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The DFNB1 subtype of autosomal recessive non-syndromic hearing impairment

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1. ABSTRACT

Inherited hearing impairment is a frequent and highly heterogeneous condition. Among the different subtypes of autosomal recessive non-syndromic hearing impairment, DFNB1 is remarkable for its high frequency in most populations. It is caused by mutations in the coding region or splice-sites of the *GJB2* gene, or by mutations affecting regulatory sequences that are essential for the expression of this gene. *GJB2* encodes connexin-26, a protein component of intercellular gap junctions, which play crucial physiological roles in the cochlea. Because of its high frequency, DFNB1 hearing impairment has received continued attention from researchers along the years, resulting in a wealth of data that is unparalleled among these disorders. Here we review our current knowledge on the genetic, molecular, and phenotypic aspects of this subtype of hearing impairment.

2. INTRODUCTION

Hearing impairment (HI), the most common sensory disorder, can be caused by environmental or genetic factors and can manifest at all ages (1). Its effects are more devastating when the onset takes place in infancy, before speech acquisition (prelingual HI). Reported incidences for bilateral hearing loss of at least 40 dB range between 1 and 2 per 1,000 newborns in different studies (2). In developed countries, genetic causes account for over 60% of the cases. In at least 70% of these, HI is not associated to clinical signs in other organs (non-syndromic HI), and the autosomal recessive inheritance pattern is preponderant (3).

Soon it was noticed that localizing genes for non-syndromic prelingual HI was a formidable task, because of a combination of factors, namely the extreme genetic

heterogeneity of HI, which results in many different etiological subtypes, the paucity of knowledge that may help to distinguish these subtypes clinically, and the endogamy between affected subjects. These problems were circumvented through the genetic study of single families, either large enough for conventional linkage analysis or amenable to homozygosity mapping because of consanguinity. By using this approach, the first locus for autosomal recessive non-syndromic HI (ARNSHI) was mapped to 13q12 (4). It was named DFNB1 (MIM# 220290), DFN being the acronym for deafness, B for autosomal recessive (the A letter was assigned to the dominant loci), and 1 for being the first to be discovered. As HI can be classified etiologically according to the gene that is mutated in each case, loci names are being used also to designate the different subtypes of ARNSHI. After the pioneer report of DFNB1, the list of DFNB loci has not stopped growing (5).

A milestone in the research on inherited HI was the finding that mutations in the *GJB2* gene, which codes for connexin-26, a protein component of intercellular gap junctions, are the molecular cause of the DFNB1 subtype of ARNSHI (6). Subsequent studies revealed the high frequency of *GJB2* mutations in most populations. Since then, molecular testing for *GJB2* mutations has become the gold standard for the genetic diagnosis of ARNSHI. Moreover, generation and study of murine models and functional analyses of normal and mutated proteins have resulted in remarkable advances in our understanding of the underlying pathogenetic mechanism. In this review we summarize the current knowledge on the genetic, molecular, and phenotypic aspects of the DFNB1 subtype of ARNSHI.

3. *GJB2* GENE AND CONNEXIN-26

3.1. Gene and protein structures

The *GJB2* gene (MIM#121011) lies within a cluster of connexin genes on 13q12, flanked on the centromeric side by *GJA3* (encoding connexin-46) and on the telomeric side by *GJB6* (encoding connexin-30). *GJB2* spans 5,513 bp and consists of just two exons. The 678-bp *GJB2* coding sequence is completely contained in the second exon. Transcription from the single known transcription start site results in the production of two mRNAs of different lengths (2.4 and 2.8 kb) due to the use of alternative polyadenylation signals (7).

The 226-amino acid-long connexin-26 (Cx26), also known as Gap junction beta-2 protein, belongs to the connexin protein family, which comprises 21 different members in humans (8). Connexins are integral membrane proteins that form hydrophilic channels spanning a lipid bilayer. The connexin channel unit is a hexamer (termed "connexon"). Two connexons lying in the plasma membranes of two adjoining cells are able to assemble head to head (a process called "docking") to form an intercellular hydrophilic channel that directly connects the cytoplasms of the two cells. Arrays of hundreds of closely packed connexin intercellular channels form the characteristic plaques identifiable as gap junctions in electron microscopy images (9).

