or parents, education level, and occupational status of parents (ANOVA  $P > 0.10$ ). Although level of hearing had improved over time, there was no statistically significant difference in this improvement between the carriers of *GJB2* mutations and the rest of the implantees ( $P = 0.18$ ). Regression in each measured frequency of the audiograms over the period of followup did not show a significant difference between the *GJB2* mutation carriers and the rest of the patients (ANOVA  $P > 0.2$ , statistical power = 77.3%). The very same statistically similar findings were seen for the SAT, SRT, and MCL measurements at the specified times (all  $P > 0.10$ , statistical power > 70% in all cases). Results remained unchanged after correcting for age at the time of operation or age at the time of diagnosis. Table 4 shows the measured SAT, SRT, and MCL values at the specified times, but further subcategorizes the group with *GJB2* mutations into two

subgroups. The time of first vocalization did not significantly differ between the two groups (2.27 months for *GJB2*-related deaf versus 2.62 for controls,  $P = 0.22$ , statistical power = 90.4%). The association between *GJB2*-mutation and time of first vocalization remained insignificant after correction for age at the time of operation ( $P = 0.345$ ) or age at the time of diagnosis ( $P = 0.215$ ).

Repeated measures analysis showed that although the level of hearing had significantly improved over time, the difference was independent of the genetic status of patients ( $P = 0.971$ ). Similarly, presence of 35delG had no effect on the improvement observed in SRT ( $P = 0.973$ ) or MCL ( $P = 0.511$ ) over time. Figures 1 and 2 depict that the trend of improvement of these variables was similar in the two groups. We repeated this analysis by restricting it to patients with homozygote 35delG mutation, and yielded similar results: the presence of homozygote 35delG was not associated with improvements observed in SRT ( $P = 0.581$ ) or MCL ( $P = 0.253$ ).

#### 4. Discussion

This is the largest study to date on prelingual nonsyndromic deaf population investigating the effect of *GJB2* mutations on the outcome of cochlear implantation. We found that *GJB2* mutations do not have any significant relation with the speech perception abilities following CI compared to non-*GJB2*-related patients. In addition, no correlation was found between the genetic mutation and time to vocalization of the first comprehensible word. Other variables which had been shown to affect the outcome of CI such as age at implantation, duration of implant use, and residual hearing before CI were kept constant in this study through the patient selection criteria or at the time of analysis. In this regard, our studied population constitutes a relatively homogenous population to study the effect of genetic factors. Our study also shows that the genetic change in a large number of patients with hereditary SNHI remains unknown in our population.

At the time of clinical introduction of *GJB2* mutation screening for deaf patients, many came to the conclusion of *GJB2* superiority in CI recipients [9]. A primary study on the CI recipients had identified a better response in the carriers of mutations in *GJB2*. An issue raised about this study was patient selection and matched controls where a clear etiology of deafness in controls is not provided [13]. The same journal published another study in the following year which showed no benefit in the carriers of *GJB2* mutations [8]. This is the year when further studies had come to a conclusion of superiority of *GJB2* mutation carriers over non-*GJB2* controls [14]. The concept of *GJB2* performance superiority in CI performance was additionally reinforced within two years [7].

Correct selection of the matched controls for any deduction is emphasized in the works of other authorities [15]. More recent studies have been cautious in making any suggestion regarding the superiority of genetic deafness over controls for the CI outcome [16]. In 2008, a study on Taiwanese patients reported the same positive effect for

TABLE 3: Percentages showing the shape of audiograms and level of hearing at the times measured.

Shape of audiogram	Before CI	1 mo	3 mo	6 mo	12 mo	12 mo <
Fragmentary	52.1	—	—	—	—	—
Descending	46.6	—	—	—	—	—
Flat	1.4	100	100	100	100	100
Level of hearing						
Profound	82.2	—	—	—	—	—
Severe-to-profound	17.8	—	—	—	—	—
Moderate-to-severe	—	13.9	2.8	—	—	—
Moderate	—	86.1	97.2	95.7	81.2	53.8
Mild	—	—	—	4.3	18.8	46.2

TABLE 4: SAT, SRT, and MCL measurements and gender distribution of patients in the sample. Patients with *GJB2* mutations are sub-categorized into two columns, based on the presence of 35delG variant. Continuous values are presented as mean ± standard deviation; dichotomous values are presented as frequency (percentage in row). Values pertain to the operated ear only. Unit of measurement: dB.

		35delG mutation	Non-35delG mutation	No mutation
Gender	Male	26 (56.5%)	8 (50%)	74 (53.2%)
	Female	20 (43.5%)	8 (50%)	65 (46.8%)
SAT	<i>N</i> = 72	<i>N</i> = 20	<i>N</i> = 5	<i>N</i> = 47
	One month after surgery	43.6 ± 3.8	41.7 ± 2.9	44.1 ± 5.1
	Three months after surgery	40.7 ± 5.3	35.0 ± 0*	39.7 ± 4.8
SRT	<i>N</i> = 65	<i>N</i> = 19	<i>N</i> = 4	<i>N</i> = 42
	Six months after surgery	40.8 ± 4.5	43.3 ± 2.9	39.2 ± 4.7
	Twelve months after surgery	38.5 ± 4.7	37.7 ± 7.6	37.3 ± 4.1
MCL	<i>N</i> = 45	<i>N</i> = 13	<i>N</i> = 4	<i>N</i> = 28
	Six months after surgery	71.8 ± 4.0	75.0 ± 0.0*	72.1 ± 4.4
	Twelve months after surgery	69.7 ± 6.0	66.7 ± 7.6	71.5 ± 4.4
Total**		46	16	139

\* All measured values were equal, hence a standard deviation equaling zero.

\*\* Not all values presented in the table were available for the total number of subjects in each group.

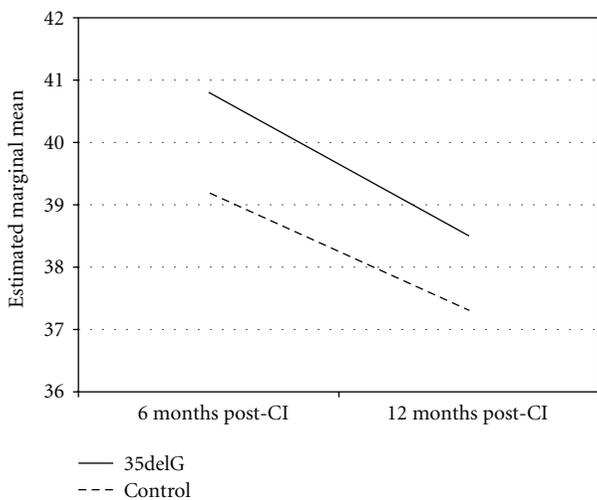


FIGURE 1: Changes in SRT following implantations in patients with 35delG mutation versus others with non-*GJB2*-related deafness.

genetic cases of hearing loss on the outcome of the CI. Although they had a very small number of *GJB2* patients and the larger proportion of identified genetic cases were due

to *SLC26A4*, they proposed an intact auditory nerve as the cause of a better outcome in their implantees but had not studied or proposed other possible causes in the rest of their population. While a syndromic cause is usually identified following a physical examination, lack of the diagnosis in the rest of the recipients brings them into the category of other nonsyndromic causes of deafness that had not been tested. In that case, the theory of an intact auditory nerve could not be readily relied on [6]. One of the advantages of our study stems from the selection of nonsyndromic patients with a prelingual diagnosis of profound hearing loss, which makes the comparison easier by keeping more variables constant. Sampling nonsyndromic patients was particularly feasible in our study setting because of high rate of consanguinity of parents in the target population [10, 11].

Justification of a better auditory perception in genetic cases—including *GJB2* mutation carriers—by other authorities mainly lies in the intactness of the auditory nerve. In a population with such a high rate of consanguineous marriages and taking into consideration the high rate of deafness presenting as a nonsyndromic disease in general, correct selection of patients through physical examination, history taking, and required imaging modalities will choose a high rate of patients with intact auditory nerves. It should

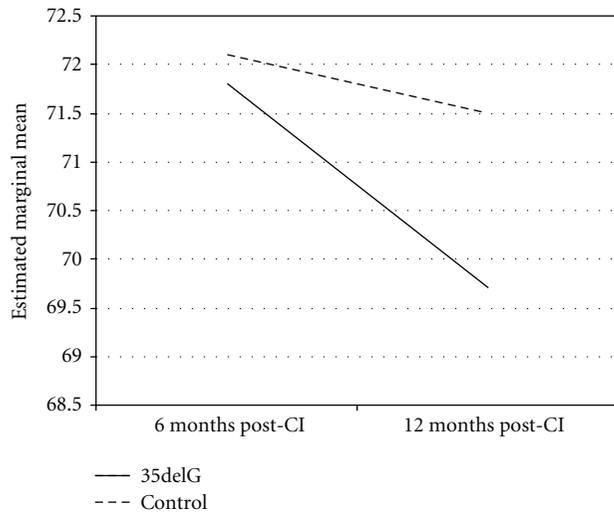


FIGURE 2: Changes in MCL following implantations in patients with 35delG mutation versus others with non-*GJB2*-related deafness.

be highlighted that if only intact auditory nerve had been the support of justifications of a better response in congenital deafness, a much more simple way to choose such patients would have been through electrophysiological tests which are much less expensive compared to genetic tests.

In many articles which suggest a genetic effect on the outcome of CI, the studied population comprises a large variety of cases in which many syndromic deaf patients are also studied [7, 9, 13, 14]. This might have been a confounding factor in their final conclusion. The other question which remains to be answered by supporters of a positive genetic effect is where the problem had been in their patients with no identified genetic cause and still a functioning auditory nerve. The answer to this question might shed light on the reported effect of genetic hearing loss on the outcome of CI.

To the contrary, a perfectly matched, yet smaller study, on CI patients found that there is no advantage in connexin-associated deafness over other patients [17]. The same results had been reported through another two-center study earlier [18]. As indicated in our study, if the patients are selected through a delicate physical examination and other necessary investigations before CI, the acceptable outcome of the implantation would be assured. This will once again emphasize the importance of selecting a comparable control group in making a final conclusion [17]. The similarity of response to CI is also claimed in a recent study [19]. Very interestingly, a current publication on *GJB2* and CI outcome had found a poorer outcome following implantation in *GJB2* carrying patients [20]. All these data are self-explanatory of the debated genetic effect on the outcome of CI.

Although our initial aim was to evaluate the effect of *GJB2* and *GJB6* mutations on the outcome of CI in children with congenital hearing loss, our patient population appeared to have no *GJB6* mutation. Previous studies had reported *GJB6* mutations to be rare or absent in the Iranian population [21, 22]. Further studies on different patient

populations might be done to assess whether our findings can be generalized to patients with *GJB6* mutations.

Our study is not without limitations. Similar to other studies discussed above, we are unable to clarify the pathophysiology of congenital hearing loss in the fraction with no *GJB2* mutation. Answering this question needs genome-wide studies which are beyond our scope. Additionally, because the collection of audiological data was based on manual review of medical records, we were unable to collect complete data on all patients. Finally, as mentioned previously, we used SAT, SRT, and MCL tests as our outcome measures due to a lack of speech-level tests standardized for Persian language. We suggest future researchers to validate open-set and close-set tests for Persian language and use them as their outcome measure.

In short, the findings of our study with a notable statistical power are not in accordance with the assumption that genetic mutations may be associated with different prognosis after CI. Given the intensive work and the expenses of CI with limited resources, particularly for countries where implants are used mainly for profoundly deaf children before the age of 3, we cannot support the hypothesis that genetic testing has a value to the CI procedure and its outcomes. When limited resources and lack of insurance coverage for the genetic tests are factored in, the costs of genetic testing can significantly outweigh its potential benefit. In the absence of larger studies with higher statistical power, a systematic review may enlighten the effect of mutations on the outcome of cochlear implantation and also investigate the reason for heterogeneous results of previous studies.

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## Research Article

# Autosomal Recessive Nonsyndromic Hearing Impairment due to a Novel Deletion in the *RDX* Gene

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The *RDX* gene anchors cytoskeletal actin of stereocilia to hair cell transmembrane and is responsible for autosomal recessive nonsyndromic hearing impairment (ARNSHI) due to DFNB24. A genome scan was performed using DNA samples from a consanguineous Pakistani family with ARNSHI. A significant maximum two-point LOD score of 4.5 ( $\theta = 0$ ) and multipoint LOD score of 5.8 were achieved at marker D11S1998 (chr11 : 117.20 Mb). The region of homozygosity is bounded by markers D11S2000 (105.06 Mb) and D11S4464 (123.13 Mb) and contains the NSHI genes *TECTA* and *RDX*. Although no potentially causal variants were identified in the *TECTA* gene, within the *RDX* gene a novel deletion c.1076\_1079delTTAA (p.Ile359Lysfs\*6) was identified. The *RDX* deletion segregates with ARNSHI within the family and was not observed in 500 control chromosomes. It is predicted to cause premature truncation of radixin at the  $\alpha$ -helical domain and to result in nonfunctional transcripts within the cochlea. *RDX* isoforms which encode the coiled-coil region of the  $\alpha$ -helical domain are deemed necessary for proper function of hair cell stereocilia.

## 1. Introduction

Within the mammalian cochlea, the most specialized cells for hearing that mediate conversion of mechanical vibration from sound energy to neural impulses in the brain (mechanotransduction) are the hair cells, so-named because of the hair-like projections at their apical surfaces, which are called stereocilia. Of the 54 nonsyndromic hearing impairment (NSHI) genes that are currently known, ~20 genes are associated with stereocilia structure or assembly (Hereditary Hearing Loss Homepage). Variants in *RDX* (MIM 179410) cause autosomal recessive (AR) NSHI (DFNB24) and can be found in all three domains of the radixin protein [1, 2]. Radixin is a member of the ERM family of highly homologous proteins including ezrin and moesin. ERM proteins link cell membrane proteins to actin, which is the basic component of the cytoskeleton of microvillar structures such as stereocilia [3]. There are three well-characterized

ERM protein domains: (1) the FERM domain, which binds to transmembrane proteins [4]; (2) the  $\alpha$ -helical domain, which masks interaction sites of the FERM domain to regulate activation [5]; (3) the C-terminal tail or ERM association domain (C-ERMAD) which has an F-actin-binding site [6]. Consistent with its anchoring function in the hair cell, radixin is mainly located at the stereociliary base [7]. This paper describes the fifth family known to date to have ARNSHI due to an *RDX* mutation.

## 2. Materials and Methods

The study was approved by the Institutional Review Boards of Quaid-I-Azam University and the Baylor College of Medicine and Affiliated Hospitals. Informed consent was obtained from all members of family 4208 who participated in the study.

