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Genetics of isolated auditory neuropathies

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1. ABSTRACT

Auditory neuropathies are disorders combining absent or abnormal auditory brainstem responses with preserved otoacoustic emissions and/or cochlear microphonics. These features indicate a normal function of cochlear outer hair cells. Thus, the primary lesion might be located in the inner hair cells, in the auditory nerve or in the intervening synapse. Auditory neuropathy is observed in up to 10% of deaf infants and children, either as part of some systemic neurodegenerative diseases or as an isolated entity. Research on the genetic causes of isolated auditory neuropathies has been remarkably successful in the last few years. Here we review the current knowledge on the structure, expression and function of the genes and proteins so far known to be involved in these disorders, as well as the clinical features that are associated with mutations in the different genes. This knowledge is permitting to classify isolated auditory neuropathies into etiologically homogeneous types, so providing clues for the better diagnosis, management and therapy of the affected subjects.

2. INTRODUCTION

The human ear is a highly specialized organ with a complex structure and finely coordinated functional mechanisms, which require the participation of many different proteins. Mutations of the genes encoding these proteins result in hearing impairment (1, 2). To date, about 60 different genes are known to be involved in non-syndromic forms of hearing impairment (3), whereas mutations of several hundreds genes underlie syndromes that associate a hearing disorder with clinical signs in other organs (4). Many more genes still remain unidentified.

Because of this enormous genetic heterogeneity, it is difficult to conjecture which gene can be responsible for hearing impairment in a given patient, especially if it is non-syndromic. Given that the proteins involved in hearing impairment play specific roles within the ear, it could be expected that deficiencies in each of them might result in specific anomalies of the hearing mechanism, which in turn might originate distinctive clinical signs (5). These signs could range from a variety of inner ear malformations (6) to

Table 1. cDNA sequences corresponding to different isoforms of human otoferlin in electronic databases

Isoform name	Isoform type	Exon composition	Coding region (bp)	Polypeptide (amino acids)	Accession numbers
a	Long	1-5 + 7-30 + long variant of 31 + 32-48	5,991	1,997	NM_194248 AF183185
b	Short type 1	5'UTRsf1 + 20-30 + short variant of 31 + 32-48	3,690	1,230	NM_004802 AF107403
c	Short type 2	5'UTRsf1 + Intron 0 + 20-30 + long variant of 31 + 32-48	3,921	1,307	NM_194322 AF183186
d	Short type 1	5'UTRsf1 + 20-30 + short variant of 31 + 32-46 + 48	3,690	1,230	NM_194323 AF183187

subtle audiological features without associated structural abnormalities. One of these clinical signs is auditory neuropathy.

Auditory neuropathy (AN) was the term coined to designate a condition combining absent or abnormal auditory brainstem responses (ABR) with preserved otoacoustic emissions (OAE) and/or cochlear microphonics (CM) (7). In patients with AN, middle-ear muscle reflexes are generally absent, although they may be present with elevated threshold. Pure-tone audiometry (PTA) may indicate normal hearing or hearing losses ranging from mild to profound (8-10). A key feature is the very poor speech intelligibility, usually out of proportion with the hearing level determined by PTA (11), which is postulated to be caused by dys-synchronous neural responses. Accordingly, AN is also termed *auditory dys-synchrony* (12).

Estimates of the prevalence of AN vary according to the different criteria of patient inclusion in the cohorts that have been studied, but it may account for up to 10% of infants and children diagnosed as deaf by ABR (13, 14). It is relevant that newborn hearing screening programs that rely solely on OAE would miss these patients. Etiology of AN is heterogeneous, as shown by the different locations of the primary lesion and by the variety of factors that can cause it. Preservation of OAE and/or CM indicates normal electromotility of cochlear outer hair cells (OHCs). Therefore, the primary lesion in AN can be located in the auditory nerve (post-synaptic or type I AN), or in the inner hair cells (IHCs) and their synapses with auditory nerve terminals (pre-synaptic or type II AN) (8-10). Note that since the auditory nerve is not affected in type II AN, the term *neuropathy* could be misleading (15). Recently, the term *auditory synaptopathy* has been proposed to designate those auditory neuropathies in which the auditory ribbon synapse is specifically affected (16). Given this etiological heterogeneity, it is not surprising that the outcomes of cochlear implantation of patients with AN have been also variable (17).

Causes of AN can be environmental, such as severe neonatal hyperbilirubinemia (kernicterus), neonatal hypoxia or prematurity, or genetic (8-10). In some cases, AN is just one among several clinical signs resulting from systemic neurodegenerative diseases (eg. Charcot-Marie Tooth peripheral neuropathy, Friedreich ataxia, mitochondrial disorders). In other cases, it is an isolated entity. Here we review the genetics of isolated auditory neuropathies, a research field that has experienced remarkable advances in the last few years.

3. *OTOF* GENE AND OTOFERLIN

The study of a large Lebanese consanguineous family segregating autosomal recessive non-syndromic hearing impairment (ARNSHI) revealed a novel genetic locus for this disorder on 2p22-p23 (18). This locus, initially reported as DFNB6, was finally renamed DFNB9 (MIM# 601071). Three years later, one of the genes within the critical interval, *OTOF*, which encodes otoferlin, was shown to carry pathogenic mutations that were responsible for ARNSHI in the original Lebanese family and in three other families from the same region (19). Finally, two independent studies reported that mutations in the *OTOF* gene are responsible for an isolated form of auditory neuropathy (20, 21).

3.1. Gene and protein structures

The *OTOF* gene (MIM# 603681) spans 101,495 bp on 2p23.1. It contains 49 exons that were named 1 to 48 and 5'UTRsf1 (for 5'-untranslated region of short form 1, an alternative exon that is located between exons 19 and 20). A variety of different protein isoforms is produced from this gene through different combinatorial mechanisms (22).

Two transcription start sites are known, one at the 5' end of exon 1 (for long isoforms), the other at the 5' end of exon 5'UTRsf1 (for short isoforms). Therefore, the first 19 exons are exclusive of the long isoforms. The translation start site for long isoforms seems to be unique (start codon at nucleotides 128-130 from the 5' end of exon 1). On the contrary, there are two possible translation start sites for short isoforms. In the first type of short isoforms, the mRNA contains exon 5'UTRsf1 followed by exon 20, with the translational start codon at nucleotides 28-30 of exon 20. In the second type of short isoforms, the mRNA includes also the intron between 5'UTRsf1 and exon 20 (intron named 0), and then translation starts at the codon in nucleotides 150-152 of exon 5'UTRsf1.