The crystal structure of the human Cx26 intercellular channel was recently determined at 3.5 Å resolution (10). Each Cx26 monomer consists of four transmembrane helical segments termed TM1 (residues 21-41), TM2 (residues 73-109), TM3 (residues 125-158) and TM4 (residues 186-216), joined by one cytosolic loop (residues 110-124) and two extracellular loops (E1, residues 42-72; and E2, residues 159-185), with N- and C-termini (residues 1-20 and 217-226, respectively) exposed to the cytosol (Figure 1 A). Overall, the Cx26 monomer forms a compact four-helix bundle in which adjacent helices are arranged in antiparallel conformation. Helices TM3 and TM4 face the hydrophobic environment of the membrane, whereas TM1 and TM2 face the interior of the channel (Figure 1 B-C). The longer helices TM2 and TM3 protrude into the cytosol. The four-helix bundle is stabilized both by different intramolecular interactions (hydrogen bonds or salt bridges) between the side chains of residues in adjacent transmembrane segments and by dipole-dipole interactions of the antiparallel helices.

Extracellular loops E1 and E2 play two distinct structural roles. Both loops have 3 cysteine residues each, with a specific spacing conserved in nearly all connexins (CX_6CX_3C in E1 and CX_4CX_5C in E2) (8), which are essential for normal folding and channel function. These cysteines form three intramolecular disulfide bonds (Cys53-Cys180, Cys60-Cys174 and Cys64-Cys169) between the E1 and E2 loops during the conformational maturation process that Cx26 undergoes when trafficking along the endoplasmic reticulum and Golgi complex. In addition, the E1 and E2 loops mediate the interactions between docked connexons in adjoining cell membranes that underlie the intercellular channel. The E1 loops of opposite monomers face each other across the extracellular gap; this is also the case with the E2 loops (Figure 1 C). Hydrogen bonds and salt bridges between polar residues in opposite E1-E1 loops and E2-E2 loops create a two-layered wall that bridges the gap between both cell membranes and separates the interior of the channel from the extracellular medium.

The actual hydrophilic pore of each connexon consists of an intracellular entrance (formed by the cytosolic protrusions of helices TM2 and TM3, with a net positive charge), a funnel (lined by the TM1 helix and the N-terminus), and an extracellular cavity (formed by the residues in the amino-terminal half of the E1 loop). Opening and closure of the channel depend on intracellular voltage-dependent interactions between residues in a short helix of the N-terminus and residues in TM1.

The structures of the cytoplasmic loop and C-terminus could not be modelled as they are disordered in electron density maps. They may participate in chemical gating or complete pore closure (10).

3.2. Gene expression and protein localization

The basal promoter of the *GJB2* gene is contained in a 128-bp region located immediately upstream of the single transcription start site (7,11). The basal promoter contains a TATA motif (TTAAAA, at -24) and two