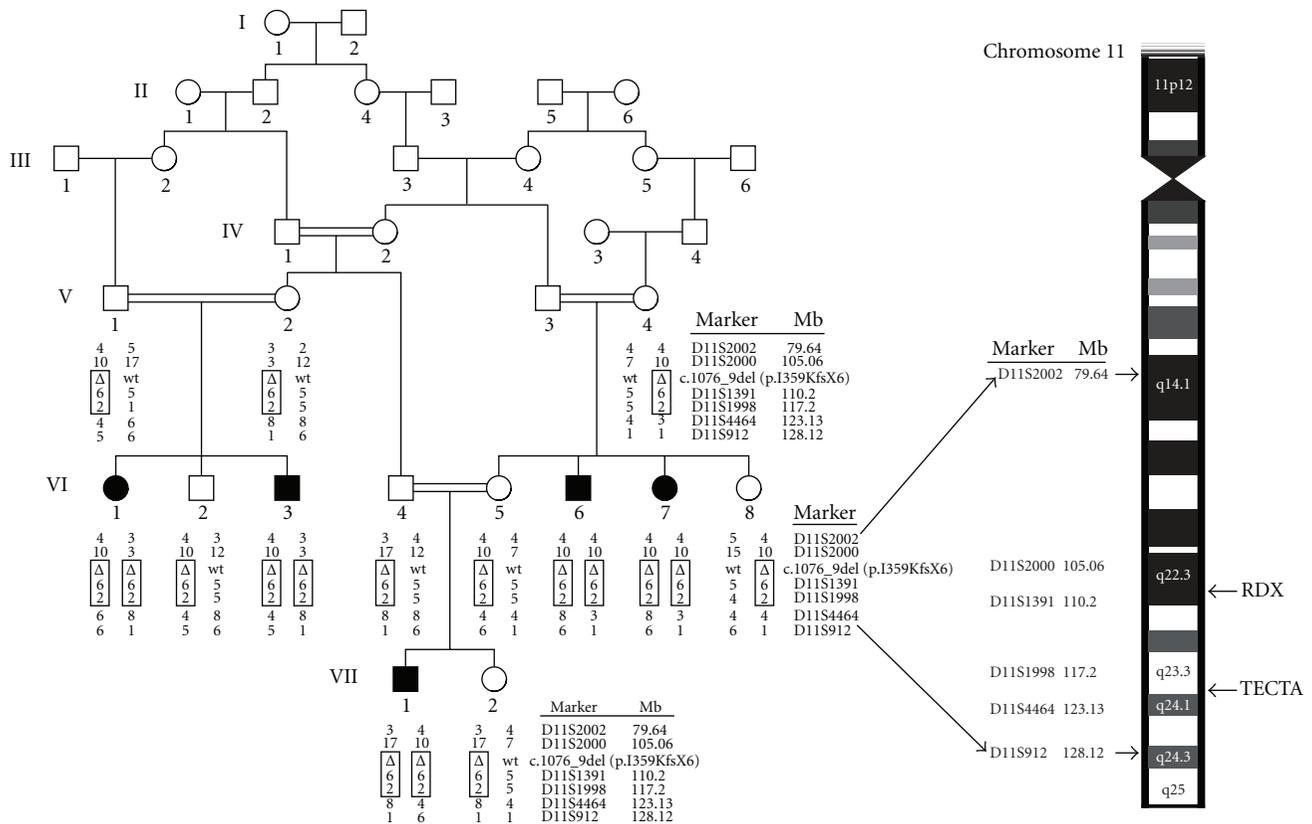


FIGURE 1: Pedigree drawing and haplotype of family 4208 with the genetic interval on chromosome 11. Filled symbols denote individuals with ARNSHI, while clear symbols represent hearing individuals. The haplotype segregating with ARNSHI is shown in a box and includes short tandem repeat markers and the *RDX* deletion c.1076\_1079delTTAA (p.Ile359Lysfs\*6) as a triangle. The positions of the *TECTA* and *RDX* genes relative to the genome scan markers are labelled. The genetic interval with genotyped marker loci and locations of *RDX* and *TECTA* genes were indicated on the cartoon of chromosome 11.

The members of family 4208 are from the Muzafar Ghar district of Punjab province. Venous blood was obtained from 13 members of family 4208, five of whom have HI (Figure 1). Genomic DNA was extracted from whole blood following a standard protocol [8]. All 13 samples underwent a whole genome linkage scan at the Center for Inherited Disease Research (CIDR) using 405 short tandem repeat markers with average spacing of 9cM. Data quality control was performed on the resulting genotype data using PedCheck [9] in order to identify Mendelian inconsistencies and MERLIN [10] to detect occurrences of double recombination events over short genetic distances, which are most likely due to genotyping error. Two-point linkage analysis was performed with MLINK of the FASTLINK package [11]. Multipoint linkage analysis was carried out using Allegro1.2c [12]. An AR mode of inheritance with complete penetrance and a disease allele frequency of 0.001 were used in the parametric linkage analysis. Marker allele frequencies were estimated from observed and reconstructed genotypes of founders from family 4208 and 35 other families who underwent genome scan at the same time at CIDR. For multipoint linkage analysis, genetic map positions were based on the Rutgers combined linkage-physical map of the human genome Build 36 version [13]. For markers used in

the analysis which are not included on the Rutgers map, the physical map position from the human reference sequence (Build 36) was used to interpolate the genetic map position on the Rutgers map. Haplotypes were reconstructed using SimWalk2 [14].

All exons of the *RDX* (MIM 179410; NM\_002906.3) and  $\alpha$ -tectorin (*TECTA* [MIM 602574; NM\_005422.2]) genes were sequenced in HI individuals VI-1 and VII-1 and hearing individual VI-5 who are members of family 4208 (Figure 1). After PCR amplification and purification, sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, Calif, USA). The resulting sequences were assembled and analysed using the Sequencher software (Gene Codes Corp., Ann Arbor Mizh, USA). After identification of the *RDX* deletion, exon 10 was sequenced using DNA samples from additional family members and 250 unrelated hearing control individuals from Pakistan.

### 3. Results and Discussion

Family 4208 (Figure 1) is a consanguineous kindred with ARNSHI from the Punjab region of Pakistan and the family

TABLE 1: Two-point and multipoint LOD scores for family 4208 at chromosome 11q22.3-q24.1.

Marker name <sup>1</sup>	Physical map position <sup>2</sup>	Genetic map position <sup>3</sup>	Multipoint LOD score	Two-point LOD score at $\theta =$					
				0.0	0.01	0.05	0.10	0.20	0.30
D11S2371	73,182,778	84.41	$-\infty$	$-\infty$	-0.35	0.24	0.37	0.31	0.15
D11S2002	79,643,050	91.48	-13.66	$-\infty$	-1.41	-0.18	0.20	0.33	0.22
<b>D11S2000</b>	<b>105,063,887</b>	<b>111.71</b>	-6.18	-6.08	-0.15	0.90	1.07	0.83	0.42
D11S1391	110,200,327	115.14	5.20	3.18	3.10	2.74	2.29	1.43	0.68
D11S1998	117,202,969	126.24	5.78	4.47	4.36	3.95	3.41	2.33	1.26
<b>D11S4464</b>	<b>123,131,592</b>	<b>136.99</b>	$-\infty$	$-\infty$	-2.84	-0.93	-0.25	0.16	0.17
D11S912	128,129,301	145.13	$-\infty$	$-\infty$	-1.52	-0.62	-0.25	0.01	0.06
D11S968	133,323,584	160.10	-4.03	-1.95	-0.06	0.54	0.64	0.45	0.18

<sup>1</sup>Markers in bold denote marker limits based on the 3-unit support interval and the homozygous region.

<sup>2</sup>Physical map positions in base pairs from Build 36 of the human reference sequence.

<sup>3</sup>Genetic map positions in cM from Rutgers combined linkage-physical map of the human genome Build 36 version.

members speak Saraiki. The hearing impairment (HI) was described by family members as prelingual in onset, bilateral, and profound. No possible environmental causes of HI such as perinatal, ototoxic, traumatic, and infectious factors were elicited from the clinical history. Evidence of syndromic and vestibular phenotypes was not found after physical examination that included balance and gait testing.

A significant maximum two-point LOD score of 4.5 was obtained at marker D11S1998 (chr11 : 117.20 Mb) at  $\theta = 0$ . At the same marker, a maximum multipoint LOD score of 5.8 was obtained. The 3-unit support interval (Table 1) and the region of homozygosity (Figure 1) completely overlap, with the proximal limit at D11S2000 (105.06 Mb) and the distal limit at D11S4464 (123.13 Mb). The linkage interval extends over a 25.28 cM region at 11q22.3-q24.1 and contains 18.07 Mb of sequence.

Within the linkage interval there are 167 RefSeq genes, including two NSHI genes, *RDX* and *TECTA*. Both genes were sequenced. No sequence variants were identified in the *TECTA* gene. On the other hand, a novel *RDX* deletion c.1076\_1079delTTAA (p.Ile359Lysfs\*6), which segregates with ARNSHI in family 4208 (Figure 2), was identified. This deletion was not found in 500 control chromosomes, in either the homozygous or in the heterozygous state. The deletion results in a frameshift and premature truncation of the radixin protein to 363 residues and, subsequently, in loss of most of the  $\alpha$ -helical domain and the whole F-actin-binding domain (Figure 3(a)). According to the PROSITE database [15], the deleted domains contain multiple phosphorylation sites for protein kinase C and casein kinase II, which are receptor specific for the ERM protein family [16].

The *TECTA* gene causes both autosomal dominant (DFNA8/12) and AR (DFNB21) NSHI [17]. When inherited recessively, *TECTA* mutations cause moderate-to-severe hearing impairment with a flat or U-shaped audiogram pattern [18]. In contrast, the HI in family 4208 is profound, which is similar to the previously reported HI pattern for *RDX* [2]. In *Rdx*<sup>-/-</sup> mice, degeneration of outer hair cell stereocilia began at onset of hearing [19], and this is consistent with the prelingual onset of HI in humans with *RDX* mutations, as was observed in family 4208. Congenital

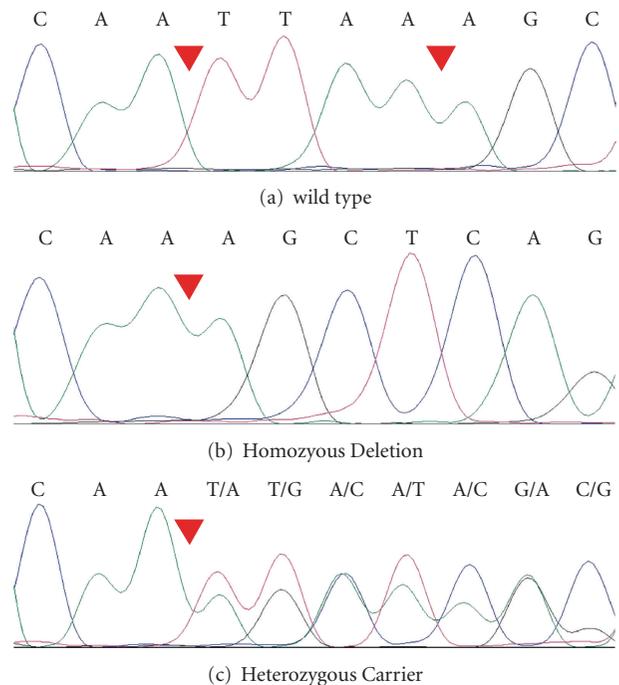


FIGURE 2: Chromatogram displaying the novel *RDX* deletion c.1076\_1079delTTAA (p.Ile359Lysfs\*6). Chromatograms shown are from sequences of the following: (a) wild type, (b) hearing-impaired homozygous individual VII-1, and (c) unaffected heterozygous individual VI-5. The deleted nucleotides TTAA are indicated with a red triangle.

jaundice was also noted in *Rdx*<sup>-/-</sup> mice [20]. However there is no evidence of hyperbilirubinemia in family 4208. Additionally in the previously reported four families that segregate *RDX* mutations there is no evidence of hyperbilirubinemia [1, 2].

The mutation c.1076\_1079delTTAA is located in exon 10 of *RDX*. Of the six isoforms of *RDX* (Figure 3(a)), isoform c does not include exons 7 to 13 of isoform a. In the original DFNB24 family, the p.Gln155\* stop codon mutation can be found in exon 5, which is not included in *RDX* isoform d [2]



(Figure 3(a)). In particular, exon 10 marks the beginning of a series of highly conserved heptad repeats in the  $\alpha$ B helix which interacts with the corresponding highly conserved heptad repeats in the  $\alpha$ C helix (Figure 3(b)) as the  $\alpha$ -helical domain folds on itself to form an antiparallel coiled coil in the dormant state [5] (Figures 3(a) and 3(b)). When active, the coiled coil is fully extended, unmasking ligand-binding sites [5]. This may indicate that the  $\alpha$ B/ $\alpha$ C coiled coil is necessary for regulation of radixin activity within hair cell stereocilia.

#### 4. Conclusion

A novel deletion c.1076\_1079delTTAA (p.Ile359Lysfs\*6) in the *RDX* gene was identified in a large Pakistani consanguineous pedigree that segregates ARNSHI. This novel *RDX* deletion is predicted to cause premature truncation of radixin at the  $\alpha$ -helical domain and to result in non-functional transcripts within the cochlea. Identification of the c.1076\_1079delTTAA (p.Ile359Lysfs\*6) deletion gives a better understanding of the role the *RDX* gene plays in hearing impairment.

#### Electronic Database Information

The following URLs were accessed for data in this paper: Hereditary Hearing Loss Homepage (<http://hereditaryhearingloss.org/>); UCSC Genome Browser (<http://genome.ucsc.edu/>); OMIM (<http://www.omim.org/>). ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

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## Review Article

# The Sound of Silence: Mouse Models for Hearing Loss

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Sensorineural hearing loss is one of the most common disabilities in humans. It is estimated that about 278 million people worldwide have slight to extreme hearing loss in both ears, which results in an economic loss for the country and personal loss for the individual. It is thus critical to have a deeper understanding of the causes for hearing loss to better manage and treat the affected individuals. The mouse serves as an excellent model to study and recapitulate some of these phenotypes, identify new genes which cause deafness, and to study their roles *in vivo* and in detail. Mutant mice have been instrumental in elucidating the function and mechanisms of the inner ear. The development and morphogenesis of the inner ear from an ectodermal layer into distinct auditory and vestibular components depends on well-coordinated gene expression and well-orchestrated signaling cascades within the otic vesicle and interactions with surrounding layers of tissues. Any disruption in these pathways can lead to hearing impairment. This review takes a look at some of the genes and their corresponding mice mutants that have shed light on the mechanism governing hearing impairment (HI) in humans.

## 1. Introduction

The mammalian inner ear is a highly organized structure divided into auditory and vestibular components that are responsible for detecting and coordinating hearing, acceleration, and balance. The auditory organ consists of the coiled cochlea which senses sound. Inside this organ is a highly specialized epithelium that converts mechanical actions into electrical potentials. These epithelia contain sensory hair cells (HCs) as well as surrounding supporting cells. The hair cells are mechanoreceptors that can trigger action potentials in response to sound or movement. Damage to this small population of hair cells is a major cause of hearing loss.

There are two types of hearing impairment (HI): conductive and sensorineural. This classification is based on which part of the ear is affected. While conductive HI results from defects in the external or middle ear, sensorineural HI is due to malformations along the inner ear from the cochlea to the auditory cerebral cortex. A conductive defect generally leads to a less severe HI and can be solved with medical treatment. In contrast, a sensorineural defect leads to a range of HI, from less severe to extreme, and though sensorineurally hearing-impaired persons may be

aided with cochlear implants or hearing aids, the problem is not completely solved [1].

In more than half of the patients with hereditary hearing loss (HHL), there are known genetic mutations. In most of these cases, a single gene is affected, resulting in the defect. About 70% of HHL cases in human are associated with a vestibular dysfunction only (nonsyndromic hearing loss (NSHL)) [2]. Various types of genes have been associated with HLL in humans. They include protein-coding genes (68) and tRNA- or rRNA- coding genes (7). (An extensive list can be found in the Hereditary Hearing Loss Homepage: <http://hereditaryhearingloss.org/>). The protein-coding genes primarily include transcription factors, ion channels and transporters, extracellular matrix components, gap junction and adhesion proteins, as well as myosins, cytoskeletal proteins, which all interact with each other to form a complex network that is critical for hearing.