This picture is complicated further by the fact that some exons experience alternative splicing. Exon 6 can be included or skipped in the long isoforms. For exon 31 there are two variants, one longer than the other. Finally, exon 47 can be included or skipped. If included, it encodes the C-terminal end of the protein, and then the whole exon 48 belongs to the 3' untranslated region. If skipped, the C-terminal end of the protein is encoded by exon 48, which

Table 2. C2 domains of human otoferlin

Domain	Domain boundaries[1]	Calcium binding[2]	Aspartate residues predicted to be involved in calcium binding
C2A	3-119	No	
C2B	256-378	Yes	272
C2C	419-542	Yes	453, 501, 503
C2D	962-1095	Yes	976, 982, 1038, 1040, 1046
C2E	1494-1622	Yes	1508, 1514, 1563, 1565, 1571
C2F	1734-1895	Yes	1748, 1759, 1834, 1836, 1842

[1] As determined in reference 25, [2] As demonstrated experimentally (16, 28, 80)

also provides the 3' untranslated region. Of note, the two alternative C-terminal 60-residue tails encoded by exons 47 and 48 can be aligned, sharing a 65% of amino acid identity, plus an additional 28% of amino acid similarity. Some of these alternative-splicing events might be tissue-specific. For example, exon 47 seems to be skipped in cochlear but not brain mRNAs (22, 23).

In spite of this wide potential variability, only four human cDNA sequences corresponding to different isoforms of otoferlin have been registered in electronic databases (Table 1). Isoform *a* is the unique long isoform, and so it serves as the reference sequence to name the different mutations found in the *OTOF* gene. However, it is important to keep in mind that this isoform lacks exon 6, which is not included in any human *OTOF* cDNA sequence in electronic databases. In fact the reality of human *OTOF* exon 6 is just supported by its existence in human genomic DNA and by the presence of the homologous sequence in murine *Otof* cDNAs.

Otoferlin received this name because it belongs to the ferlin family of proteins and it is expressed in the inner ear (19). Proteins of the ferlin family share similarity with the *Caenorhabiditis elegans* sperm vesicle fusion protein FER-1. Other remarkable members in humans are myoferlin and dysferlin, which are expressed in muscle. They all are membrane-anchored cytosolic proteins that contain several repeats of a structural module called C2 domain (24). A canonical C2 domain comprises approximately 130 residues and folds into an eight-stranded beta-sandwich. Many C2 domains can bind calcium and phospholipids. The calcium-binding region is located in the loops at one end of the fold, where five aspartate residues serve as ligands for two or three calcium ions (24, 25). Like myoferlin and dysferlin, otoferlin long isoforms have six C2 domains (Table 2). Human otoferlin short isoforms are unique in having only three C2 domains (C2D to C2F). Otoferlin short isoforms were not detected in mice (22).

Otoferlin is anchored to membrane through a transmembrane segment that is located near its C-terminus (residues 1965 to 1983 in long isoform *a*). As discussed above, otoferlin has two alternative transmembrane segments, encoded by either exon 47 or exon 48 (22).

3.2. Gene expression and protein localization

OTOF gene expression has been investigated through Northern blotting, RT-PCR and *in situ* hybridization (19, 22, 26). Northern blot experiments detected a 5-kb short isoform transcript in several adult human tissues (skeletal muscle, heart, liver, pancreas, kidney, placenta and brain), as well as a 7-kb long isoform transcript in adult human brain. In murine tissues, only the long isoform transcript was detected, and solely in adult brain (22). RT-PCR experiments performed on murine RNA were positive in all tissues tested (cochlea, vestibule, eye, brain, heart, skeletal muscle, liver, kidney, lung and testis), but the strongest bands were obtained from cochlea, vestibule and brain (19). Further RT-PCR testing revealed a ubiquitous expression in rat brain, including strong signals from cerebellum, cortex, superior colliculus, inferior colliculus, and hippocampus, and a weaker band in brainstem (26). *In situ* hybridization performed on rat brain revealed barely detectable signals at P6, but intense signals at P19 in the cerebellum (granular and Purkinje cell layers), hippocampus (granule cells of the dentate gyrus and pyramidal cells of the CA1-CA3 region) and cortex (layers IV and V). Weaker signals were observed in the inferior and superior colliculus (26).

The localization of otoferlin has been investigated by immunohistofluorescence. The protein is present in cochlea and vestibule, cochlear and vestibular nuclei, cerebellum, hippocampus and cortex (16, 26). In cochlea, otoferlin is first detected at E16 in the IHCs, and at E18 in the OHCs. The intensity of the immunolabeling signals increases in IHCs and OHCs until P6. The strongest staining is found in the basolateral region of these cells, always more intense in the IHCs than in the OHCs. From P6 onward the signal vanishes in most of the OHCs, persisting only in the OHCs of the most apical region of the mature cochlea (16, 26). In vestibule, otoferlin is first detected in the hair cells at E14, and at P30 it is found in the hair cells of crista ampullaris, utricular macula and saccular macula (16, 26).

The remarkable correlation between the otoferlin expression profile and afferent synaptogenesis of the hair cells suggested that otoferlin might be a component of the hair cell presynaptic machinery of the auditory ribbon synapse. Consistently, immunogold electron microscopy on sections of the murine organ of Corti showed that more than half of the gold particles were found in association with the synaptic vesicles surrounding the ribbon, and an additional 20% were present close to the presynaptic plasma membrane (16).

3.3. Function of otoferlin

Members of the ferlin family participate in mechanisms of membrane fusion. On this basis, it was soon proposed that otoferlin could be involved in synaptic vesicle exocytosis at the hair cell afferent ribbon synapse (19). Unlike conventional synapses, these specialized structures have an osmiophilic body (the ribbon) with a monolayer of synaptic vesicles tethered to its surface, some

of which are docked onto the presynaptic membrane. The unique feature of ribbon synapses is their ability to respond to graded changes in membrane potential, and so they must sustain high rates of exocytosis for relatively long periods. Two kinetic components (slow and fast) are discriminated in hair cell synaptic exocytosis (27). The role of otoferlin in this complex machinery is being progressively elucidated through the identification of its ligands and the study of murine models.

Bioinformatic analysis predicted that only three of the six C2 domains of otoferlin (C2D, E and F), which have all five required aspartate residues at the appropriate positions, would bind calcium (25). However, recent studies indicate that some C2 domains from other proteins bind calcium although lacking several of those aspartate residues. Indeed, experimental analysis of recombinant proteins has finally established that all C2 domains of otoferlin, except C2A (which has an unconventional domain structure) (81), are able to bind calcium, although with different apparent dissociation constants (16, 28, 80) (Table 2). Coimmunoprecipitation experiments and pull-down assays with full-length otoferlin, as well as *in vitro* binding assays with an otoferlin construct containing residues 762 to 1992 (lacking C2 domains A to C) demonstrated interactions with syntaxin-1 and SNAP25, two proteins of the SNARE complex, in a calcium-dependent manner (16). In addition, separate C2 domains of mouse otoferlin were tested through yeast two-hybrid assays, pull-down experiments and coimmunoprecipitation (28, 80). Calcium-promoted binding to syntaxin-1A and SNAP25 was shown for C2C, C2D, C2E and C2F (28, 80), although only the C2F interaction was strictly calcium-dependent (28). Domain C2D also interacted with $Ca_V1.3$, the major L-type calcium channel that controls mammalian hair cell exocytosis, in a calcium-dependent manner as well (28). Furthermore, yeast two-hybrid assays using the C2D domain as bait identified other otoferlin interacting partners, namely Rab8b GTPase (29) and myosin VI (30). As regards the interaction of otoferlin with membranes, it has been demonstrated that five of the separate C2 domains (B to F) are able to aggregate liposomes and accelerate SNARE-mediated membrane fusion, but only in a calcium-dependent manner (80).