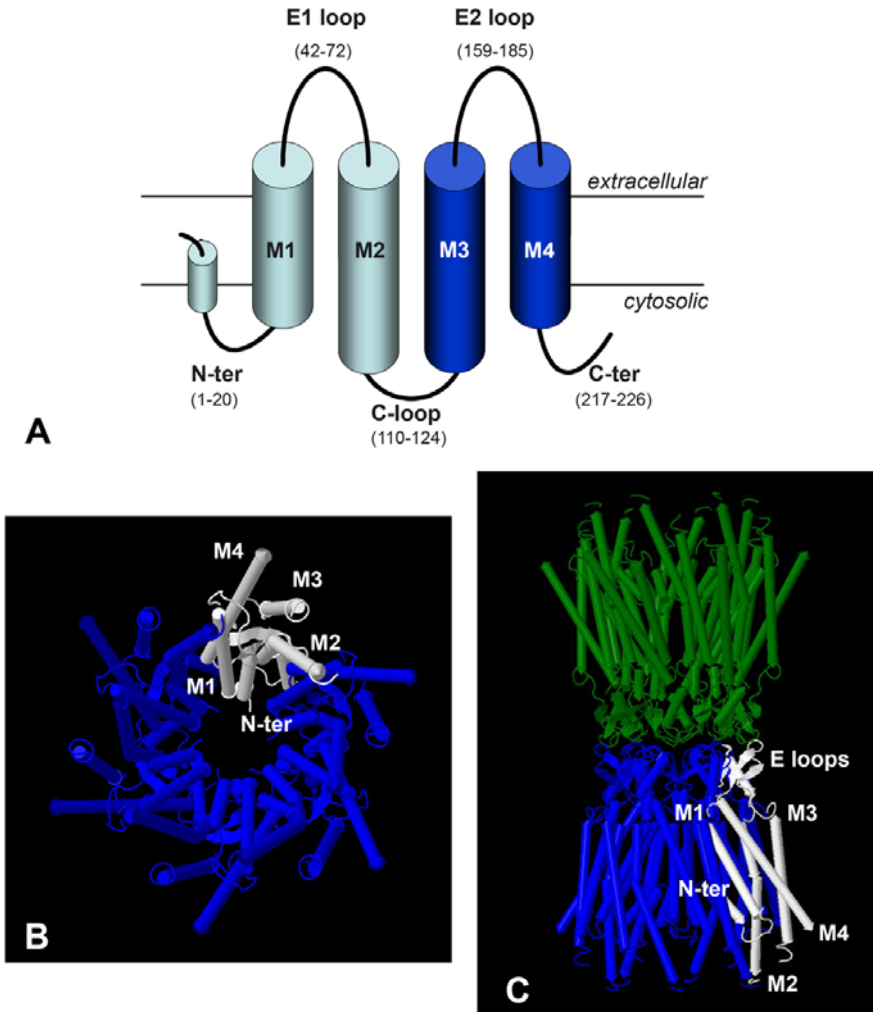

Figure 1. Structure of Cx26. (A) Topological organization of the Cx26 monomer, showing the disposition of the different structural elements. Helices (cylinders) in light grey face the channel, whereas those in blue face the hydrophobic environment of the membrane. Note that the longer helices M2 and M3 protrude into the cytosol. (B) Structure of a Cx26 hexameric connexon as viewed from the cytosolic side. One of the monomers has been highlighted to show the spatial organization of the transmembrane helices. (C) Lateral view of a Cx26 intercellular channel, which consists of two connexons (green and blue) docked head to head. One monomer of the bottom connexon has been highlighted; note the antiparallel arrangement of the helices in the four-helix bundle of each monomer. The images in (B) and (C) were obtained from the spatial coordinates of PDB file 2ZW3 (10) with the program JMOL (190).

identical GC boxes (CCGCCC, at -81 and -93), which are the core recognition sequences for transcription factors of the Sp1 family. These motifs are conserved between the human and murine basal promoter sequences, which are 81% identical (7). Electrophoretic mobility shift and supershift assays as well as transactivation experiments demonstrated that the Sp1 and Sp3 transcription factors bind the GC boxes; moreover, *in vitro* engineered mutations that destroy the consensus sequence for the Sp1 family factors in either of the GC boxes drastically reduce transcription from the *GJB2* basal promoter, indicating that these two transcription factors indeed regulate the basal expression of *GJB2* (11). Intriguingly, expression of the *Gjb2* gene in mouse cochlear supporting cells is directly co-regulated with that of the neighbouring *Gjb6* gene by a mechanism dependent on the activation of NF-κB (12). However, the relevance of this finding for the expression of human *GJB2* is uncertain, as the human basal promoter lacks the NF-κB consensus binding site present in the murine basal promoter (11).

Northern blotting, RT-PCR, quantitative RT-PCR and *in situ* hybridization experiments in rodents have shown that *GJB2* expression follows a tissue-specific pattern, with transcripts being detected in the mammary gland, inner ear (cochlea and vestibular organs), brain and hair follicles (13-17). Similar experiments performed with human material detected either *GJB2* transcripts or Cx26 protein in preimplantation embryos (18), myometrium (19), lactating mammary gland (13, 20), corneal epithelium (21),