## 2. One Stone Two Birds: Auditory and Vestibular Function of Inner Ear

To have a better understanding of the hearing loss, it is important to have a clearer picture of the formation of the inner ear. The mature inner ear, with its acoustic and

vestibular components, is encased in the dense bone of the skull. Research over the years from multiple labs has helped us to have a better understanding of how the inner ear is shaped from a simple otocyst during embryonic development.

While the otocyst initially consists of simple pseudo-stratified epithelium, it soon undergoes extensive proliferation and differentiation that will eventually establish the ventrally derived auditory component, the cochlea, and the dorsally derived vestibular apparatus. In mammals, hearing is initially mediated through sensory cells located within the coiled cochlea. Almost all of the cell types within the membranous labyrinth of the inner ear are derived from multipotent epithelial progenitor cells initially located in the otocyst. Otocyst-derived cells develop into three major lineages, prosensory (cells that will develop as either hair cells or associated supporting cells), proneural (cells that will develop as auditory or vestibular neurons) and nonsensory (all other otocyst derived cells) with cells within each lineage developing in a precise spatiotemporal manner. Gene knockout studies have identified specific signaling molecules and pathways, including *Notch*, *Hedgehog*, *Sox2*, and *Fgfs*, that guide progenitor cells to develop first as a sensory precursor and subsequently as one of the more specialized cell types. For a detailed review on cochlear development, see [3, 4]. Highly differentiated sensory hair cells develop within the coiled cochlear duct to form the organ of Corti, which is responsible for detecting sound. Likewise, sensory hair cells arise within the vestibular apparatus to form the maculae in the utricle and saccule and the cristae in the semicircular ducts. Collectively, they are responsible for detecting gravity as well as maintaining balance [5, 6], for review see [7]. Generally, genes expressed in the ventral otocyst are implicated in the formation of auditory structures, while genes expressed in the dorsal otocyst are implicated in the formation of the vestibular apparatus.

*Pax2*, which is an early marker of otic fate, is subsequently required for cochlear development, and its inactivation in mice leads to cochlear agenesis [8]. *Pax2* is also expressed in the endolymphatic duct [9]. However, the vestibular apparatus and endolymphatic duct develop normally in the *Pax2* null mice, maybe due to compensation from other *Pax* genes, possibly *Pax8*. Detailed analysis of *Pax2/Pax8* double null mice could substantiate and clarify the roles of both genes during inner ear development.

In the *Otx1* null mice, both the lateral semicircular duct and the lateral sensory cristae are absent, similar to *Prx1/Prx2* double null mice, suggesting that *Otx* and *Prx* genes may interact with each other via other genes during inner ear development. The ventral-apical expression domain of *Otx2* in the otic epithelium gives rise to the saccule and a portion of the cochlea. Since *Otx2* null mice die prior to morphogenesis of the inner ear beyond the otic vesicle, *Otx2* function during inner ear development has been inferred by the analysis of *Otx1* null mice that are also heterozygous for *Otx2* [10–12]. The inner ear defects in these mice are more extensive than those reported for the *Otx1* null mice [10]. The defects to the cochlea are expanded ventrally and the saccule, which is unaffected in *Otx1* null mice, is

dysmorphic. Therefore, *Otx2* expression appears to function both redundantly and independently of *Otx1* in establishing proper specification of the cochlea and saccule. Unlike *Otx1*, *Otx2* expression does not appear to be mediated by *Shh* signaling as ectopic expression of *Shh* in mice does not induce concomitant ectopic expression of *Otx2* [13].

*Gbx2* expression eventually becomes restricted to the endolymphatic duct and ceases in the inner ear by E15.5 [14]. The knockout analysis showed a key role of *Gbx2* in patterning of the dorsomedial regions (endolymphatic duct, vertical pouch) [14] leaving *Gbx2* null mice with a phenotype similar to that described for *kreisler* mice [15], mainly the absence of the endolymphatic duct and swelling of the membranous labyrinth. In the most severe cases, even the ventral inner ear structures were affected [14]. For a more detailed review of the inner ear development, see [16].

### 3. Of Mice and Men

The study of sensorineural HHL in humans is severely hampered by the absence of tools to study inner ear development *in vivo* as well as absence of cell lines, which recapitulate this process. Though genetic linkage analysis of HHL in humans is one possibility, it is limited by the requirement of a large family with many HI patients. Over the years, mouse has been a model system of choice for human diseases due to its evolutionary closeness. Hence, mutant mouse models that exhibit HHL may help to identify genes that have a role in the development or function of the inner ear. Many hearing-impaired mutant mice have arisen spontaneously during the last century. Also X-ray, chemical (by ENU, N-ethyl-N-nitrosourea and chlorambucil), gene trapped or targeted mutants have resulted in many new hearing-impaired mouse strains. Mutations in about 180 different genes have been reported as responsible for inner ear malformations in mice (<http://hearingimpairment.jax.org/>). After locating the affected gene, the mutant mice can be used for a spatiotemporal study of the gene, in an attempt to pinpoint the exact role of the gene in various pathways and processes involved in shaping the inner ear. But as with any model system, the mice do not and cannot recapitulate all the genes and pathways in human as only 44 of these genes have been linked to human HHL. In addition, two genes that were linked with human HHL were found as not crucial for inner ear development and function in knockout mice [2].

Over the years, the many assays used to evaluate the phenotype of deafness-related mutations have been available in the mouse model, making it an ideal system to study human deafness. These assays include, but are not limited to microscopy—bright field, transmission and scanning electron [17–19] (Figures 1(a)–1(h)), as well as by paint-fill analysis [23]. Physiological test, such as patch clamp assays, may be used to measure currents or membrane potentials in a single cell. Change in length of individual cells may be used to measure electromotility of outer hair cells [20] (Figure 1(i)). Temporal and spatial expression patterns of specific mRNAs can be observed by *in situ* hybridization in wild type and mutant inner ears [21, 24] (Figure 1(j)), and localization

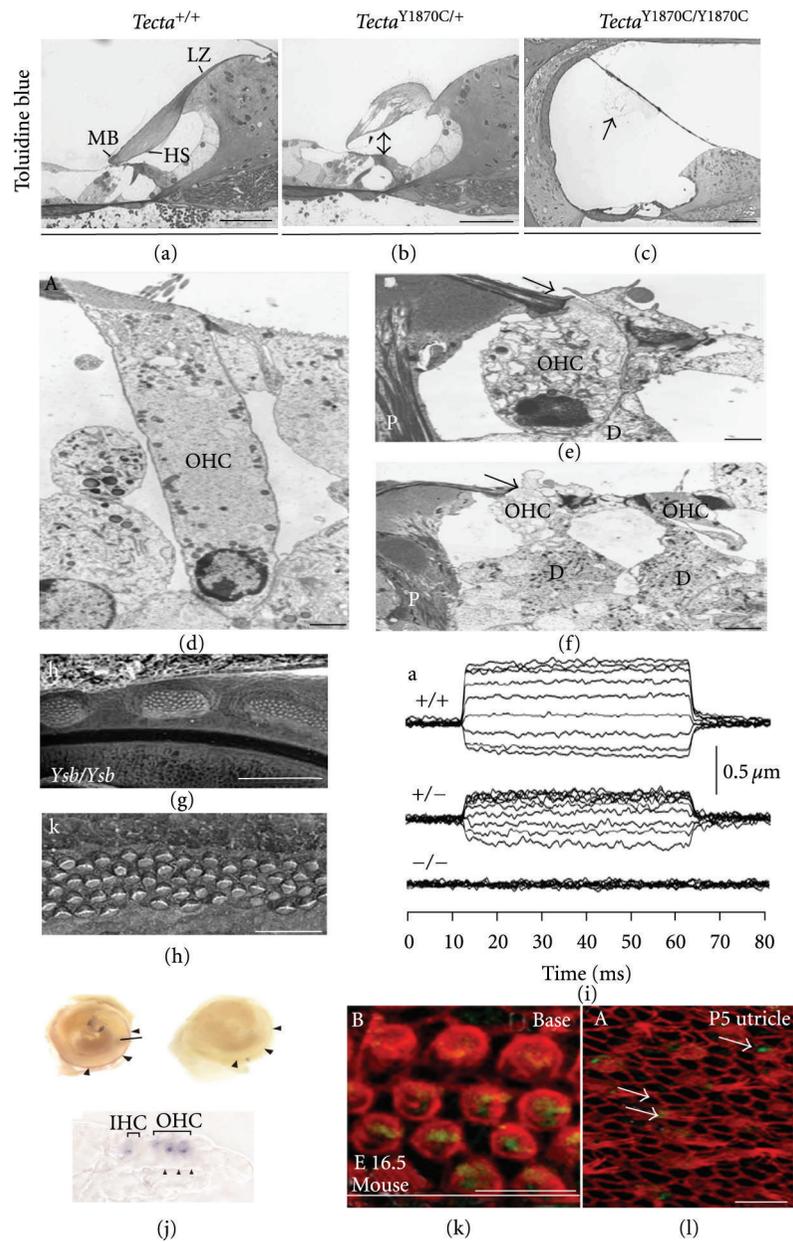


FIGURE 1: Assays to detect phenotypes of deafness-related mutations in mice. ((a)–(c)) Light microscopy analysis of sections of cochlear duct from (a) wild type, (b) *Tecta*<sup>Y1870C/+</sup>, and (c) *Tecta*<sup>Y1870C/Y1870C</sup>. LZ: limbal zone, and MB: marginal band, HS: Hensen's stripe. Arrowhead in (b) Kimura's membrane, arrow in (c) tectorial membrane. Reprinted with permission from [17]. ((d)–(f)) Transmission electron micrograph of the organ of Corti in wild type versus when *Cx26* was deleted (e–f). D: deiters cell, P: outer pilla cells. Reprinted with permission from [18]. ((g)–(h)) Scanning electron micrograph of Organ of Corti basal portion at P0 from yellow submarine (*Ysb*) homozygous. Reprinted with permission from [19]. (i) In vitro analysis of OHC electromotility in wild type and mutant (*Slc26a5*) mice. It shows length changes of OHC in response to voltage steps (–120–60 mV in 20 mV steps) in whole-cell, voltage-clamp recordings. Reprinted with permission from [20]. (j) In situ hybridization detects expression of *Cdh23* in the neurosensory epithelium of 4-day neonates. Antisense probe specifically labels the neuroepithelium along the cochlea duct (arrowheads). Cross-section through the cochlear duct identifies specific labeling in three outer (OHC) and one inner hair cells (IHCs). Reprinted with permission from [21]. (k) Immunohistochemistry on whole mount shows *Cdh2* (green) is detected at E16.5 in stereocilia (stained for F-actin, red) of hair cells from the basal turn of the cochlea in wild-type mice. (l) Immunolocalization of cadherin 23 (shown in green) in the inner ear of homozygous waltzer *v<sup>6j</sup>* mice. Cadherin 23 (green) was not detected in utricle stereocilia (stained for F-actin, red), but was detected in the cuticular plate of hair cells (arrows). Reprinted with permission from [22].

of various proteins can be detected by immunofluorescence [21, 22, 25, 26] (Figures 1(k)-1(l)).

#### 4. Mutant Mice for Genes of the Hair Bundle

Five genes have been implicated in NSHL in humans that directly have a role in either development and/or maintenance of the hair cell bundle. They are *MYOVI*, *USH1C*/*Harmonin*, *CDH23*, *PCDH15*, and *VLGR1*/*MASS*. These genes also contribute towards the progression of Usher syndrome, which is a combination of hereditary deafness and blindness. It is marked by sensorineural hearing loss and loss of visual field due to retinitis pigmentosa.

It has been shown that all the Usher-related proteins are bound to each other through the harmonin PDZ sites and form a multiprotein unit that can move along the hair cell actin filaments to their site of action within the stereocilia [27]. In the hair bundle, the stereocilia are arranged in rows with a special staircase pattern. The Usher-related proteins participate in interstereociliar links essential for mechanotransduction, a process where the cochlear and vestibular hair cells translate mechanical movements of their hair bundles to electrochemical signals.

Cadherin 23 (*Cdh23*) and protocadherin 15 (*Pcdh15*) are transmembrane proteins with a short intracellular and a long extracellular domain. In their cytoplasmic end, *Cdh23* and *Pcdh15* contain class-I-PDZ-binding motifs (PBM) that can bind PDZ-containing proteins like Harmonin. In the wild-type mouse, *Cdh23* is localized to the hair cell stereocilia and Reissner's membrane [22, 28]. *Pcdh15* is widely expressed in multiple tissues, including the brain, cochlea, and vestibule [29]. In the developing cochlea, *Pcdh15* was localized to the apical surface of hair cells, supporting cells, outer sulcus cells, and spiral ganglion cells, while mature cochleae express protocadherin 15 only in hair cell stereocilia [30].

The first reported mutant for *Pcdh15* was Ames-waltzer (*av*). Ames-waltzer mice were known to harbor a recessive spontaneous mutation causing deafness, circling behavior, head tossing, and hyperactivity, which were very similar to the waltzer (*v*) phenotype. Subsequently, many mutations in the same locus arose independently, resulting in similar phenotypes. The mutated gene was found to be *Pcdh15* in an Ames-waltzer allele that was raised in transgenic mice following insertional mutagenesis [31]. Circling behavior and a reduced AM1-43 dye uptake, that correlates with normal transduction in hair cells, preceded structural defects in the vestibule that could be observed by light or scanning electron microscopy. While inner ears of P10 homozygotes displayed only abnormal stereocilia in the cochlea, by P30 the saccular stereocilia began to be affected. Adult homozygous mice had an almost complete degeneration of the organ of Corti and vestibular saccular macula. In the cochlea, degeneration of the spiral ganglion neurons was also observed. The neuroepithelia of the utricle and the semicircular canals cristae had a normal morphology, but the utricular otoconia were large and malformed.

There are four different spontaneous mutants for *Cdh23* in mice: *waltzer* [21, 28], *waltzer niigata* [32], modifier

of deafwaddler—*mdfw* [33], and age-related hearing loss—*Ahl* [34]. Chemical mutagenesis by chlorambucil—deletion mutations—(*Albany-waltzer* [35]) and ENU point mutations have also given rise to *Cdh23* mutated mice. All but one (*Ahl*) of these-*Cdh23*-mutated mouse strains displayed a similar phenotype: NSHL with circling behavior, head tossing, and erratic movements that appear in homozygotes from birth. Heterozygotes appeared normal at birth, but developed a progressive hearing loss with aging and had a higher sensitivity for noise-induced hearing loss [36]. Waltzer mouse mutants also display a progressive disorganization of the hair bundle, which is first noted at the beginning of the bundle formation at E18.5.