Further insight into the otoferlin function has been provided by the studies on mouse models. These include the *Otof*$^{-/-}$ mouse, generated by engineered deletion of *Otof* exons 14 and 15 (16), and two ENU-induced mutants, *pachanga* (31) and *deaf5Jcs* (32), which carry homozygous missense mutations affecting the C2F and C2B domains, respectively. In *Otof*$^{-/-}$ and *deaf5Jcs* mice, otoferlin was not detected; in contrast, the protein was detected at reduced levels in the *pachanga* mutant (35). All these mice are profoundly deaf (16, 31, 32), but normal OHC function has been reported only in *Otof*$^{-/-}$ and *pachanga*, because the *deaf5Jcs* mice were not tested for OAE. In *Otof*$^{-/-}$ mice, IHC ribbon synapses developed and assembled normally. On the contrary, calcium-triggered exocytosis at these synapses was almost completely abolished. Specifically, the fast component of exocytosis was lost, whereas the slow component was partially preserved (16). The study of *Otof*$^{-/-}$ mice also showed that synaptic exocytosis was abolished in immature OHCs (33), whereas it was reduced and altered (slower kinetics, reduced calcium sensitivity and nonlinear calcium dependence) at vestibular hair cell ribbon synapses (34).

Taking into account all these data, it has been proposed that otoferlin might act as the major calcium sensor triggering membrane fusion at the IHC ribbon synapse, playing a role similar to that of synaptotagmin I in conventional synapses (16). However, recent research has revealed another putative function of otoferlin. The IHC ribbon synapse, because of its high rate of exocytosis, requires an efficient mechanism of vesicle replenishment. Interestingly, this is impaired in *pachanga* mice (35). Then, otoferlin might play a dual role in IHC exocytosis, i.e. calcium triggered fusion and vesicle replenishment.

3.4. Mutations of the *OTOF* gene in auditory neuropathy

In a pioneer study, genome-wide linkage analysis was performed on four families segregating autosomal recessive isolated AN. Significantly, results were consistent with linkage to markers of the 2p23 region, which contained *OTOF*, one of the genes known to be involved in ARNSHI. The screening of the *OTOF* gene revealed four mutations. In one of the families, affected subjects were compound heterozygotes for two *OTOF* mutations. In two other families, affected subjects carried only one mutant allele (20). In parallel, an independent study was being conducted on subjects with ARNSHI in order to explore the nature and frequency of *OTOF* mutations in the Spanish population (21, 36). The high frequency of a single mutation, due to a local founder effect (see below), led to establishing a collection of 37 subjects with ARNSHI caused by mutations in *OTOF*. A thorough clinical study of this genetically homogeneous cohort of subjects revealed AN in 11 subjects (21). So *OTOF* became the first gene whose mutations were reported to be involved in isolated AN. According to the expression sites and proposed roles of otoferlin, AN caused by *OTOF* mutations must be classified as pre-synaptic.

Before these two studies (20, 21), several works had reported mutations in *OTOF* in subjects with ARNSHI (19, 22, 36-40). Except in one study (36), whose affected subjects were reevaluated (21), no data regarding the presence/absence of AN in those subjects are available. More recently, some reports on the spectrum and frequencies of *OTOF* mutations in subjects with ARNSHI did not explore this issue either (23, 41, 42). On the contrary, other genetic studies have focused on subjects with a previous diagnosis of AN (43-52). The underlying question is whether the DFNB9 type of ARNSHI is associated to AN in all cases, OHCs losing their function secondarily in some subjects. Or, alternatively, there might be DFNB9 patients whose OHCs are not functional from the very onset of their hearing impairment. If this were the case, specific mutations could be involved in each disorder. As detailed below, the distributions of mutations found in subjects with DFNB9 hearing impairment, with or without AN, along the *OTOF* gene, are similar. Furthermore,

Table 3. Screenings of subjects with isolated AN for mutations in *OTOF*

Origin of subjects	Characteristics of the cohort	Subjects with AN	Subjects carrying *OTOF* mutations	Reference
USA	Familial cases	9	5 (55.5 %)	45
Spain	Familial cases Sporadic cases not attributable to environmental causes	15	13 (86.7 %)	46
Germany, Italy, France, Austria, Libya, Lebanon and Argentina	Familial cases Sporadic cases not attributable to environmental causes	20	11 (55.0 %)	46
Brazil	Familial cases Sporadic cases	11	7 (63.6 %)	47
Taiwan	Familial cases Sporadic cases not attributable to environmental causes	22	5 (22.7 %)	49
China	Sporadic cases	73	4 (5.5 %)	51

different subjects with the same combination of *OTOF* mutations display or not AN. In a few patients, OAE could be recorded bilaterally at early ages, but through transient stages of unilaterally present OAE, and absent OAE but present CM, the activity of OHCs was finally lost. The variability in the preservation of the OHCs function among DFNB9 patients may result from environmental or genetic modifier factors, which remain to be identified. In conclusion, it is very likely that DFNB9 hearing impairment always begins as an isolated AN.

Mutations in *OTOF* have been found in variable proportions in different cohorts of subjects with isolated AN (Table 3). Although it cannot be excluded that these differences might be due in part to an actual diversity between populations, it should be remarked that the criteria for including subjects in the different cohorts do not coincide, so preventing a proper comparison. Different proportions of familial and sporadic cases, inclusion/exclusion of cases with AN attributable to environmental causes, and other factors such as differences in the age of onset of AN in the patients, could strongly bias the results. Anyway, the current available data suggest that mutations in *OTOF* are a major cause of isolated auditory neuropathy in many populations.