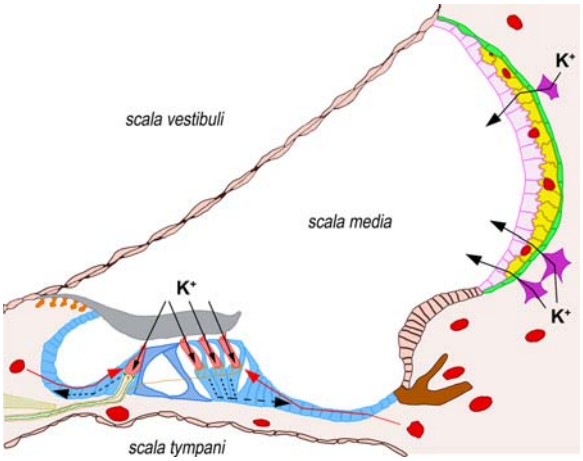

Figure 2. Transverse section of the cochlear duct showing the proposed roles of gap junctions in inner ear physiology. Cx26-containing gap junctions define two networks of coupled cells. The epithelial network comprises all supporting cells from the organ of Corti (blue), interdental cells of the spiral limbus (orange) and root cells in the spiral ligament (brown). The connective tissue network comprises fibrocytes in the spiral ligament (purple), as well as basal (green) and intermediate (yellow) cells in the stria vascularis. In the organ of Corti, K^+ ions and the neurotransmitter glutamate (both excitotoxic), released by hair cells upon sound stimulation, are taken up by supporting cells and dispersed by gap junction-mediated intercellular transport (dashed arrows). In addition, glucose from blood capillaries (red) reaches supporting cells by gap junction-mediated diffusion (red arrows). Secretion of K^+ (black thick arrows) into the endolymph-filled scala media by the stria vascularis depends on gap junction-mediated intercellular transport of K^+ ions from fibrocytes of the spiral ligament to basal and intermediate cells.

skin keratinocytes throughout wound healing (22), sweat glands from normal skin (23) and inner ear structures (24).

Most of the data concerning the localization of Cx26 within the cochlea have been obtained from immunohistochemical experiments performed on mice or rats, because of the scarcity of fresh human cochlear material. Cx26 is one of the two major cochlear connexins, the other being Cx30, both in rodents (14, 16, 17) and humans (24). Both connexins are expressed in the same cochlear structures and co-localize (14, 16). In the adult, Cx26-containing cochlear gap junctions define two independent networks of coupled cells (Figure 2). The epithelial network couples all supporting cells of the organ of Corti and adjacent epithelial cells (25-27). The connective tissue network comprises fibrocytes and mesenchymal cells, and also includes basal and intermediate cells of the stria vascularis (25, 26, 28).

3.3. Function of connexin-26

Experiments performed on cultured cells that synthesize exogenous Cx26 have shown that Cx26 channels may play two different functional roles in tissue homeostasis. Cx26 intercellular gap-junction channels participate in the transfer from cell to cell of most hydrophilic compounds with a molecular mass below 1 kDa (including ions, metabolites and second messengers), down their electrochemical gradient. This kind of intercellular communication allows cells that are coupled by means of Cx26-gap junctions to act as a single functional unit. In addition to this transport role common to nearly all connexins, Cx26 so-called “hemichannels” (i.e. Cx26 connexons located at the plasma membrane that are not docked and thus connect the cytosol and the extracellular medium) participate in paracrine and autocrine signalling through the release or uptake of specific second messengers.

Within the cochlea, auditory transduction critically depends on the maintenance of the composition of endolymph and perilymph, on preserving the functionality of hair cells and on the production of the endocochlear potential. These homeostatic processes require the existence of specialized ion and solute transport mechanisms, in which gap junction networks are believed to be major players. Indeed, nearly all cochlear cells (except hair cells and marginal cells of the stria vascularis) are extensively coupled to their neighbours by numerous gap junctions, whose plaques are among the largest in the body (16, 25). On the basis of data gleaned from the analysis of cochlear organotypic cultures and of mouse models, four different roles for Cx26 channels in cochlear homeostasis have been postulated, three of them in the organ of Corti (spatial buffering of excitotoxic substances, supply of glucose to hair and supporting cells, calcium and ATP signalling in supporting cells) and one in the stria vascularis (transport of potassium ions for secretion into the endolymph) (Figure 2).