The first spontaneous mouse mutant for *Vlgr1* was *Mass1<sup>Frings</sup>*. This is used as a model for epilepsy due to its susceptibility to loud-noise-induced seizures. The BUB/BnJ inbred mouse strain is naturally homozygous for the *Mass1<sup>Frings</sup>* mutation and is prone to sound-induced seizures and progressive hearing loss leading to complete deafness [37, 38]. Interestingly, BUB/BnJ mice are also homozygous for the *Ahl* allele of *Cdh23*, but unfortunately this is not enough to explain the deafness of these mice because other strains that are homozygous to *Ahl* do not display the severity of hearing loss. The association of *VLGR1* mutations with HHL in Usher type II syndrome in humans [39] pointed to the fact that the *Mass1<sup>Frings</sup>* mutation underlies hearing loss in BUB/BnJ mice. Indeed, it was shown that the double mutants of *Cdh23* and *Vlgr1* were responsible for most of the hearing loss in BUB/BnJ mice. In young BUB/BnJ mice, the cochlear stereocilia developed abnormally and remained in an underdeveloped state. Stereocilia were disconnected and detached, the most severely affected bundles lost their polarity and graded height. At older ages, hair cells and spiral ganglion cells were degenerated [40].

There are two mutant *Vlgr1* mice, a knockout mouse that expresses no *Vlgr1* proteins [41] and *Vlgr1/del7TM* mice, in which the transmembrane domain is deleted [42]. In both models, the absence of *Vlgr1* receptors resulted in cochlear abnormalities. Homozygous mice did not display ankle links between the hair cell stereocilia. Although the hair bundles appeared normal at birth, they became disorganized thereafter. Homozygous mutant for *Vlgr1* developed acute deafness by the third week of birth and henceforth displayed disorganized hair bundles, including displaced kinocilia, resulting in distorted stereocilia development.

#### 5. Mutant Mice for Genes Responsible for Endolymph Production

The cochlea is an interesting organ with regards to electrophysiology. The perilymphatic space contains a fluid (perilymph) with a high  $\text{Na}^+$  and low  $\text{K}^+$  concentration. The apices of the hair cell are facing the endolymph, which is just the opposite in its ionic composition (low  $\text{Na}^+$ , high  $\text{K}^+$ ), but its basal surface is immersed in perilymph. Movement of  $\text{K}^+$  in the cochlea from perilymph to endolymph through the cochlear lateral wall and maintenance of the ionic composition of the endolymph are critical for auditory

function. Mutant mice for genes involved in K<sup>+</sup> movement and recycling have been instrumental in the understanding of this critical process.

There are several genes known to account for HLL in human which have a role to play in K<sup>+</sup> recycling, and mutant mouse were developed for the some of them which include *Gjb2/Cx26*, *Gjb6/Cx30*, and *Cldn14* that encode intercellular adhesion proteins. *Gjb2* and *Gjb6* genes produce the gap junction proteins, connexin 26 (Cx26) and connexin 30 (Cx30), while *Cldn14* codes for a tight-junction protein; *Kcne1*, *Kcnq1*, and *Kcnq4*, all encoding for potassium ion channels; and finally *Slc26a4* that encodes an anion transporter.

In humans, *GJB2* and *GJB6* genes are located in the same chromosomal locus (13q11-12). Mutations in this locus account for a very high incidence of congenital hereditary NSHL with slight variability over populations. The causative mutations are small deletions in the *GJB2* gene and they are autosomal recessive in nature. Over a hundred deafness-related different mutations in *GJB2* have been identified in humans so far.

Both targeted mutagenesis [43] and chemical (ENU-induced) mutagenesis [44] have been used to knock out the *Gjb2/Cx26* gene in mice. Both of these methods resulted in an identical phenotype. While the heterozygotes had no auditory defects, the homozygotes had prenatal death due to placental defects in the mother. The absence of any auditory phenotype led to the employment of two additional strategies to generate hearing-impaired viable mutant *Gjb2* mouse models. *Gjb2* was conditionally knocked out in the cochlear epithelial network, using the *cre-loxP* system by crossing the *Gjb2-loxP* mouse to a mouse carrying the *Cre* under the *Otog* promoter, which is expressed only in cochlear epithelial cells [18]. In the other approach, point mutagenesis was employed to create the *Cx26<sup>R75W</sup>* mutation [45] that is causative of autosomal dominant SHL (HHL and skin disease) in human heterozygotes [46]. The dominant inheritance was attributed to the ability of the mutant Cx26 to inhibit the function of gap junctions that coassemble wild-type and mutant Cx26 molecules.

Both homozygotes for *Gjb2* conditional knockout and heterozygotes *Cx26<sup>R75W</sup>* exhibited similar auditory defects in adults and histological profiles, although the point mutant displayed a much more severe phenotype. In both mice, the inner ear developed normally until postnatal day 14 (P14). Only after the onset of auditory function, at P15-P16, epithelial cells began to die mostly due to apoptosis. The IHC-neighboring supporting cells were first damaged, followed by OHC and their supporting cells. The tunnel of Corti was collapsed. Heterozygotes for *Cx26<sup>R75W</sup>* displayed degeneration of all organs of Corti that began at P14 and led to a complete degradation of both hair cells and supporting cells by seven weeks after birth. It is interesting to note that in *Gjb2* knockout mice, IHC died only in the severely hearing-impaired mice, and some of the intradental cells of the spiral limbus were degenerated at a much older age (P60). The reticular lamina at the apical surface of the sensory epithelium was disrupted from an early stage in *Gjb2* knockout mice. Therefore, it appears that, though Cx26 is

not required for the normal development of the organ of Corti, it is essential for its survival and function.

Following reports of recessive mutations of human *CLDN14* as causative of extreme NSHL in humans [47], *Cldn14*-null mutant mice were created to explore the role this gene plays in the inner ear function and morphogenesis. *Cldn14*-null mice had a normal EP, but were deaf. There were no discernible vestibular phenotypes. Although the reticular lamina tight junctions seemed normal microscopically in null mice, the hair cell stereocilia were lost or disintegrated during the first 3 weeks of life, rapidly followed by hair cell degeneration. Unlike the Cx26 mutant, here OHCs were degenerated before IHC.

The genes *Kcne1*, *Kcnq1*, and *Kcnq4* encode for subunits of slow voltage activated potassium channels, which are critical components of cellular repolarization in excitable cells. They open during depolarization and facilitate selective efflux of K<sup>+</sup> across semipermeable membranes. *Kcne1* [48] or *Kcnq1* [49, 50] knockout mice exhibited a classic waltzer-like phenotype with severe hearing loss and vestibular symptoms, leading to complete deafness in adult mice. Although these mice display normal anatomical structure of the inner ear at birth, there are rapid changes later. The strial marginal cells and the vestibular dark cells were unable to secrete K<sup>+</sup> ions, leading to degeneration of the neuroepithelium including the hair cells and complete collapse of the endolymphatic space.

M type channels are very slow-voltage-dependent K<sup>+</sup> channels, and *Kcnq4* is an alpha subunit of such a channel. In humans, *KCNQ4* mutations induce autosomal dominant NSHL, suggesting that the mutated gene has a dominant negative effect when it is coexpressed with the wild-type allele [51]. Two mutant mouse models for *Kcnq4* are available: a homozygous knockout mouse and a mouse with a point mutation that mimics the dominant negative mutation in humans. Surprisingly, no vestibular phenotypes were observed in both these mutants, although *Kcnq4* is strongly expressed in vestibular hair cells. The mice had normal hearing at and after birth, but displayed a progressive hearing impairment that was accompanied with a degeneration of OHC. The progression of both deafness and OHC loss was much faster in homozygotes (both for knockout as well as point mutant) over the heterozygotes. In the homozygotes the phenotype was detected within weeks as compared to months in heterozygotes. A selective inhibitor of Kcnq channels was used to isolate Kcnq-dependent K<sup>+</sup> currents, and both from the OHC homozygous, or dominant negative heterozygous Kcnq-dependent K<sup>+</sup> currents were detected. The absence of the K<sup>+</sup> current led to depolarized resting membrane potentials of the OHC. IHCs were not significantly affected. Therefore, it was proposed that *Kcnq4* mutations induce a progressive HHL due to chronic depolarization of OHC, leading to their degeneration [52].

The transportation of several anions, including chloride, iodide, sulfate, nitrate, bicarbonate, hydroxyl, oxalate, and formate constitutes the main function of the SLC26 (solute carrier protein 26) family of anion exchangers. These are integral proteins with 10–12 transmembrane domains. Each member in this family has different affinity and specificity for different anions. Two members of the SLC26 have been

associated with HHL in humans: SLC26A4/pendrin and SLC26A5/prestin. *SLC26A4* mutations were associated with both SHL (Pendred syndrome) [53] and NSHL [54], while *SLC26A5* was associated only with NSHL [55].

*Slc26a4* knockout mice (*Pds*<sup>-/-</sup>) exhibited waltzer-like vestibular dysfunction and complete deafness. Their inner ears developed normally only until E15, which is two days after the start of pendrin expression in wild-type mice. Thereafter, endolymphatic cavities were severely dilated, both in cochlea and vestibule. This dilatation was proposed to be secondary effect due to a changed osmotic condition and a much increased volume of the endolymphatic fluid. During the second postnatal week, hair cells began to degenerate. In the vestibule, the otoconia and otoconial membranes were also destructed [56]. After weaning, the stria vascularis marginal cells of *Pds*<sup>-/-</sup> mice displayed irregular shapes and sizes, resulting in a thinner stria vascularis.

## 6. Mutant Mice for Extracellular Matrix Components (ECM)

The basilar membrane, composed of collagen, is the connective tissue on which sits the cochlea harboring the organ of Corti. The basilar membrane is of various stiffness along the cochlea and resonates due to sound-induced movements of the cochlear fluids. These vibrations are detected by two types of hair cells, included in the sensory epithelium of the organ of Corti and the inner and outer hair cells (IHC and OHC), respectively. Though so far seven known collagens have been linked with Human HLL: COL2A1, COL4A3, COL4A4, COL4A5, COL9A1, COL11A1, and COL11A2, only five of these have a mutant mouse model (*Col11a1*, *Col11a2*, *Col2a1*, *Col4a3*, *Col9a1*). A *COL11A2* mutation was linked to autosomal dominant NSHL and Stickler syndrome in human. The other collagen genes were only associated with SHL in humans, mainly Stickler (*COL2A1*, *COL9A1* and *COL11A1*) and Alport (*COL4A3-5*) syndromes. Because the human *COL4A3* gene is causative for Alport syndrome [57], a mouse knockout for *Col4a3* was generated [58]. Homozygotes died at about 14 weeks of age due to renal failure. Postmortem analysis revealed that renal glomeruli had defective basement membranes and cochlear membranous labyrinth was highly degenerated, mimicking the human condition. Both *Col4a3* and *Col4a4* were found to be completely absent in the cochlear labyrinth. Basement membranes of different regions of the membranous labyrinth were significantly different in thickness or were completely absent when compared to wild type cochleae, and there were collapsed capillaries nearby. Renal and cochlear defects were highly progressive and HI was detected only after 6 weeks.

In addition to sensorineural HHL, Stickler syndrome also causes degenerative changes in various joints with abnormal bone development, vertebral abnormalities, osteoarthritis, and in severe cases unusual cleft palate. The Stickler syndrome-related collagens, *Col2a1*, *Col11a1*, and *Col11a2*, are important components of both the cochlear TM and

cartilage. COL2A1 was found to be involved in sensorineural deafness that is associated with hereditary syndromes in humans, like Stickler syndrome, spondyloepiphyseal dysplasia congenita (SEDC), and chondrodysplasia. A *Col2a1* mutant mouse was generated by irradiation in 1966. This mutant had a three-nucleotide deletion in the region encoding the C-propeptide globular domain of *Col2a1* recapitulating the *Dmm* condition in humans. *Dmm* mice expressed a reduced level of collagen II and suffered from cartilage defects that affect inner ear development as well [59].

The *cho* mutation is a single nucleotide deletion in the *Col11a1* gene that causes a frameshift and a premature stop codon. *Cho* mice are spontaneously arisen mutant mice [60]. Homozygotes had a cleft palate and had postnatal death due to lethal chondrodysplasia. The premature termination of translation leads to an incomplete protein that is incapable of assembling with other collagen molecules. Homozygotes were severely hearing-impaired at birth due to underdevelopment of the organ of Corti in the lower turn of the cochlea, with no hair cells, supporting cells, nerve endings, and pillar cells [61].

A *Col11a2* knockout mouse was generated by insertional mutagenesis by inserting a neomycin resistance cassette in exons 27 and 28 of the gene. The inserted sequence included a premature termination codon, resulting in a truncated protein product. The phenotype was much less severe compared to *cho* mice. The only observable phenotype in the inner ear was a larger and less compact TM with disintegrated collagen fibrils [62, 63].

## 7. Micro-RNAs in Deafness

In recent years, there is an increasing focus on noncoding RNAs and their contribution to regulation of normal development as well as disease. It is now established that the miR-183 family of miRNAs is expressed specifically in the inner ear hair cells and the eye retina in mammals [64, 65], and other studies have reported that at least 100 different miRNAs are present in the developing mouse inner ear [66]. Recently, two groups have located point mutations in the seed region of the *miR-96* with autosomal dominant progressive NSHL in humans [67] and mice [68]. In human, two different mutations (transversion and transition) in neighboring nucleotides (13G > A and 14C > A) of the *MIR96* gene were observed in two unrelated Spanish families. The hearing loss in the two families was not identical. These mutations significantly affected the biogenesis of mature miR-96 or increased its degradation. In addition, these mutations changed the target mRNA population which was targeted by the miRNA, and the mutated miR-96 had an impairment in its ability to downregulate the translation of several mRNAs that are targeted by wild-type miR-96. Other substitutions in the pre-miRNA sequences of *MIR96* and *MIR182* genes, outside the mature miRNA sequences, were found in 10 and 22 families [67], respectively, but did not segregate with the hearing loss phenotype. Although from the mouse study the link between the direct targets of the miRNA and the phenotype are not clear, several genes

known to be important for hair cell function are specifically downregulated in the diminuendo mutant and any one could account for the hair cell dysfunction.

## 8. Conclusion

The sense of hearing is one of the most crucial senses endowed to a living organism and its loss can have many ramifications. Finding the causes, both genetic and environmental, goes a long way in understanding this common disability. The mouse has been an invaluable ally in our understanding of the various genetic components which underlie hearing impairment. Efforts are underway to knock out all the genes in the mouse genome and this will undoubtedly give rise to many more models for HLL. The future requires research in complex hearing impairment and a study of complex/multiple mutants to obtain a better molecular handle of the various genetic interactions occurring in both the normal, as well as diseased ear. There is also a growing need to look at this problem from a systems biology angle by simultaneously deciphering the multiple genes and their regulatory networks involved in hearing loss and to capture the earliest set of genes involved in the auditory processes and their downstream targets. With the knowledge accumulated, it will eventually make hearing impairment easier to detect, manage and rectify at the genetic level.

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## Research Article

# Assessing Noncoding Sequence Variants of *GJB2* for Hearing Loss Association

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Involvement of *GJB2* noncoding regions in hearing loss (HL) has not been extensively investigated. However, three noncoding mutations, c.-259C>T, c.-23G>T, and c.-23+1G>A, were reported. Also, c.-684\_-675del, of uncertain pathogenicity, was found upstream of the basal promoter. We performed a detailed analysis of *GJB2* noncoding regions in Portuguese HL patients (previously screened for *GJB2* coding mutations and the common *GJB6* deletions) and in control subjects, by sequencing the basal promoter and flanking upstream region, exon 1, and 3'UTR. All individuals were genotyped for c.-684\_-675del and 14 SNPs. Novel variants (c.-731C>T, c.-26G>T, c.\*45G>A, and c.\*985A>T) were found in controls. A hearing individual homozygous for c.-684\_-675del was for the first time identified, supporting the nonpathogenicity of this deletion. Our data indicate linkage disequilibrium (LD) between SNPs rs55704559 (c.\*168A>G) and rs5030700 (c.\*931C>T) and suggest the association of c.[\*168G;\*931T] allele with HL. The c.\*168A>G change, predicted to alter mRNA folding, might be involved in HL.