Currently, the known spectrum of sequence variants of the *OTOF* gene includes near 70 pathogenic mutations (Figure 1) and more than 50 neutral variants (19-23, 36-52). There is not a hot spot for pathogenic mutations in the gene, but most of them are clustered between exons 13 and 30, and between exons 35 and 48. Non-pathogenic sequence variants follow a relatively similar distribution, with a majority of changes between exons 16 and 45, but also with a cluster in the first five exons. Most of the pathogenic mutations were described each in only one family (private mutations). Only a few have been reported repeatedly. As observed in other types of non-syndromic hearing impairment, the spectra of mutations and their frequencies are largely variable among populations. Local founder effects are major contributors to these differences, the most frequent mutations being c.4491T>A (p.Tyr1497X) in the Lebanese population (19, 46), c.2485C>T (p.Gln829X) in Spain and populations of Hispanic ancestry (21, 36, 44-46), c.2905_2923delinsCTCCGAGCGCA in Argentina (46), and c.5098G>C (p.Glu1700Gln) in Taiwan (49).

Most of the pathogenic mutations in *OTOF*, including nonsense mutations, small and large deletions, insertions-deletions (indels), duplications, and splice site mutations, are predicted to result in the synthesis of truncated polypeptides, in the synthesis of polypeptides carrying longer and abnormal C-terminal tails, or in no protein synthesis at all because of the action of nonsense-mediated mRNA decay mechanisms (19-23, 36-52). Consequently, these sequence variants are expected to be inactivating mutations. On the other hand, 23 non-truncating pathogenic mutations have been described in *OTOF* so far, including 20 missense mutations and 3 in-frame deletions removing single amino acid residues. Interestingly, most of these mutations affect the predicted functional domains of otoferlin. Fourteen mutations affect conserved residues in the C2 domains that are involved in calcium binding: C2B (1 mutation), C2C (2 mutations), C2D (4 mutations), C2E (2 mutations), and C2F (5 mutations). Four other mutations affect the transmembrane domain of otoferlin encoded by exon 48. On the contrary, most of the non-pathogenic missense sequence variants do not affect the predicted functional domains of otoferlin. This distribution of non-truncating pathogenic mutations and missense neutral polymorphisms confirms the importance of the C2 domains B to F and the transmembrane anchor for the function of otoferlin. Consistently, the cause of deafness in the *deaf5Jcs* and *pachanga* mice are *Otof* missense mutations that affect the C2B and C2F domains, respectively (31, 32). Apparently, there is not a specific type of mutation in the *OTOF* gene leading to AN (Figure 1), since this disorder has been observed in subjects carrying any combination of mutations, i.e. truncating/truncating (the most prevalent subgroup), truncating/non-truncating, and non-truncating/non-truncating, as well as in subjects carrying two mutations affecting all otoferlin isoforms, or one mutation affecting all isoforms and the other affecting only the long isoforms (19-23, 36-52).

Most of subjects with isolated AN caused by *OTOF* mutations have prelingual severe or profound hearing impairment. To date there are few exceptions to this rule. Among subjects who are homozygous for the p.Glu1700Gln mutation, a prelingual progressive mild to moderate hearing impairment has been reported (49). Of note, this missense mutation does not affect any of the C2 domains of otoferlin. In addition, three cases with AN induced by high body temperature (temperature-sensitive

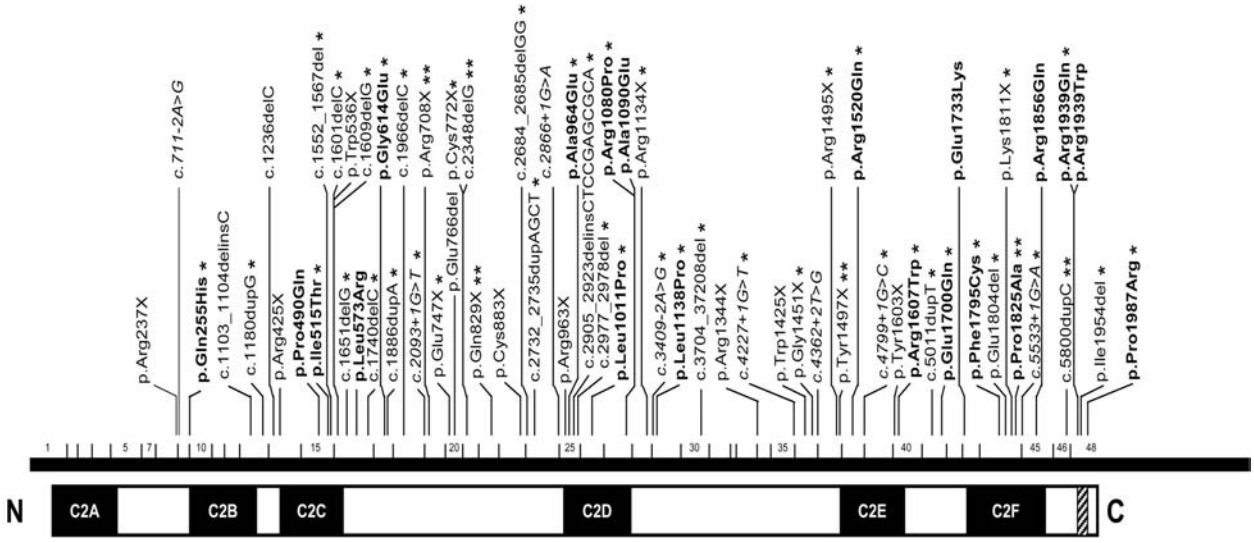

Figure 1. Location of pathogenic mutations in the *OTOF* gene. Vertical lines indicate the position of each mutation on the reference cDNA (accession number AF183185.1) and on the correlated position in the protein. A horizontal bar depicts the cDNA, small vertical bars delimiting the exons, which are numbered. The reference cDNA does not contain exon 6. Exon 47 has been removed from the figure to show the effects of the mutations in exon 48 on the cochlear isoforms of the protein. Black boxes represent the C2 domains, whereas the cross-hatched box represents the transmembrane domain. Missense mutations are shown in bold, splice site mutations are in italics. The position of splice site mutations is shown in the border between the exons flanking the affected intron. Two asterisks indicate mutations that have been reported in subjects with ARNSHI and in subjects with AN. One asterisk indicates mutations found only in subjects with AN. No asterisk indicates mutations found only in subjects with ARNSHI (note that most of these subjects were not tested for OHC function).

AN) have been reported to carry *OTOF* mutations. When afebrile, these subjects have normal or mildly elevated hearing thresholds. As the body temperature raises, their hearing losses become severe or profound and speech intelligibility decreases dramatically. The first case was familiar, all three affected subjects being heterozygous for the p.Ile515Thr mutation (in the C2C domain), but the mutation in the other alelle was not found (45, 53). The second case was a compound heterozygote for p.Gly614Glu (not in any C2 domain) and p.Arg1080Pro (in C2D) (47). The third case was also familiar, all three affected subjects being homozygous for p.Glu1804del (in C2F) (50). Remarkably, at least one of the alleles found in these cases carries a non-truncating mutation altering a C2 domain. It is likely that these amino acid substitutions may confer thermosensitivity to these mutant otoferlins by weakening the structure of their C2 domains, but this hypothesis needs to be confirmed by further experimental work. Anyway, the study of the mutations found in subjects with milder phenotypes, by means of knock-in models and in *vitro* assays, should help to gain further insight into the molecular mechanisms by which otoferlin plays its physiological roles.

Recently, transtympanic electrocochleography was used to record sound-evoked cochlear potentials from four profoundly deaf subjects with mutations in *OTOF*, and from 16 normally hearing children (48). In this pioneer study, CM of the four affected subjects were recorded with normal amplitudes from all but one ear. After CM cancellation, cochlear potentials obtained were of negative polarity, with reduced amplitude and prolonged duration compared to controls. These results are consistent with decreased neurotransmitter release resulting in abnormal dendritic activation and impairment of auditory nerve firing (48).

Given the pattern of expression of otoferlin in the inner ear, it could be expected that subjects with mutations in *OTOF* might have vestibular dysfunctions. However, no subject complained of vertigo or dizziness, and in the few subjects who were specifically tested (rotary chair, bithermal calorics) the results were normal (20), except for two unrelated children with a putative hypoactive vestibular function (45). Consistently, no vestibular dysfunction has been observed in murine models. *Otof* $^{-/-}$ mice were evaluated at P30 for the reaching response, air-righting reflex, contact-righting reflex, elevated platform test, and swimming tests, and their scores were similar to those of their wild-type littermates. Results of these behavioural tests do not exclude the possibility of compensation from visual or proprioceptive inputs (16). However, when *deaf5Jcs* mice were examined by vestibular-evoked potentials, their results were also similar to those of wild-type mice (32). Apparently, although expressed in vestibular hair cells, otoferlin is not essential for a normal vestibular function.

The indication of cochlear implantation for subjects with isolated AN has been relatively controversial (17) because different etiologies of the disorder result in different outcomes of the implants. AN due to mutations in *OTOF* is properly pre-synaptic and, as it could be expected, cochlear implants provided to subjects with mutations in

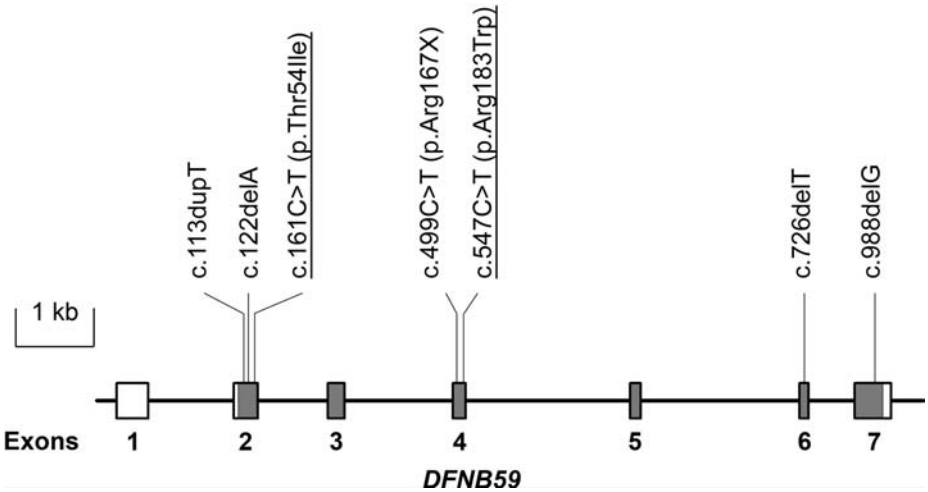

Figure 2. Exon-intron structure of the *DFNB59* gene, showing the location of pathogenic sequence variants. The *DFNB59* coding sequence, spanning exons 2-7, is shaded. Mutations that cause auditory neuropathy are underlined.

this gene consistently yielded good outcomes (21, 44, 45, 49, 52).

4. *DFNB59* GENE AND PEJVAKIN

Analysis of a large consanguineous kindred from Iran with profound ARNSHI led to the identification of a novel deafness locus, DFNB59 (MIM# 610220), on chromosomal region 2q31 (54). Sequencing of all genes and expressed sequence tags within the 3.7 Mb interval defined for the locus showed homozygous mutations in a hypothetical gene, later named *DFNB59*, in the original family and in three additional Iranian families segregating severe-to-profound ARNSHI. Further audiologic examination of affected individuals from two of these families indicated that their hearing impairment met the diagnostic criteria for auditory neuropathy (54).

4.1. Gene and protein structures

The *DFNB59* gene (MIM# 610219) is located on 2q31.2. Its structure was elucidated by cloning the full length cDNA of the mouse *Dfnb59* orthologue and aligning the murine cDNA sequence on human genomic sequences to reveal potential exons; this tentative structure was verified thereafter by cloning and sequencing the actual complete *DFNB59* cDNA from a human testis expression library (54). The *DFNB59* gene consists of 7 exons, spanning 9,950 bp of genomic sequence (Figure 2). A unique transcription start site is known and there are no evidences of alternative splicing. So, a single 1,534 bp-long mRNA is produced. The *DFNB59* coding sequence extends from exons 2 to 7.

Because of the Iranian origin of the affected families, the protein encoded by *DFNB59* was named pejvakin after the Persian word for “echo”. Pejvakin is a 352-residue protein with deduced molecular weight of 39.9 kDa. Bioinformatic analyses indicate that pejvakin is a soluble protein with putative motifs for nuclear localization (residues 249-258) and, with low scoring, for interaction with DNA (residues 305-331). These characteristics, whose functional signification is unknown, are shared by all members of the protein family to which pejvakin belongs, which includes DFNA5, gasdermins and the MLZE protein (54, 55). Within this family, pejvakin is most closely related (32% residue identity and 54% similarity) to DFNA5, a protein of unknown function altered in autosomal dominant non-syndromic hearing loss (MIM# 600994) (56).

4.2. Gene expression and protein localization

The expression of the mouse *Dfnb59* orthologue was investigated by RT-PCR and *in situ* hybridization. *Dfnb59* transcripts were detected by RT-PCR in all adult mouse organs tested, namely brain, eye, inner ear, heart, lung, kidney, liver, intestine, testis and skeletal muscle (54). Within the inner ear, *in situ* hybridization detected strong expression of *Dfnb59* in hair cells from the cochlea and vestibule at postnatal days P1 and P4 (31).