## 1. Introduction

About two hundred *GJB2* mutations causing nonsyndromic hearing loss (NSHL) have been reported (<http://www.hgmd.org/>) [1]. Most *GJB2* mutations described so far localize to the coding region (totally included within exon 2), which is routinely analysed upon the study of *GJB2* in HL patients. Also involved in HL, two deletions, del(*GJB6*-D13S1830) [2–4] and del(*GJB6*-D13S1854) [5] disrupt the *GJB6* gene which codes for connexin-30, and it is thought that they may ablate a *GJB2* cis-regulatory sequence [5–7]. This putative element is likely to be ablated by a third deletion, del(chr13:19,837,344–19,968,698), localized upstream of *GJB2* and *GJB6* [8, 9].

Along with *GJB2* coding region, the noncoding first exon and donor splice site have been analysed in several studies, and two pathogenic mutations, c.-23G>T (exon 1) [10] and c.-23+1G>A (intron) [11], both in the donor splice site, have been identified. The c.-23+1G>A mutation (commonly known as IVS1+1G>A), shown to impair splicing [12], has been identified in several cases, being particularly frequent in Czech Republic, Turkey, and Hungary [13–15].

A few studies have investigated, in addition to exon 1, the noncoding region immediately upstream of this exon, including the basal promoter [14, 16–21].

Houseman and coworkers [16] analysed HL patients heterozygous for c.101T>C (p.Met34Thr), in which no second *GJB2* coding mutation had been detected, and identified a

monoallelic 10 bp deletion, c.-684\_-675del (firstly designated -493del10), upstream of the basal promoter. The deletion was also present in other hearing impaired individuals as well as in control individuals, with or without c.101T>C. However, c.-684\_-675del homozygosity was only observed in c.101T>C homozygous patients. The fact that in the control population 22 of the 25 (88%) c.101T>C heterozygotes carried the deletion suggested the existence of LD between c.101T>C and c.-684\_-675del, later demonstrated by Zoll and coworkers [22]. Transcription was observed from alleles harbouring in *cis* the deletion and the variant c.101T>C, derived from keratinocytes and cell lines. However, eventual subtle differences would not have been detected, since this was not a quantitative analysis [16]. To date, the role of c.-684\_-675del in HL has remained uncertain.

More recently, a pathogenic basal promoter mutation, c.-259C>T (firstly designated -3438C>T) was identified, in *trans* with c.250G>A (p.Val84Met), in a Portuguese HL patient, highlighting the relevance of screening *GJB2* noncoding regions in nonelucidated cases [18].

In the present study, we have analysed the basal promoter and the flanking upstream region, as well as the exon 1 and the 3'UTR of the *GJB2* gene in 89 Portuguese HL patients. The same analysis was conducted on 91 normal hearing control individuals from the Portuguese population.

## 2. Methods

**2.1. Subjects.** Eighty-nine Portuguese HL patients previously screened for mutations in the *GJB2* coding region and acceptor splice site (by SSCP and/or sequencing) and for the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) *GJB6* deletions (using the methodology described in [5]) were enrolled in this study. Eight patients were heterozygous for a *GJB2* coding mutation: c.71G>A (p.Trp24X; *n*=1), c.35delG (*n*=3), c.109G>A (p.Val37Ile; *n*=1), c.380G>A (p.Arg127His; *n*=1), c.457G>A (p.Val153Ile; *n*=2), and one patient was heterozygous for the c.-22-12C>T variant (apparently a polymorphism; dbSNP accession number rs9578260). No patient harboured either of the known *GJB6* deletions. The HL was nonsyndromic in all patients, except for one of them, who presented with Waardenburg syndrome. The patient was heterozygous for the controversial c.457G>A mutation and was thus included in the study. The patients presented with bilateral, mild to profound HL, and were either familial or sporadic cases. The familial cases predominantly showed a recessive pattern of inheritance. All patients were audiotologically evaluated by pure tone audiometry.

The control sample was composed of 91 Portuguese individuals with apparent normal hearing. The status regarding c.101T>C *GJB2* variant of those control individuals harbouring the c.-684\_-675del, here referred, had been previously investigated, by sequencing, as part of an unpublished work. The status of the entire *GJB2* coding region is not known for the vast majority of the 91 control individuals, which were blindly included in this study (and

not based on their eventually available *GJB2* coding region status).

Informed consent was obtained from all the participants.

**2.2. Genetic Analysis.** In all individuals, we have sequenced a region of about 0.7 kb immediately upstream of the exon 1 (which includes the basal promoter), the exon 1, and the whole 3'UTR. The region upstream of the exon 1, plus exon 1 and donor splice site, was amplified in a 1009 bp amplicon, using the pair of primers PF2 5'-CgTTCgTTCggATTg-gTgAg-3' and PR1 5'-CAGAAACgCCCgCTCCAgAA-3', as previously described [18]. The amplicons were sequenced using the primers PF2 and PF1 5'-ggCTCAAaggAACTaggAgATCg-3'. When necessary, primers PR1 and PR2 5'-ggAgACTgggAAAgtTACgg-3' were used for sequencing. The 3'UTR (plus the last 90 nucleotides and stop codon) was amplified in 3 overlapping fragments using the following three pairs of primers: 3UTRaF 5'-gCagTgTCTggAATT-TgCATC-3', 3UTRaR 5'-AggCACTggTAACTTTgTCC-3', 3UTRbF 5'-CACgTAAAgTgAACATTgg-3', 3UTRbR 5'-CgACAgAACTTCTCCCTC-3', 3UTRcF 5'-gTAgCCAgC-ATCggAAAgtAAC-3', 3UTRcR 5'-ACTCTggCAACTTACCATTg-3'. The 3'UTR PCR products were sequenced using the respective amplification forward primers.

**2.3. DNA Sequence Variants and SNPs Description.** Description of variants follows the HGVS recommendations, and is based on the *GJB2* reference sequences accessed through the following links:

- (1) [https://research.cchmc.org/LOVD/refseq/GJB2\\_codingDNA.html](https://research.cchmc.org/LOVD/refseq/GJB2_codingDNA.html);
- (2) [https://research.cchmc.org/LOVD/refseq/GJB2\\_intron\\_01.html](https://research.cchmc.org/LOVD/refseq/GJB2_intron_01.html);
- (3) [https://research.cchmc.org/LOVD/refseq/GJB2\\_upstream.html](https://research.cchmc.org/LOVD/refseq/GJB2_upstream.html);
- (4) [https://research.cchmc.org/LOVD/refseq/GJB2\\_downstream.html](https://research.cchmc.org/LOVD/refseq/GJB2_downstream.html).

These sequences show 100% identity with the NM\_004004.5 (link 1) and NG\_008358.1 (links 2, 3, and 4) NCBI reference sequences.

Novel variants were submitted to dbSNP and the respective reference SNP (rs) accession numbers are provided within the text.

SNPs are referred to by the dbSNP reference SNP (rs) accession number whenever it was available, and by the HGVS recommended designation, relative to the forementioned reference sequences.

**2.4. Genotyping and Statistical Analysis.** We have genotyped all individuals for the c.-684\_-675del deletion; three SNPs in the promoter (rs9550621 (c.-484T>C), rs73431557 (c.-410T>C), rs9552101 (c.-369A>G)); ten SNPs in the 3'UTR (c.\*1C>T, rs3751385 (c.\*84T>C), rs7337074 (c.\*104A>T), rs7329857 (c.\*111C>T), rs55704559 (c.\*168A>G), rs5030700 (c.\*931C>T), rs1050960 (c.\*1067G>T), rs7623 (c.\*1152G>A), rs11841182 (c.\*1197T>A), and rs7988691

(c.\*1277T>C)); one SNP downstream of the 3'UTR (rs11839674 (c.\*1447G>A)).

For the sake of simplicity, when describing the composite genotypes regarding SNPs rs73431557 (c.-410T>C), rs3751385 (c.\*84T>C), rs55704559 (c.\*168A>G), and rs5030700 (c.\*931C>T), the genotype at each position, indicated in order from 5' to 3', is designated by A, C, G, or T if homozygous, or by a code letter, according to IUPAC nucleotide ambiguity code, if heterozygous.

The allelic frequencies regarding deletion c.-684\_-675del and the 14 SNPs, were determined in the control population and used to test for Hardy-Weinberg equilibrium. The chi-square test was used to compare the allelic frequencies of the patients with those of the normal hearing individuals. Allelic frequencies of the control sample for the 14 SNPs were used to calculate pairwise linkage disequilibrium values. Testing for Hardy-Weinberg equilibrium, calculation of pairwise linkage disequilibrium values, and haplotype estimation (through the expectation maximization algorithm), were performed using SNPAnalyzer 1.2A online software (<http://snp.istech.info/snp/SNPAnalyzer.html>).

**2.5. Analysis of mRNA Folding.** Mfold (<http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form>) [23] was used to assess the effect of alleles c.[\*168A;\*931C], c.[\*168G;\*931T], c.[\*168A;\*931T], and c.[\*168G;\*931C] on the folding of *GJB2* mRNA (template sequence: ENST00000382848, retrieved from Ensembl). For each sequence the lowest free-energy structure was considered.

### 3. Results and Discussion

In the current study, 89 Portuguese HL patients, previously screened for mutations in the *GJB2* coding region and acceptor splice site (80 patients presenting no mutation, plus eight heterozygous for coding mutations and one heterozygous for the noncoding variant c.-22-12C>T), and 91 hearing individuals were analyzed as regards the noncoding region immediately upstream of the exon 1 (which includes the basal promoter), the exon 1, and the whole 3'UTR of *GJB2* gene. All individuals were also genotyped for c.-684\_-675del and 14 SNPs localized therein.

**3.1. DNA Sequence Variants.** No additional *GJB2* variant was found in any of the eight patients previously found to be heterozygous for a coding *GJB2* mutation or in the patient heterozygous for the c.-22-12C>T noncoding variant.

Among the remaining 80 patients, six of them presented noncoding variants, which had already been reported (Table 1).

One patient, presenting with profound HL was heterozygous for the donor splice site c.-23+1G>A mutation. The patient may just be a carrier, or other *GJB2* or *GJB6* mutation might remain undetected. One other patient, presenting with moderate to severe HL, harboured in heterozygosity the c.-216T>G variant, located within the basal promoter, between two GT boxes [24, 25]. This

variant was previously identified in two HL patients, also in heterozygosity [26]. The c.-45C>A variant in exon 1 was found in heterozygosity in one individual with severe HL. This variant was referred, by Wilch and coworkers [8], as an SNP at position +94 in exon 1. These authors observed expression of the *GJB2* allele harbouring the variant but, since a quantitative comparison with wild-type allele was not performed, a possible contribution to HL cannot be excluded. Three affected individuals (two heterozygous and one homozygous) harboured the deletion c.-684\_-675del.

No novel putative pathogenic noncoding mutation has been found in the patients, which might be due to the low number of monoallelic individuals and the small sample size. It is also possible that, simply, such mutations are very rare in our population.

Among controls, four novel noncoding variants were identified: c.-731C>T, c.-26G>T, c.\*45G>A, and c.\*985A>T (rs112400198, rs112875543, rs112399473, and rs111729919, resp.). Each of these variants was identified only once, in heterozygosity, and in different individuals (Table 1). The hearing individual harbouring the novel c.-731C>T variant was also heterozygous for the recessive c.670A>C (p.Lys224Gln) 1 mutation (<https://cchmc.org/LOVD/>; phase unknown). One control individual harboured the c.-45C>A exon 1 variant in heterozygosity (Table 1). Interestingly, we found one control subject homozygous for c.-684\_-675del (Table 1), which is, to our knowledge, the first case described to date of a normal hearing individual presenting this genotype. This individual did not harbour the c.101T>C mutation. Our finding, together with the previous report of transcription from alleles harbouring c.-684\_-675del [16] suggests the nonpathogenicity of the deletion. In addition, six normal hearing heterozygotes for the deletion were also identified (Table 1), with one also heterozygous for c.101T>C.

It should be noted that the pathogenic basal promoter mutation c.-259C>T, identified for the first time in a Portuguese family [18], was not found among the 89 patients and 91 normal hearing individuals here analysed, and neither was it identified in the other studies which analysed the basal promoter [14, 16–21]. Therefore, known occurrence of c.-259C>T continues to be restricted to that Portuguese family.

**3.2. Genotypic Data and Statistical Analysis.** The allelic frequencies and Hardy-Weinberg equilibrium status regarding the deletion c.-684\_-675del and the 14 noncoding SNPs were determined (Table 2; see Supplementary Table 1 in Supplementary Material available online at doi:10.4061/2011/827469).

The allelic frequencies of the deletion c.-684\_-675del in patients and controls are not statistically different (Table 2). The allelic frequency observed for this deletion in our control population is close to the one found among the British control population [16], and higher than the one determined in the German control population [22].

The allelic frequencies regarding SNPs c.-410T>C, c.\*84T>C, c.\*168A>G, and c.\*931C>T, were statistically

TABLE 1: *GJB2* variants identified in this study. Novel variants are in italic. BP: basal promoter; Ex 1: exon 1; DSS: donor splice site; Ex 2: exon 2; CR: coding region; 3'UTR: 3' untranslated region.

Variant	Location	Patients ( <i>n</i> = 89)		Controls ( <i>n</i> = 91)	
		Heterozygote	Homozygote	Heterozygote	Homozygote
c.-731C>T	5' of the BP	0	0	1	0
c.-684_-675del	5' of the BP	2	1	6	1
c.-216T>G	BP	1	0	0	0
c.-45C>A	Ex 1	1	0	1	0
c.-26G>T	Ex 1	0	0	1	0
c.-23+1G>A	DSS	1	0	0	0
c.670A>C (p.Lys224Gln)	Ex 2 (CR)	0	0	1	0
c.*45G>A	3'UTR	0	0	1	0
c.*985A>T	3'UTR	0	0	1	0

TABLE 2: Differences in the allelic frequencies, regarding c.-684\_-675del and 14 SNPs, between patient and control samples (chi-square test). ND: not determined: chi-square test could not be performed for SNPs with an expected value <5, or for SNPs which both alleles were observed in only one sample. Four SNPs present statistically significant differences in allelic frequencies between patients and controls (*P* values < 0.05, in bold).