The latter expression pattern of *Dfnb59* largely coincides with the data on the inner ear localization of pejvakin, obtained by immunostaining with rabbit and guinea pig polyclonal antibodies directed against peptides of mouse pejvakin (54). In the vestibule, pejvakin labelling is associated with the kinocilium of hair cells from all the sensory areas (cristae ampullares of the semicircular canals and maculae of the saccule and utricle). In the organ of Corti, pejvakin is detected in pillar cells (from P1 onwards, in the apical surface and stalk portion of both pillars) and hair cells. In developing cochlear hair cells (P4-P8), pejvakin staining is also associated with the transient kinocilium. From P12 onward, however, pejvakin staining gradually disappears in IHCs, whereas in OHCs a strong, persistent labelling becomes apparent in the cuticular plate, the apical filamentous network that supports the hair bundle. Remarkably, pejvakin is also detected in neurons of the spiral ganglion and of the first three relays of the afferent auditory pathway (cochlear nuclei, superior olive and inferior colliculus). In all those neurons, pejvakin

labelling is always observed in the cell bodies and not in fiber bundles.

4.3. Function of pejvakin

Since the putative cellular activities of pejvakin, and of all other members of the same family of proteins, remain unknown, information on the physiological roles of pejvakin is limited to what can be deduced from the effects of known disease-causing mutations in patients and model mice. These murine models include *Dfnb59*$^{tm1Ugds/tm1Ugds}$ knock-in mice (54), and ENU mutagenesis-generated *sirtaki* mice that are homozygous for a nonsense mutation in the *Dfnb59* gene (31). On the one hand, the distorted ABR with increased wave latencies observed in DFNB59 auditory neuropathy subjects and in *Dfnb59* $^{tm1Ugds/tm1Ugds}$ knock-in mice argue for essential roles of pejvakin in the activity of neurons of the afferent auditory pathway, which may be related to the propagation of signals along the nerve. On the other hand, the hearing impairment with loss of OHC function observed in some patients (57-59) and in *sirtaki* mice, together with the persistent expression of pejvakin in OHCs of adult mice, suggests roles for pejvakin in OHC physiology. In both cases, the roles of pejvakin must be related to the activity of neurons or hair cells and not to their development or maintenance, as no structural defects or cell losses were apparent in *sirtaki* (31) and *Dfnb59* $^{tm1Ugds/tm1Ugds}$ mice (54).

No proteins that interact with pejvakin have been identified so far. The similarity of pejvakin and DFNA5 and the fact that mutations in both *DFNB59* and *DFNA5* result in hearing impairment suggested the possibility that the two proteins might act in a common pathway or functionally interact somehow (31). However, this is considered unlikely because the characteristics of the hearing impairment in DFNA5 and DFNB59 patients are different (54, 60, 61). Moreover, the auditory phenotype of *Dfnb59* $^{tm1Ugds/tm1Ugds}$ mice is indistinguishable from that of *Dfnb59*$^{tm1Ugds/tm1Ugds}$ *Dfna5*$^{-/-}$ double homozygous mice (54).

4.4. Mutations of the *DFNB59* gene in auditory neuropathy

Seven pathogenic mutations have been identified in the *DFNB59* gene so far (31, 54, 57-59) (Figure 2); in every case, affected subjects were homozygous for a given mutation, without any reported compound heterozygous patient. One of these mutations, c.547C>T (p.Arg183Trp), was identified in 3 Iranian families (54) and 1 Turkish family (58). However, haplotype analysis of individuals from the 4 kindreds indicates that it is very unlikely that p.Arg183Trp is a founder mutation (58) and probably arose more than once on a CpG dinucleotide. The remaining mutations have been reported in just one family each.

p.Thr54Ile and p.Arg183Trp were the first pathogenic mutations identified in *DFNB59* (54). Both are missense mutations that affect residues conserved across all known pejvakin orthologous proteins. Eight p.Thr54Ile homozygotes (with severe hearing impairment) and four p.Arg183Trp homozygotes (with profound hearing impairment) underwent detailed audiological examination that showed preserved, high-amplitude spontaneous synchronized OAE (SSOAE) bilaterally in 11 of 12 subjects. SSOAE are long-lasting, ringing sounds, synchronized to an external stimulus, that are emitted spontaneously by the cochlea; recording of SSOAE indicates that OHC function is preserved. In contrast, ABR were absent in all p.Arg183Trp homozygotes and were severely distorted, with pathological increases in wave latency, in p.Thr54Ile homozygotes. Altogether, these results are consistent with preserved OHC function and impaired neural transmission of auditory information and thus fulfill the criteria for a diagnosis of auditory neuropathy.

The knock-in mouse model *Dfnb59* $^{tm1Ugds/tm1Ugds}$, harbouring the same p.Arg183Trp mutation detected in patients, was generated to gain further insights into the pathomechanisms underlying DFNB59 auditory neuropathy (54). *Dfnb59* $^{tm1Ugds/tm1Ugds}$ mice present high-frequency, non-progressive hearing impairment, distorted ABR with pathologic increases in wave latencies, and normal distortion-product OAE. In homozygous mutant mice there are no detectable anomalies in the structure of the cochlea (including hair cell morphology and ultrastructure) or of the nuclei of the afferent auditory pathway. Since the p.Arg183Trp mutation does not alter the pejvakin labelling patterns observed in wild-type mice, it is currently thought that this mutation causes a dysfunction in neurons of the afferent auditory pathway, while sparing OHC function.

Later on, mutations that presumably result in null pejvakin alleles (one nonsense and five small insertion or deletion mutations) (31, 57-59) were identified in *DFNB59* in deaf subjects. When subjects were available for audiological evaluation, it was realized that the severity of hearing impairment was moderate-profound or severe-profound. Progression was clearly documented in two families (c.113dupT and c.122delA homozygotes) (31, 57) and a central vestibular dysfunction compatible with vestibular neuropathy was reported for c.113dupT homozygotes (57); it must be remembered that the posturography tests required to evaluate central vestibular dysfunction are very specialized, and thus it is likely that other DFNB59 patients have not been tested for such impairment. However, ABR were absent or distorted but OHC function was lost in all the patients who underwent OAE testing (c.113dupT, p.Arg167X and c.988delG homozygotes), indicating a cochlear pathology distinct from auditory neuropathy (57-59). Notably, a similar phenotype of progressive hearing impairment with distorted ABR and absent OAE is observed in *sirtaki* mice, which harbour the p.Lys290X nonsense mutation in *Dfnb59* (31). As in the case of *Dfnb59* $^{tm1Ugds/tm1Ugds}$ mice, *sirtaki* mice show no structural anomalies of hair cells or nuclei of the afferent auditory pathway.

It might be tempting to conclude from these data that pathogenic mutations in *DFNB59* result in two different clinical conditions, with missense mutations being responsible for auditory neuropathy and fully inactivating variants, such as nonsense mutations and frame-shifting small insertions or deletions, causing a more severe hearing

impairment phenotype that includes the loss of OHC function. However, the finding that p.Arg183Trp homozygotes from a Turkish family lack transiently-evoked OAE (58) argues against such a clear-cut correlation. Indeed, the fact that this same mutation may result in intact (54) or impaired (58) OHC function suggests that other genetic or environmental factors modulate the observed phenotypes. Identification of more *DFNB59* pathogenic variants and thorough characterization of the audiological and vestibular phenotypes of patients are necessary for a clear pattern to emerge.

Little is known about the contribution of *DFNB59* to the total of cases of isolated auditory neuropathy with genetic origin. In the single published study (47), *DFNB59* was screened for mutations in a cohort of 11 auditory neuropathy subjects from Brazil, without any pathogenic variants being found. This result suggests that mutations in *DFNB59* are not a common cause of auditory neuropathy in the Brazilian population, although it is uncertain whether these data could be extrapolated to other populations.

5. *DIAPH3* GENE AND DIAPHANOUS-3.

The AUNA1 locus (MIM# 609129) was defined by studying a large American family, of European descent, which segregated autosomal dominant isolated AN (62, 63). AUNA1 mapped to a 5.47 cM interval on chromosome 13q14-21. The interval was narrowed down to a 3.28-cM gene-poor region by genotyping additional family members. Sequencing of all four genes comprised in this region led to the identification of a heterozygous mutation in the *DIAPH3* gene, which segregated with the disease, whereas no pathogenic variants were detected in the remaining genes (64).

5.1. Gene and protein structures

The *DIAPH3* (*Diaphanous homologue 3*) gene consists of 29 exons (named 1, 1b and from 2 to 28) spanning 498,402 bp of genomic sequence on 13q21.2 (64, 65). The transcription start site used in all tissues is located at the 5' end of exon 1, although a second start site used solely in testis is located at the 5' end of alternative exon 1b. The longest *DIAPH3* mRNA consists of exons 1-28 and encodes the full-length diaphanous-3 protein (1,193 amino acids long, with a deduced molecular weight of 136.9 kDa). The testis-specific mRNA consists of exons 1b and 8-28 and codes for a shorter diaphanous-3 protein of 849 residues. In addition, several minor isoforms, generated by skipping of diverse exons present in the longest mRNA, have been detected (64). Translation start codons are located in exon 1 (for all isoforms save the testis-specific mRNA) and exon 1b (for the testis-specific mRNA). The unique translation stop codon is in exon 28.

DIAPH3 is one of the three human orthologues of *diaphanous* from *Drosophila.* These genes code for diaphanous-related formins, soluble modular proteins that consist of an N-terminal Rho GTPase-binding domain (residues 115-297 in the full-length diaphanous-3 protein), a formin homology (FH) 3 domain (residues 302-494), an actin-binding FH2 domain (residues 636-1009), and a C-terminal diaphanous autoinhibitory domain (residues 1060-1074) (66).

5.2. Gene expression and protein localization

DIAPH3 is ubiquitously expressed, as indicated by RT-PCR and Northern blot hybridization experiments (64). In all organs but testis, the most abundant mRNA is that consisting of exons 1-28. No information is currently available about precise expression patterns of *DIAPH3* or localization of diaphanous-3 within the inner ear.

5.3. Function of diaphanous-3

Diaphanous-related formins are factors that regulate actin polymerization by affecting nucleation, elongation and capping, although they also bind to microtubules, modulating their growth. They participate in the maintenance of cell polarity and cell shape, as well as in intracellular vesicular traffic. The activity of the diaphanous-related formins is regulated by means of their N-terminal GTPase-binding domain. In the inactive conformation, the FH3 domain is bound to the diaphanous autoinhibitory domain, impeding binding of the FH2 domain to actin. Interaction of the GTPase-binding domain with Rho-GTP releases the diaphanous autoinhibitory domain from the FH3 domain, and, as a result, the actin-binding site in the FH2 domain becomes unmasked (66).

The functions of diaphanous-3 in the cochlea are uncertain. Because diaphanous-3 participates in actin dynamics, it might play a role either in specifying the precise shape or polarity of the hair bundle, the apical specialization of hair cells that houses the mechanotransduction machinery, or in controlling the length of stereocilia, the actin-filled microvilli that compose the hair bundle. The recent discovery that the paralogous diaphanous-2 protein from mouse is essential for the proper activity of dendritic spines in neurons from the cortex and hippocampus (67) suggests an alternative role for diaphanous-3 in the postsynaptic elements of hair cell synapses.

5.4. Mutations of the *DIAPH3* gene in auditory neuropathy

Only a single mutation in *DIAPH3* is known, underlying the auditory neuropathy observed in affected members of the family that defined the AUNA1 locus. It is a point mutation in the 5'-untranslated region (UTR) of the gene, c.-172G>A, which appears heterozygously in most affected subjects, homozygously in two siblings from the family that were offspring of a consanguineous marriage, and was not found in 758 chromosomes from normal-hearing controls (64).

The c.-172G>A mutation affects a GC-box element in the 5'-UTR that is conserved among vertebrate *DIAPH3* orthologues. These elements are bound by proteins from the Sp1 and Krüppel-like families of transcription factors to activate or repress gene expression. Indeed, 2-3 fold overexpression of *DIAPH3* mRNA and 1.5-fold increase in diaphanous-3 protein levels were detected in lymphoblastoid cell lines from AUNA1 patients as indicated by experiments with expression microarrays, quantitative RT-PCR and immunoblotting. Luciferase

reporter expression assays confirmed that the cause of this *DIAPH3* overexpression is the c.-172G>A mutation (64). The effect of increased diaphanous-3 levels was further investigated in Johnston's organ, the auditory organ of *Drosophila melanogaster*. Johnston's organ consists of an array of mechanosensory neurons that respond to sound, gravity and wind movements, and are similar in development and function to mammalian IHCs. Mutant flies expressing a constitutively active form of the *Drosophila* diaphanous protein exhibited significantly reduced sound-evoked potentials (64), which is reminiscent of the phenotype observed in AUNA1 patients.

The first audiological finding in AUNA1 patients is late-onset auditory neuropathy (63). Affected individuals initially present with abnormal ABR but preserved OHC function (normal OAE and CM) at an average age of onset of 18.6 years. The hearing loss progresses thereafter over a period of 10-20 years to become a profound sensorineural impairment that eventually involves OHCs, with absent ABR and OAE (62, 63). It is interesting to note that cochlear implants significantly improved ABR and speech recognition scores in the three AUNA1 patients that received such devices, which supports that the pathological process initially involves IHCs, the synapses or the terminal dendrites of spiral ganglion neurons (62). In case that *DIAPH3* overexpression had an effect on actin dynamics of the hair bundle, it remains to be elucidated how it would affect IHC function while initially sparing OHCs.

6. OTHER GENES REPORTED TO BE INVOLVED IN ISOLATED AUDITORY NEUROPATHY

6.1. *GJB2* (connexin-26) gene

In 1997, mutations in the *GJB2* gene, encoding the gap junction protein connexin-26 were shown to underlie autosomal recessive non-syndromic hearing impairment (68). Soon it was noticed that mutations in this gene were frequent in most populations, accounting for up to 50% of all cases of ARNSHI (69). Consequently, testing for *GJB2* mutations has become the gold standard for genetic diagnosis of ARNSHI (70). Unexpectedly, two different studies reported the finding of isolated AN in deaf subjects carrying *GJB2* mutations (71, 72).