Variant/SNP	Alleles	Patients ( <i>n</i> = 178 alleles)		Controls ( <i>n</i> = 182 alleles)	<i>P</i> value
		Observed	Expected		
c.-684_-675del	wt	174	170.18	174	0.162043
	c.-684_-675del	4	7.82	8	
rs9550621	c.-484 C	162	165.29	169	0.338941
	c.-484 T	16	12.71	13	
rs73431557	c.-410 C	41	26.41	27	<b>0.002089</b>
	c.-410 T	137	151.59	155	
rs9552101	c.-369 A	20	19.56	20	0.916103
	c.-369 G	158	158.44	162	
c.*1C>T	c.*1 C	177	178	182	ND
	c.*1 T	1	0	0	
rs3751385	c.*84 C	129	139.86	143	<b>0.04734</b>
	c.*84 T	49	38.14	39	
rs7337074	c.*104 A	174	177.02	181	ND
	c.*104 T	4	0.98	1	
rs7329857	c.*111 C	174	177.02	181	ND
	c.*111 T	4	0.98	1	
rs55704559	c.*168 A	154	170.18	174	<b>3.33E-09</b>
	c.*168 G	24	7.82	8	
rs5030700	c.*931 C	155	169.20	173	<b>9.18E-07</b>
	c.*931 T	23	8.80	9	
rs1050960	c.*1067 G	19	19.56	20	0.893155
	c.*1067 T	159	158.44	162	
rs7623	c.*1152 A	165	165.29	169	0.93373
	c.*1152 G	13	12.71	13	
rs11841182	c.*1197 A	2	0	0	ND
	c.*1197 T	176	178	182	
rs7988691	c.*1277 C	178	176.04	180	ND
	c.*1277 T	0	1.96	2	
rs11839674	c.*1447 A	2	0	0	ND
	c.*1447 G	176	178	182	

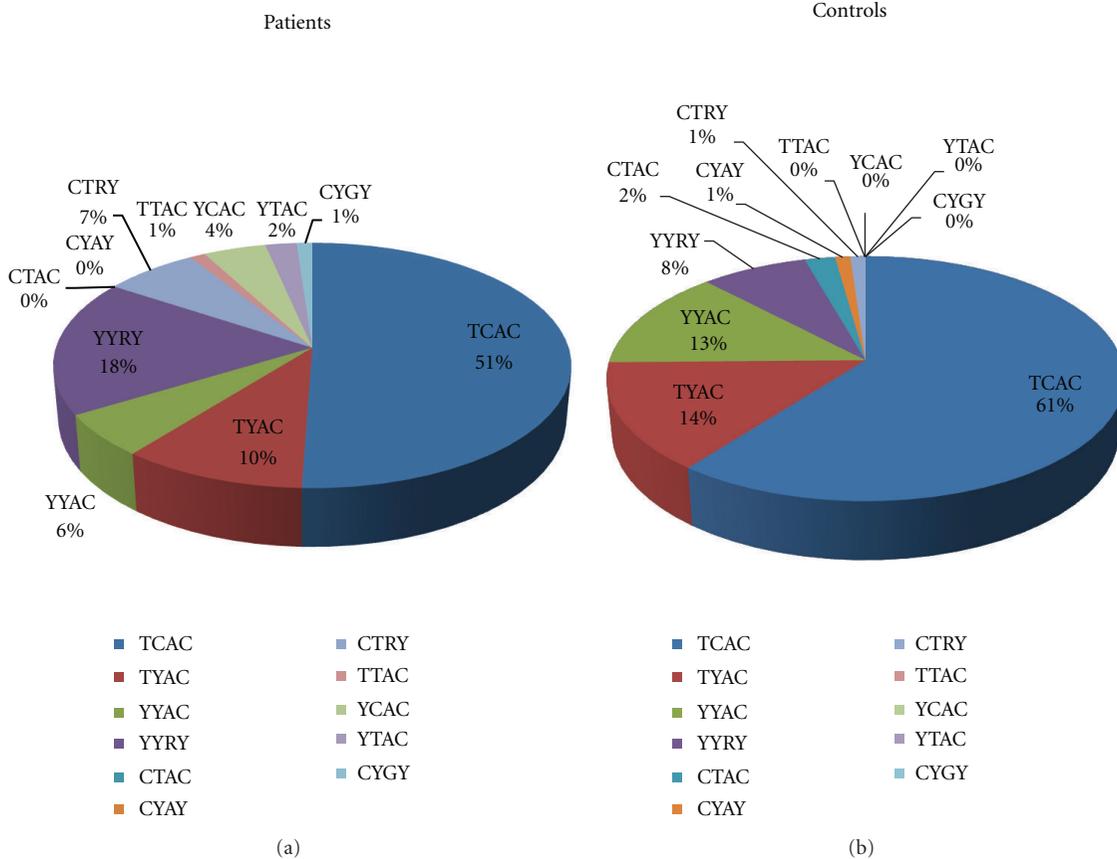


FIGURE 1: Frequencies, in patients ( $n = 89$ ) and controls ( $n = 91$ ), of the composite genotypes concerning SNPs c.-410T>C, c.\*84T>C, c.\*168A>G, and c.\*931C>T; R = A/G heterozygosity; Y = C/T heterozygosity (based on IUPAC nucleotide ambiguity code).

TABLE 3: (a) Estimated haplotype frequencies, based on the control population, concerning SNPs c.-410 T>C, c.\*84T>C, c.\*168A>G, and c.\*931C>T. (b) Relationship between the estimated prevalent alleles regarding positions c.\*168 and c.\*931, and SNPs c.-410T>C and c.\*84T>C.

(a)		
Haplotype	Frequency	
TCAC	0.7802	
CTAC	0.0989	
TTAC	0.0714	
CTGT	0.0440	
CCAT	0.0055	

(b)		
Allele (c.*168; c.*931)	c.-410 T>C	c.*84T>C
AC	89.6% T	82.1% C
GT	100% C	100% T

different between patient and control groups (Table 2).

By sorting both patients and controls into groups reflecting the genotypes for these four SNPs altogether, eleven composite genotypes were evidenced (Figure 1). Comparison of the genotypic frequencies in controls and patients

promptly revealed an increased frequency in patients of the genotypes YYRY and CTRY, both heterozygous for SNPs c.\*168A>G and c.\*931C>T. Also, the genotype YCAC was identified in four patients but not found in controls. On the contrary, a decrease was observed in the frequency of the three genotypes that are most represented in controls—TCAC, TYAC, and YYAC. Each of the remaining genotypes was scarcely represented in both controls and patients (0%–2%), and their frequency did not vary more than 2% between the two groups; only 3% of controls and 4% of patients belong to one of these genotypes.

We also observed that, regarding SNPs c.\*168A>G and c.\*931C>T, nearly all individuals analysed (178/180) were either c.[=;=]+[=;=] or c.[\*168A>G(+)\*931C>T], which results from LD between these two SNPs (Supplementary Table 2, SNP pair 8:9). Interestingly, the overrepresentation of c.[\*168A>G(+)\*931C>T] genotype among patients, when comparing to hearing controls, is statistically very significant ( $\chi^2 = 28.159$ ;  $P = 3.4 \text{ E-}06$ ), thus accounting for the statistically significant differences in the allelic frequencies of these two SNPs between patients and hearing controls.

The statistically significant differences also observed in the allelic frequencies of SNPs c.-410T>C and c.\*84T>C seems to be due to the differential association

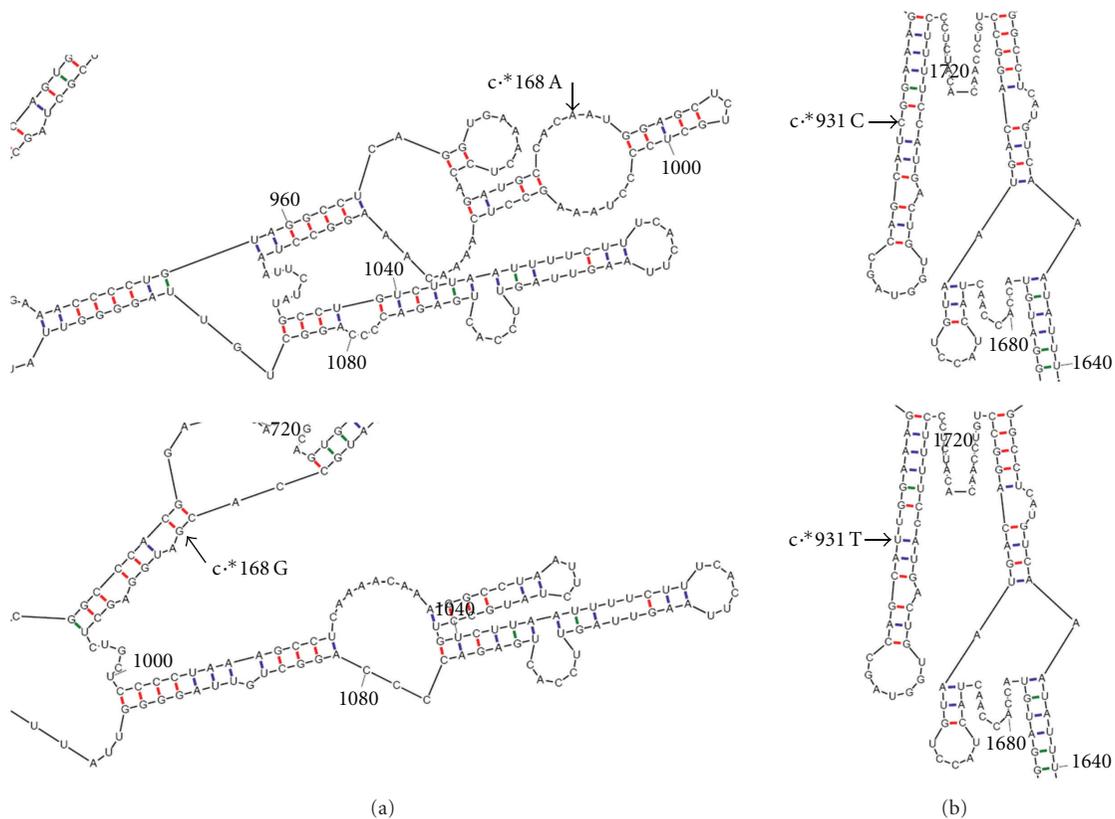


FIGURE 2: Effect of c.\*168A>G and c.\*931C>T changes in the 3'UTR on *GJB2* mRNA folding. (a) c.\*168A and c.\*168G; (b) c.\*931C and c.\*931T. The mRNA folding associated with each variant is the same regardless of the allele present at the other position, therefore only one example for each variant is provided.

of their variants with the estimated predominant alleles c.[\*168A+\*931C] and c.[\*168G+\*931T] (Tables 3(a) and 3(b)). This fact is in accordance with the observed LD between the two SNPs and SNPs c.\*168A>G and c.\*931C>T (Supplementary Table 2, SNP pairs 2:8, 2:9, 5:8, and 5:9).

It should be noticed that the presence of genotype YCAC among patients lends some contribute to the difference in allelic frequencies between patients and controls regarding SNP c.-410T>C.

The fact that YCAC genotype is not represented in 91 control individuals while it occurs in 4/89 patients is noteworthy. The presence of genotype YCAC implies the presence of haplotype CCAC, which frequency is of at least 2,2% among patients, and estimated to be null in the control population (as inferred from Table 3(a)). In order to validate a possible association of haplotype CCAC with HL analysis of larger samples of patients and normal hearing individuals is necessary. Interestingly, one of the four patients with the referred composite genotype is a c.457G>A heterozygote (phase unknown).

**3.3. 3'UTR Variants and mRNA Folding.** Our findings suggest that the c.[\*168G;\*931T] allele might have a deleterious effect, contributing to HL. We have used Mfold [23] to predict the effect of alleles c.[\*168A;\*931C], c.[\*168G;\*931T], c.[\*168A;\*931T], and c.[\*168G;\*931C] on mRNA folding.

The change c.\*168A>G, regardless of genotype at position c.\*931, was predicted to alter mRNA folding. On the contrary, the change c.\*931C>T, regardless of genotype at position c.\*168, is not predicted to alter mRNA folding (Figure 2).

The c.\*168A was predicted to be located in an internal loop of a stem-loop structure (Figure 2). Regulatory motifs in mRNA 3'UTR seem to function in the context of specific secondary structure [27]. Stem-loop structures occurring in the 3'UTR have been implicated in gene expression, with roles at the level of mRNA stability (e.g., the SLDE of G-CSF gene [28], the CDE of TNF-alpha gene [27, 29], the complex structure integrating three C-rich elements of alpha-globin gene, the histone mRNA 3' terminal stem-loops, and the IRE of TFRC gene [27]) or translation (e.g., the common 30–37 nucleotide long element present in the target mRNAs of TIA-1, a translational repressor [30], and the SECIS element [27]). The disruption of the predicted stem-loop structure and/or other adjacent stem-loop structures (Figure 2), induced by the c.\*168A>G change, might lead to deregulation of the *GJB2* gene expression, thus being a contributor to the hearing loss phenotype. It should be stressed that mRNA folding predictions are fallible. This fact notwithstanding, the simple change of sequence, without affecting the secondary structure, could conceivably disrupt a binding site for a trans-acting factor, also leading to gene expression deregulation. Regarding the

c.\*931C>T variant, despite the predictions that c.\*931C occurs in a helix and that the change from C to T does not have structural implications, the in vivo situation might be different. Functional studies involving constructs containing a reporter gene's coding sequence fused with *GJB2* 3'UTR could help elucidating the functional significance of these two sequence variants.

In this study, of a total of 15 patients presenting either a *GJB2* coding mutation or a noncoding variant, 14 do not harbour either the c.\*168A>G or the c.\*931C>T changes, whereas one patient, heterozygous for the controversial c.380G>A mutation, is a compound heterozygote regarding SNPs c.\*168A>G and c.\*931C>T (phase unknown). Therefore, our data do not allow withdrawal of conclusions concerning a putative role of the two 3'UTR variants in the HL of some monoallelic patients. In this regard, the investigation of the genotypes regarding c.\*168A>G and c.931C>T variants in larger samples of monoallelic patients would be interesting. Finally, the finding of one c.\*168G homozygote (a c.\*931C>T heterozygote, and carrying no *GJB2* sequence variant) in our patient cohort, might further support a possible role of c.\*168G in HL.

#### 4. Conclusion

This study suggests the association of the noncoding SNPs c.\*168A>G and c.\*931C>T with HL. The c.\*168A>G change is predicted to alter mRNA folding, suggesting a putative role of this SNP in the pathology. Our data also point to a possible association with HL of the haplotype CCAC, comprising SNPs c.-410T>C, c.\*84T>C, c.\*168A>G, and c.\*931C>T, respectively. However, this observation requires validation through analysis of a larger number of subjects. The technique of targeted sequence capture and massively parallel sequencing makes it very easy and cost-effective to screen large numbers of genes, and might cover noncoding sequences of some of them, such as *GJB2*. This approach could prove to be very useful for genetic diagnosis in cases of NSHL [31], with predictable benefits for genetic counselling of the affected families.

#### Acknowledgments

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## Research Article

# Molecular Investigation of Pediatric Portuguese Patients with Sensorineural Hearing Loss

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The understanding of the molecular genetics in sensorineural hearing loss (SNHL) has advanced rapidly during the last decade, but the molecular etiology of hearing impairment in the Portuguese population has not been investigated thoroughly. To provide appropriate genetic testing and counseling to families, we analyzed the whole mitochondrial genome in 95 unrelated children with SNHL (53 nonsyndromic and 42 syndromic) and searched for variations in two frequent genes, *GJB2* and *GJB6*, in the non-syndromic patients. Mutations in mtDNA were detected in 4.2% of the cases, including a hitherto undescribed change in the mtDNA-tRNA<sup>TTP</sup> gene (namely, m.5558A>G). We also identified mono- or biallelic *GJB2* mutations in 20 of 53 non-syndromic cases and also detected two novel mutations (p.P70R and p.R127QfsX84). Our data further reinforce the notion that genetic heterogeneity is paramount in children with SNHL.

## 1. Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities in human, and genetics is an important aspect in research and clinical practice for SNHL. One in 1000 children is born with bilateral SNHL, and 50–70% of them have monogenic causes for their deafness [1]. In addition, 10% of the people over 65 years have SNHL that limits considerably speech communication. Although the etiology is polygenic in most of the cases, with different contribution of ageing and environment, discovering monogenic causes has important clinical implications in terms of better counseling and management.

Hereditary hearing loss can be classified into syndromic and nonsyndromic depending on the associated features. Whilst over 400 genetic syndromes have been described in association with mono- or bilateral deafness, syndromic conditions account for about 30% of hereditary congenital hearing loss, whereas the relative contribution to all deaf people is much higher (>70%) for nonsyndromic subtypes [1].

Nonsyndromic sensorineural hearing loss (NSSNHL) is predominantly inherited in an autosomal recessive patterns

(DFNB loci) (80%) but can be also autosomal dominantly (DFNA) (15–20%), X-linked (DFN) (2–3%), or maternally (1%) transmitted. A polygenic or multifactorial pattern of inheritance should be postulated for late onset cases of hearing impairment [2, 3]. To date, 134 deafness loci (77 DFNB and 57 DFNA) have been reported, with more than 40 genes cloned [4]. Further heterogeneity is, however, expected to emerge.

Mutations in the *GJB2* and *GJB6* genes on the DFNB1 locus at chromosome 13q11-q12 are responsible for up to 50% of autosomal recessive (AR) NSSNHL [5, 6]. *GJB2* and *GJB6* encode gap junction proteins connexin 26 (Cx26) and connexin 30 (Cx30), respectively, which are expressed in the cochlea where they colocalize, form heteromeric gap junctions [7], and play a role in cochlear homeostasis [8]. The list of allelic variants in *GJB2* is wide with more than 100 variants being detected, mainly in congenital AR deafness, but also in dominant forms. The deletion of a single guanine (c.35delG) individually account for up to 50% of cases of NSSNHL among populations in Europe, North America, and Asia [9, 10]. The common 342-Kb genomic deletion in *GJB6* (termed GJB6-D13S1830) occurs in up to 20% of

the hearing-impaired American population and may account for ~10% of all DFNB1 alleles with an extremely wide range based on ethnic origin, oftentimes in dysgenic association with the c.35delG/*GJB2* variant [11, 12].

Maternally inherited hearing loss account for approximately 1% of cases, but accurate diagnosis is important because of its unique implications for affected individuals and their family members. Hearing loss may be an early manifestation of a more complex mitochondrial disorder such as the Kearns-Sayre (KSS) [13–15], the MERRF (myoclonic epilepsy with ragged-red fibers) [16, 17], or the MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) [18, 19] syndromes. In addition, SNHL can be the main, or sole, presenting feature of a mtDNA defect. In particular, mutations in the mtDNA-encoded 12S rRNA and tRNA<sup>Ser(UCN)</sup> genes are frequent. The m.1555A>G mutation in 12S rRNA has a worldwide distribution, and it is localized in a highly conserved region which is involved in decoding small ribosomal subunit [20]. The new G–C pair in 12S rRNA created by the A>G transition facilitates the binding of aminoglycosides antibiotics such as gentamycin and streptomycin, causing aminoglycoside-induced NSSNHL or worsening hearing loss in individuals harboring this mutation. The prevalence of the m.1555A>G mutation has been shown to be between 20–30% in deaf individuals in Spain and Asia of which 15% of them had the history of aminoglycoside ototoxicity [21]. Additional mutations in 12S rRNA (m.961delT, m.961insC(n), m.1005T>C, m.1095T>C, and m.1494 C>T) have been associated with impaired hearing [22–25], although their pathogenic significance remains controversial. All this information indicates that the mitochondrial 12S rRNA gene is a hotspot for mutations causing nonsyndromic hearing loss as well as increased sensitivity to aminoglycoside ototoxicity [26]. Mutations in the tRNA<sup>Ser(UCN)</sup> gene are additional genetic hotspot in Caucasian and Asian pedigrees with nonsyndromic and aminoglycoside-induced hearing loss. In the vast majority of patients, mtDNA mutations are nearly homoplasmic, indicating the requirement of a high mutation load to exert cochlear dysfunction [27].

We performed a comprehensive analysis of *GJB2* and *GJB6* in 53 non-syndromic patients and scanned the whole mitochondrial genome for mutations in a cohort of 95 paediatric Portuguese SNHL patients recruited in a hearing clinic to investigate the relative contribution of common genetic etiologies to their disorder, provide effective assessment of genetic risk, and to better counsel their relatives.

## 2. Patients and Methods

**2.1. Subjects.** Ninety-five Portuguese SNHL children from unrelated families were included in this study. A diagnosis of hearing deficit was performed in a Hearing Clinic according to international guidelines [28]. Fifty-three cases (56%) showed a non-syndromic hearing loss, profound in 80% and mild to moderate in 20%, and 42 (44%) patients had a syndromic disorder. Frequent accompanying manifestations involved the nervous system, eye, external ear, and musculoskeletal system. Parents were interviewed with regard to

age of onset, family history, mother's health during pregnancy, and patient's past clinical history, including infection, possible head or brain injury, and the use of aminoglycoside antibiotics. Written informed consent was obtained from all patients or their parents prior to blood sampling and all clinical and molecular studies accomplished with the ethical issues of Declaration of Helsinki.

**2.2. DNA Extraction.** Total genomic DNA was extracted from peripheral blood (B) or skeletal muscle (M) using commercially available procedures which employed the *EZ1 DNA Blood 350 µL Kit (QIAGEN)* and *Puregene Tissue kit (Gentra)* kits, respectively.

**2.3. Mutational Analyses.** We performed polymerase chain reaction (PCR) amplifications and direct gene sequencing of the coding exon and flanking regions of *GJB2* in 53 non-syndromic patients, as described elsewhere [5]. The common *GJB6-D13S1830* deletion [29] was searched in patients harbouring a heterozygous sequence variation in *GJB2*. All 95 patients were screened for mutations in the entire mtDNA by using a commercially available kit, mitoSEQr Resequencing System for the Human Mitochondrial Genome (Applied Biosystems, Foster City, Calif), according to the procedure recommended by the manufacturer.

For the description of the mutations, we used the latest conventions of the Human Genome Variation Society nomenclature. Synonymous, missense, and splice site variations were systematically evaluated for modifications of exonic splicing enhancers (Polyphen analysis, <http://genetics.bwh.harvard.edu/pph/>; ESEfinder, [http://rulai.cshl.edu/cgi-bin/tools/ESE/ese\\_finder.cgi](http://rulai.cshl.edu/cgi-bin/tools/ESE/ese_finder.cgi)) or consensus splicing sequences in order to determine the splice site score (<http://www.cbs.dtu.dk/services/NetGene2/> and [http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)). Multiple alignments with *GJB2* orthologs were performed using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) to evaluate the degree of conservation of missense variants. MtDNA mutations and polymorphisms were searched in MITOMAP data base (<http://mitomap.org/MITOMAP>).

## 3. Results

Table 1 summarizes molecular data detected in children with SNHL. Sequence analysis of *GJB2* indicated that eight patients carried two mutated alleles with already described pathogenic mutations and one presented the p.M34T mutation in heterozygosity, which has been reported to cause AD-NSSNHL [30]. The frequent c.35delG was homozygous in four children and was detected on a single allele in eight patients, three of whom harbored a wild-type sequence on the other allele and two children harbored the heterozygous common (*GJB6-D13S1830*) deletion. Two additional patients presented novel variations in *GJB2*: a homozygous p.R127QfsX84 mutation was found in one case whereas another patient presented the p.P70R on a single allele. Four patients presented with heteroplasmic (two individuals) or homoplasmic (two cases) mutations in mtDNA, including

TABLE 1: Genotype of patients with mutations in nuclear genes (*GJB2* and *GJB6*) and in mitochondrial genome.

Number of patients	<i>GJB2</i>	Reference	<i>GJB6</i>	Reference	mtDNA	Reference
4	c.35delG/c.35delG	[9]	—	—	—	—
1	p.W24X/p.W24X	[30]	—	—	—	—
1	c.35delG/p.E47X	[9, 31]	—	—	—	—
1	c.35delG/p.L76P	[9, 32]	—	—	—	—
1	c.35delG/p.V95M	[9, 33]	—	—	—	—
1	p.M34T/wt	[30]	—	—	—	—
1	p.R127QfsX84/p.R127QfsX84	This study	—	—	—	—
3	c.35delG/wt	[9]	—	—	—	—
1	p.P70R/wt	This study	—	—	—	—
2	c.35delG	[9]	del( <i>GJB6</i> -D13S1830)	[29]	—	—
1	—	—	—	—	m.7445A>G	[34]
1	—	—	—	—	m.1555A>G	[20]
1	—	—	—	—	m.3243 A>G	[19]
1	—	—	—	—	m.5558 A>G	This study

the hitherto unreported m.5558A>G in the mtDNA-encoded tRNA<sup>Trp</sup> gene.

Collectively, about 17% (16/95) of the Portuguese children analyzed in the present study were molecularly characterized, whereas a single variant was identified in four patients. The c.35delG accounted for 40% (16/40) of all mutant alleles, and up to half of mutations occurring in connexin-related genes. Additionally, 4.2% of studied patients (4/95) presented mutations in mtDNA, two of them (m.5558A>G and m.7445A>G) were identified in non-syndromic patients and the remaining mutations (m.1555A>G and m.3243A>G) in syndromic cases.

#### 4. Discussion

Despite increasing evidence of mutations associated with SNHL, there have been thus far no studies reporting on the relative frequency of mutations in the hearing-impaired Portuguese pediatric population. Although limited to a single center and to a relatively small number of cases, our study demonstrated that screening of two common etiologies (*GJB2* and *GJB6*) can characterize one in five patients with NSSNHL of unknown etiology. The relative frequency of the recurrent c.35delG mutation—64% of all mutated *GJB2* alleles—is in agreement with data reported by others [9, 10], as well as, the whole mtDNA in all SNHL patients.

In the present investigation, mutations in the *GJB2* gene were very frequent among NSSNHL patients, accounted for 23.6% (25/106) of mutated alleles and appear particularly frequent in South European patients [9, 10]. Among the DFNB-causing *GJB2* gene mutations reported so far, the c.35delG mutation accounts for most of mutant alleles (60–85%) in Caucasians, from which 10–50% was present in only one allele. Our data confirms the presence of the common *GJB6*-D13S1830 deletion in two patients, showing a digenic

inheritance in keeping with the severity of their hearing deficit.

We also identified two possibly pathogenic novel variants in *GJB2*. The new p.P70R that substitutes a proline (iminoacid) at codon 70 to an arginine (basic aminoacid) was predicted to be probably damaging by PolyPhen (The score is 2.108), and arginine was not tolerated when analyzed with the prediction software SIFT. Whether the mutation results in NSSNHL inherited in a dominant fashion remains a possibility. Conversely, the novel p.R127QfsX84 predicts a frameshift with a shorter, prematurely truncated connexin-26.

We also detected four mutations in mtDNA, corresponding to 4.2% of the all cohort. The m.1555A>G mutation has been reported in families whose members presented with aminoglycoside-induced deafness as the sole pathologic feature, but it may cause hearing loss even without aminoglycoside exposure, as in our case, which represents ~1% of this cohort. In our study, this mutation was found in a patient with ataxia and failure to thrive. The m.3243A>G mutation determines not only nonsyndromic, but also syndromic SNHL, such as in MELAS and MIDD (maternally inherited diabetes and deafness) [1]. The case identified in the present work was a 12-year-old boy diagnosed at the age of 8 years with syndromic SNHL; he developed hypertrophic cardiomyopathy and generalized muscle atrophy four years later. The m.7445A>G mutation was detected in a 13-year-old boy who presented a history of moderate progressive hearing loss. This mutation is believed to have led to failure in the processing of the L-strand RNA precursor, thereby reducing the steady-state levels of tRNA<sup>Ser(UCN)</sup> and ND6 mRNA [27].

We also found a novel mutation (m.5558A>G) in the tRNA<sup>Trp</sup> in a 10-year-old boy with NSSNHL. The mutation affects the conserved A49 nucleotide in the T-stem of

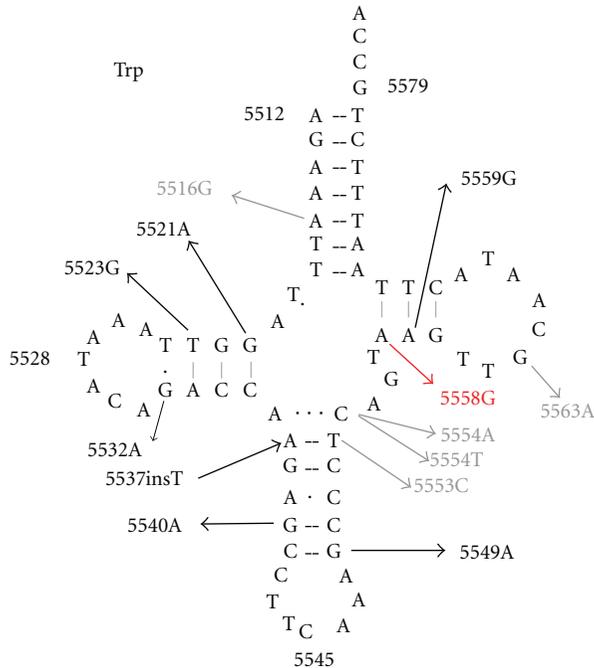


FIGURE 1: Schematic secondary structure of the wild-type human mitochondrial tRNA<sup>Trp</sup> gene showing the reported variants found in this gene. Black stands for pathogenic mutations, gray stands for polymorphic mutations, and red stands for the novel mutation described in our study.

the tRNA (Figure 1) leading to a possible disruption of its secondary structure after the loss of the T-stem. This modification could also affect the tertiary structure of the tRNA<sup>Trp</sup> and has probably a consequence on the aminoacylation. Another mutation (m.5559A>G) has been reported in the T-stem of the tRNA<sup>Trp</sup> [35], and two additional tRNA<sup>Trp</sup> mutations (namely, m.5540A>G and m.5568A>G) have been associated with SNHL [36, 37].

## 5. Conclusions

Although the contribution of less common genes remains to be determined, our results suggest that analysis of the *GJB2* gene may have clinical implications in the diagnosis of deaf Portuguese children. Also, it would make feasible early rehabilitation and prevention in affected families. The relatively higher incidence of mtDNA mutation also suggests that screening for variations in the mitochondrial genome should always be considered unless mitochondrial inheritance can be excluded for certain. The molecular diagnosis will permit more accurate genetic counseling for family members, monitor possible multisystem complications, and avoid usage of aminoglycosides if infections occur.

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## Research Article

# A Novel *ESRRB* Deletion Is a Rare Cause of Autosomal Recessive Nonsyndromic Hearing Impairment among Pakistani Families

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Mutations in the estrogen-related receptor beta (*ESRRB*) gene is the underlying cause of autosomal recessive nonsyndromic hearing impairment (ARNSHI) due to the *DFNB35* locus which maps to 14q24.3. A genome scan of a large consanguineous Pakistani pedigree with ARNSHI established linkage with a maximum multipoint LOD score of 4.2 to the 14q24 region and the region of homozygosity contained the *ESRRB* gene. Sequencing of the *ESRRB* gene using DNA samples from hearing-impaired family members uncovered a novel three-nucleotide deletion c.1018\_1020delGAG (p.Glu340del). The deletion segregates with hearing impairment in the pedigree and was not observed in 500 control chromosomes. The deletion of glutamic acid residue occurs in the ligand-binding domain of *ESRRB* protein. It is expected that the deletion affects the ligand-binding activity of the domain in *ESRRB*, which leads to the ARNSHI.