In the first study, over 700 subjects from schools for the deaf having prelingual non-syndromic hearing impairment were tested for OAE. They were present in about 10% of these cases. Five cases with preserved OAE carried mutations in the *GJB2* gene, either in the homozygous state (c.35delG or p.Trp77X) or in compound heterozygous state (c.35delG/c.360delGAG, c.35delG/p.Val95Met, and p.Met34Thr/p.Val84Met). In most of the cases, OAE were found only for the low frequencies, suggesting the presence of functional OHCs in the apex of the cochlea (71).

The second study reported three subjects who showed OAE and had hearing losses exceeding 30 dB. Two of them were homozygous for c.35delG, the third one being heterozygous for p.Met34Thr (72). The latter should not be taken into account, because the second mutant allele was not found, and p.Met34Thr is a hypomorphic allele with a weak pathogenic potential even in the compound heterozygous state. One of the two c.35delG homozygotes had an asymmetric hearing loss (profound in the right ear, moderate in the left ear). Further investigation revealed that ABR and OAE were absent in the right ear, whereas OAE and ABR with normal latencies and morphology were recorded from the left ear, so the diagnosis of AN was ruled out (72). The other c.35delG homozygote had bilateral profound hearing impairment with bilaterally preserved OAE and CM. ABR were absent, and electrocochleography revealed that the summating potential and the CAP were also absent. These data pointed to a lesion involving the inner hair cells (72).

It is currently thought that the deficiency in connexin-26 disrupts the ion homeostasis of the inner ear, which in turns leads to a local extracellular accumulation of potassium and ultimately to cell death (73). This conclusion is also supported by the histopathological analysis of the temporal bone of a patient carrying two mutations in the *GJB2* gene, which revealed degeneration of cochlear hair cells with preservation of auditory nerve fibers (74). It is very likely that the OAE that were recorded in the low frequencies from those subjects with two *GJB2* mutant alleles represent the residual activity of the few OHCs still alive in the apical part of the cochlea. If so, the hearing disorder observed in these subjects would not properly fit into a diagnosis of AN. Anyway, these findings are of concern to the newborn hearing screening programmes that rely only on OAE, given the high frequency of *GJB2* mutations among subjects with ARNSHI. Data inputs from these programmes should help to identify more cases like those reviewed above, and to clarify this issue.

6.2. Mitochondrial 12S rRNA gene

It is well established that several mutations in the mitochondrial 12S ribosomal RNA gene confer increased sensitivity to the ototoxic action of aminoglycoside antibiotics, as well as they can result in maternally-inherited non-syndromic hearing loss without exposure to aminoglycosides (75). One of these mutations is m.1095T>C, originally reported in two Italian families (76, 77). In one family, five of seven affected subjects had sensorineural deafness, one other had deafness and levo-dopa responsive parkinsonism, and the remaining subject had deafness, parkinsonism and neuropathy. Molecular analysis of the latter and of one maternal relative with isolated deafness revealed that the mutation was in heteroplasmy, with mutation loads higher than 95% in blood (76). The other family segregated maternally-inherited non-syndromic hearing loss with a history of aminoglycoside treatments. The mutation was found in homoplasmy in one affected subject, and in heteroplasmy in three other affected subjects (mutation loads in blood ranging from 2% to 45%) (77). Later on, the mutation was reported in several Chinese families with non-syndromic hearing impairment, and also in a Chinese patient with absent ABR, preserved OAE and poor speech discrimination (78). Other authors have claimed that the pathogenicity of this mutation in these Chinese families

seems unsupported, because this sequence variant is specific to mitochondrial haplogroup M11 in East Asia (79). The phenotypic differences observed in m.1095T>C carriers might mean that the mutation is not actually responsible for the observed clinical signs or, alternatively, the differences could be due to variations in mutation load in the affected tissues or to modifier effects of the nuclear and mitochondrial DNA backgrounds. Of note, other deafness-causing mutations in the mitochondrial 12S rRNA gene have not been involved in AN to date. The identification and the clinical and molecular characterization of novel cases with the m.1095T>C mutation should throw light on this question.

7. FUTURE PROSPECTS

The investigation of the molecular causes of isolated AN in humans and murine models is expected to continue improving our understanding of the structure and function of the auditory ribbon synapse and the auditory neural pathways.

Mutations in the *OTOF* gene seem to be a major cause of isolated AN in many populations. Screening additional cohorts of patients from different origins should provide confirmation of this point, and should broaden the spectrum of mutations in the gene. Investigation of those resulting in milder or conditional phenotypes, by generating the appropriate knock-in murine models and performing *in vivo* and *in vitro* assays, should provide further insight into the complex roles of otoferlin in the ribbon synapse. The individual and coordinated functions that are played by the six otoferlin C2 domains, as well as the nature of interactions of otoferlin with its known ligands need further investigation. A putative more ubiquitous role of otoferlin in the endocytic or exocytic pathways should be explored. As regards the clinical characterization of subjects with mutations in *OTOF*, it would be interesting to get accurate data on the vestibular function of larger series of patients. Transtympanic electrocochleography could be also a very useful tool to search for specific features of each genetically homogeneous type of AN.

As regards *DFNB59* and *DIAPH3*, the number of reported cases with mutations in these genes is still too small to extract general conclusions. Further investigation of the roles played by these two proteins and of their pathophysiological mechanisms should be accompanied by additional screenings of cohorts of subjects with isolated AN in which mutations in the *OTOF* gene have been excluded. These cohorts are also a precious material to search for novel genes involved in isolated AN. Good candidates are those encoding proteins of the auditory ribbon synapse, as well as those coding for otoferlin ligands. Further comprehension of the etiologies of the different subtypes of isolated AN is still needed to improve the diagnosis, management and therapy of the affected subjects.

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Abbreviations: ABR: auditory brainstem responses; AN: auditory neuropathy; ARNSHI: autosomal recessive non-syndromic hearing impairment; CAP: compound action potential; CM: cochlear microphonics; dB: decibels; FH: formin homology; IHCs: inner hair cells; MLZE: melanoma-expressed, leucine zipper-containing extranuclear factor; OAE: otoacoustic emissions; OHCs: outer hair cells; PTA: pure-tone audiometry; SNARE: soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor; SSOAE: spontaneous synchronized otoacoustic emissions; UTR: untranslated region.

Key Words Auditory neuropathy, Otoferlin, Pejvakin, Diaphanous-3, Connexin-26, Mitochondrial 12S rRNA, DFNB9, DFNB59, AUNA1, Review

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