## 1. Introduction

Hearing impairment (HI) has been associated with estrogen physiology for decades. Turner's syndrome in females and Noonan's syndrome in males are both marked by hypoestrinism and include the HI phenotype. Variations in hearing function have also been found according to gender, menstrual cycle or menopausal state, estrogen therapy, and oral contraceptive use, which implicate estrogen as a primary requirement for hearing preservation [1]. Recently, autosomal recessive nonsyndromic hearing impairment (ARNSHI) locus *DFNB35* was shown to be due to mutations in the estrogen-related receptor beta or *ESRRB* gene (MIM 602167; 2). *ESRRB* belongs to a subfamily of orphan nuclear receptors that are structurally related to estrogen receptors (ER) but do not directly bind estrogen. The *ESRR* proteins have DNA-binding and ligand-binding domains, which are both required for transcriptional regulation of ER target genes. The ligand-binding domain (LBD) provides structural stability and influences the binding affinity of the *ESRRB*

protein [2]. Here, we report a novel deletion mutation of glutamic acid residue in LBD domain of *ESRRB* gene which is responsible for ARNSHI.

## 2. Material and Methods

The study was approved by the Institutional Review Boards of the Quaid-I-Azam University and the Baylor College of Medicine and Affiliated Hospitals prior to initiation. Informed consent was obtained from all members from family 4243 who participated in the study. Family 4243 (Figure 1) is a consanguineous kindred from the Sairiki-speaking region of Punjab, Pakistan. The family segregates ARNSHI of prelingual onset and has no history of environmental exposure to factors that may cause HI, for example, infection, ototoxic medication, and trauma. Syndromic and vestibular features were ruled out through careful physical examination. To determine the severity of HI, air conduction testing was performed using a portable audiometer.

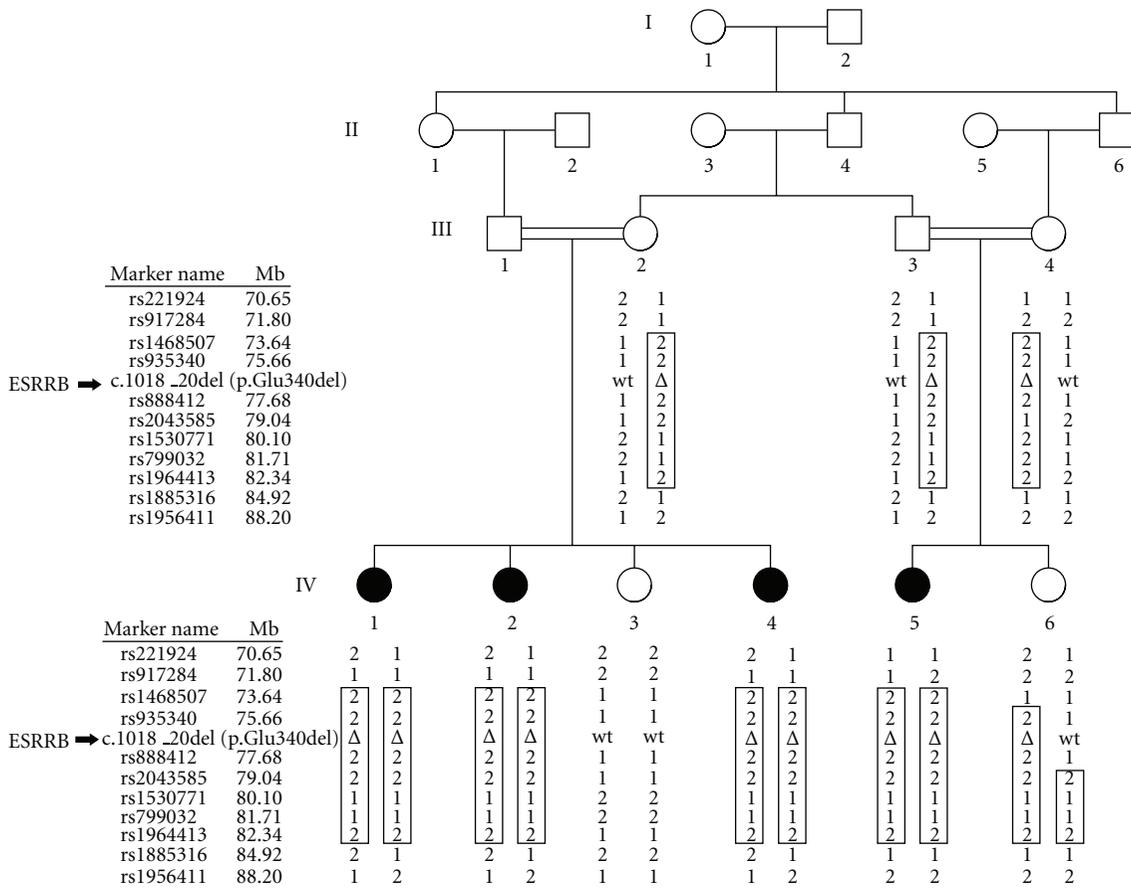


FIGURE 1: Pedigree drawing of family 4243. Filled symbols represent individuals with ARNSHI, and clear symbols represent hearing individuals. Displayed under each individual are the SNP markers within the region of the *ESRRB* gene and the p.Glu340del mutation. The haplotype segregating with the ARNSHI phenotype is shown within a box. The homozygous region that is found only in ARNSHI individuals is bounded by rs917284 (chr14: 71.8 Mb) and rs2043585 (chr14: 79.0 Mb). The black arrow indicates the position of *ESRRB* gene amid the SNP marker loci.

Genomic DNA was extracted from venous blood which was provided by five hearing and four HI family members (Figure 1). The nine DNA samples from this family underwent a whole genome linkage scan at the Center for Inherited Disease Research (CIDR) using the Illumina Linkage Panel IV-b which contains 6,090 SNP markers. Data quality control was performed using PedCheck [3] to check for genotyping error which resulted in Mendelian inconsistencies, while MERLIN [4] was used to detect occurrence of double recombination events over short genetic distances, which are most likely due to genotyping error. Two-point linkage analysis was carried out using MLINK of the FASTLINK package [5], and multipoint linkage analysis was performed with Allegro [6], while haplotypes were reconstructed using SimWalk2 [7]. An autosomal recessive mode of inheritance with complete penetrance and a disease allele frequency of 0.001 were used in the analysis. Marker allele frequencies were estimated from observed and reconstructed founders from family 4243 and 73 additional families who underwent genome scan at the same time at CIDR. For the multipoint linkage analysis, genetic map positions were determined according to the Rutgers combined linkage-physical map of the human genome [8] using the human reference sequence

(Build 36) to determine the physical map position, and then, interpolation was performed to place the markers on the Rutgers map.

Exon primers for *ESRRB* (RefSeq NM\_004452.3) gene were designed using Primer3 software [9]. PCR-amplified products were purified with ExoSAP-IT (USB Corp., Cleveland, Ohio, USA) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI 3730 DNA Analyzer (Applied Biosystems Inc, Foster City, Calif, USA). Sequencher software V4.9 (Gene Codes Corp., Ann Arbor, Mich, USA, M) was used to assemble and analyze DNA sequences.

### 3. Results and Discussion

The audiogram of HI individual IV-4 displays bilateral, severe-to-profound HI affecting all frequencies (Figure 2). This is consistent with the previous description of prelingual, bilateral profound hearing loss across all frequencies for *ESRRB*-related HI [2].

For family 4243, a maximum two-point LOD score of 3.04 ( $\theta = 0$ ) was observed at marker rs935340 (chr14: 75.66 Mb; see Table 1). A significant maximum multipoint

TABLE 1: Two-point and multipoint LOD scores for family 4243.

Marker name <sup>1</sup>	Physical map position <sup>2</sup>	Genetic map position <sup>3</sup>	Multipoint LOD score	Two-point LOD score at $\theta =$					
				0.00	0.01	0.05	0.10	0.20	0.30
rs221924	70,650,531	66.96	-4.52	-2.02	-0.26	0.28	0.39	0.33	0.20
<b>rs917284</b>	<b>71,806,864</b>	<b>67.80</b>	<b>-2.56</b>	-1.41	0.34	0.84	0.90	0.70	0.36
rs1468507	73,641,851	70.09	4.15	2.67	2.62	2.40	2.12	1.54	0.94
rs935340	75,660,959	72.79	4.16	3.04	2.98	2.72	2.40	1.73	1.06
rs888412	77,676,577	75.73	4.16	1.34	1.30	1.13	0.91	0.46	0.02
<b>rs2043585</b>	<b>79,039,998</b>	<b>77.88</b>	<b>-17.85</b>	$-\infty$	0.58	1.08	1.13	0.92	0.56
rs1530771	80,095,275	78.71	$-\infty$	$-\infty$	0.47	0.98	1.04	0.84	0.50
rs799032	81,707,098	79.67	$-\infty$	$-\infty$	0.84	1.32	1.35	1.09	0.69
rs1964413	82,338,459	80.75	-1.27	2.08	2.04	1.88	1.66	1.22	0.76
rs1885316	84,924,908	82.25	-6.89	-2.37	-0.60	-0.03	0.12	0.15	0.09
rs1956411	88,204,113	85.49	-5.94	-2.10	-0.33	0.22	0.36	0.34	0.22

<sup>1</sup>Markers in bold denote marker limits based on 3-unit support interval and homozygous region.

<sup>2</sup>Physical map positions from Build 36.1 of human reference sequence.

<sup>3</sup>Genetic map positions based on Rutgers linkage physical map of the human genome.

LOD score of 4.16 was obtained at two marker loci, rs935340 and rs888412 (77.68 Mb). The 3-unit support interval and the region of homozygosity that was observed only in HI individuals (Table 1; Figure 1) fall between markers rs917284 (71.81 Mb) and rs2043585 (79.04 Mb). The linkage region spans 10.08 cM within 14q24.2–q31.1 and contains 7.23 Mb of sequence. A total of 83 genes are found within the linkage interval, including the known ARNSHI gene, *ESRRB*. Since variants within the *ESRRB* gene are known to be involved in NSHI [2], the exon regions of *ESRRB* gene were sequenced.

Sequencing of the *ESRRB* gene in family 4243 revealed a novel deletion c.1018\_1020delGAG (p.Glu340del) in exon 8 which segregates with ARNSHI (Figure 1). The deletion was not annotated in the dbSNP database and 1000 Genomes project. Additionally, exon 8 of *ESRRB* was sequenced in 250 unrelated hearing individuals from Pakistan; the deletion was not found in 500 control chromosomes.

The glutamic acid residue at position 340 is the second residue within  $\alpha$ -helix 8 at the LBD of the *ESRRB* protein. In order to form a hypothesis on the effect of its deletion to protein structure, *ESRRB*-like proteins from both human and nonhuman species were identified from the UniProt Knowledgebase [10] using blastp [11] and aligned via ClustalW [12]. The Glu340 residue is invariant for *ESRRB* and other steroid receptor proteins in 39 nonhuman species, including 7 mammalian, 1 avian, 2 reptilian, 3 amphibian, 17 fish, 2 ascidian, 3 mollusk, and 4 arthropod. It is also conserved in 32 human nuclear receptor proteins, which indicates that this residue is essential to the structure of the LBD. Using the SWISS-MODEL Workspace [13], the LBD of *ESRRB* was modeled after the LBD of human proteins *ESRRG* (PDB ID: 2GPO) [14] and *PPARG* (PDB ID: 3DZY) [15]. The Glu340 residue forms a hydrogen bond with Glu337, the last of three residues of the  $\alpha$ - $\alpha$  corner between  $\alpha$ -helices 7 and 8. This hydrogen bond marks the beginning of the formation of  $\alpha$ -helix 8. Glu340 also forms

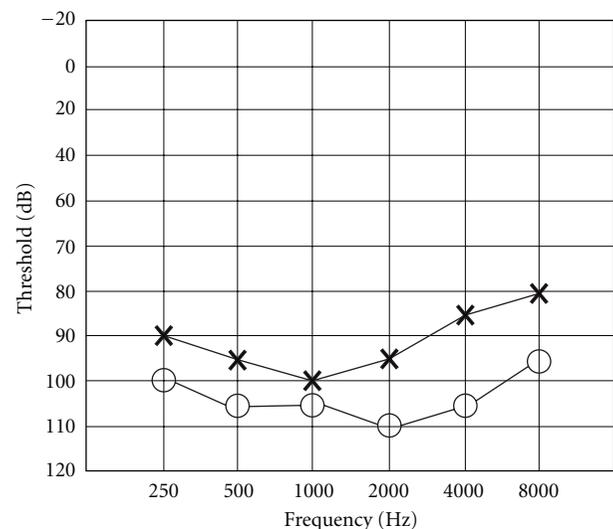


FIGURE 2: Audiogram of individual IV-4 demonstrates bilateral severe-to-profound HI at all frequencies. Air conduction threshold testing was performed for the right (circles) and left (crosses) ears.

two hydrogen bonds with Arg388, the first residue of  $\alpha$ -helix 10. Removal of Glu340 dissolves these hydrogen bonds and also results in the rotation of the side chains of residues 336–339 along the helical axis. Residues at positions 336–340 do not have direct contact with ligand. However, the removal of hydrogen bonds due to p.Glu340del is expected to affect the stability of  $\alpha$ -helix 8 and its conformation relative to other helices, in particular  $\alpha$ -helices 7 and 10. Notably, the side chain of Lys338 is moved out of the hydrophobic pocket formed by Leu286, Tyr290, Tyr331, and Phe341. In the known crystal structure of the LBD of *ESRRG*, a similar pocket is contiguous with the second pocket of the LBD, which receives agonist ligand GSK4716 and coactivator

RIP140 [14]. The ESRRG-RIP140 interaction is facilitated by conformational changes in the charged residues on the LBD surface. The hydrophobic pocket which includes Lys338 of ESRRB may have a similar function of allowing agonist ligand and coactivator binding by increasing the volume for ligand interaction and providing charged residues that are required for ligand binding.

Three of five reported *ESRRB* mutations that cause ARNSHI are also predicted to result in structural defects at the LBD [1]. In particular, two *ESRRB* mutations, namely, p.Val342Leu and p.Leu347Pro [2], also occur within  $\alpha$ -helix 8. Both substitutions are predicted to disrupt hydrophobic interactions of  $\alpha$ -helix 8 with other helices, thus resulting in conformational change and decreased stability of the LBD [2]. Mutations in  $\alpha$ -helix 8 that decrease ligand-binding affinity have been identified in other nuclear receptor proteins, ESR1 and HNF4 $\alpha$ , which have structures similar to ESRRB [16, 17]. The p.Glu276Gln mutation for HNF4 $\alpha$ , which is one of the underlying causes of mature-onset diabetes in the young, results in unstable protein with no DNA-binding ability and, hence, no transcriptional activity [16]. The Glu276 residue of HNF4 $\alpha$  aligns with Glu340 of ESRRB. It is, therefore, highly plausible that p.Glu340del affects the stability and ligand-binding activity of the ESRRB protein in order to result in the HI phenotype.

#### 4. Conclusion

A genome-wide linkage scan performed using DNA samples from a large consanguineous ARNSHI pedigree and the subsequent sequencing of the *ESRRB* gene led to the identification of a novel deletion mutation c.1018\_1020delGAG (p.Glu340del). The glutamic acid residue 340 is located in the ligand-binding domain of the ESRRB protein. The identification of the deletion mutation of *ESRRB* gene will expand our understanding of hearing impairment due to mutations in the estrogen-related gene *ESRRB*.

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