(i) Spatial buffering in the organ of Corti. Hair cells become depolarized when K^+ ions flow into them upon sound stimulation. Hair cell repolarization involves K^+ ion efflux through potassium channels at the basolateral domain of the hair cell membrane. K^+ ions are then actively taken up by the supporting cells, what avoids the toxic effects of high extracellular K^+ concentrations. It is believed that gap junctions coupling all supporting cells provide a pathway to disperse the excess K^+ ions and funnel them away from the hair cells (Figure 2). This hypothesis is supported by the fact that deletion of *GJB2* in the epithelial gap junction network (29, 30) results in death of hair cells on the onset of hearing, leading to the degeneration of the organ of Corti. Cx26 gap junctions in supporting cells would also help disperse other excitotoxic substances released by the receptor activity of hair cells, such as the neurotransmitter glutamate (29).

(ii) Gap junction-dependent metabolite transport. Energy consumption in the cells of the organ of Corti increases dramatically during sound transduction because of all the active transport processes that it entails. However, the organ of Corti is an avascular epithelium and, in consequence, hair cells and supporting cells lack a direct access to energy metabolites supplied by blood vessels. It was shown that glucose from the blood reaches the supporting cells by gap junction-mediated diffusion (31) (Figure 2).

(iii) Cx26-mediated Ca^{2+} and ATP signaling in the organ of Corti. Study of inositol 1,4,5-trisphosphate (IP_3) transport in the organ of Corti showed that cochlear Cx26 participated in paracrine signalling (32). IP_3 is a second messenger that causes intracellular release of Ca^{2+} ions and the spreading of Ca^{2+} oscillations to coupled supporting cells via gap junctions; these signaling processes are probably involved in the coordination of the activity of supporting cells, for instance to accomplish effective spatial buffering of K^+ ions. Further characterization of this signaling route led to the identification of Cx26 hemichannel activity in supporting cells. Such hemichannels mediate the release of ATP, modulated by Ca^{2+} ions, to organ of Corti fluids. Extracellular binding of ATP by the purinergic P2X receptors of supporting cells elicits intracellular IP_3 release and the propagation of a Ca^{2+} oscillation among coupled cells (33). In addition, ATP signaling through P2X receptors on outer hair cells modulates their electromotility, which regulates auditory transduction (34).

(iv) Transport of K^+ ions within the stria vascularis for their secretion into endolymph. The stria vascularis generates the endocochlear potential and actively secretes K^+ ions from the perilymph into the endolymph. The stria vascularis consists of two distinct epithelial cell layers (basal and marginal) that surround an extracellular space termed intrastrial space. Intermediate cells and capillaries are located in the intrastrial space. K^+ ions are believed to be transferred to basal cells and intermediate cells, with the help of Cx26-containing gap junctions coupling these two cell types to fibrocytes within the connective tissue network (Figure 2). Other specialized transport mechanisms subsequently secrete K^+ ions to the intrastrial space (which generates the endocochlear potential) and transfer them through the marginal cell layer into the endolymph (35-38).

4. MUTATIONS IN THE CODING REGION OF *GJB2*

Since the identification of the first DFNB1 pathogenic mutations, p.Trp24X and p.Trp77X (6), more than 100 sequence variants in the coding region of *GJB2* have been reported. Most of these variants are responsible for DFNB1 hearing impairment, but mutations causing DFNA3 autosomal dominant non-syndromic HI or various skin disorders with or without HI have also been described. The sequence variants include nucleotide substitutions (nonsense, missense or silent mutations), and short insertions, duplications or deletions (either in-frame or causing frameshifts that lead to premature stop codons). It is not the purpose of this review to provide an exhaustive list of *GJB2* variants that can be found in electronic databases (39). Instead, we focus our attention on the effects that the different classes of mutations causing DFNB1 HI exert on Cx26 function.

Given that the complete coding sequence of *GJB2* is contained within the last exon of the gene, mutations generating premature stop codons are expected to escape the nonsense-mediated mRNA decay pathway (40, 41) and so truncated Cx26 proteins should actually be synthesized. This has been demonstrated for the c.235delC (p.Leu79CysfsX3) mutation, i.e. the truncated polypeptide was visualized by immunocytochemical methods in HeLa cells transiently transfected with a *GJB2*-c.235delC expression plasmid (42). Nevertheless, most of these truncated proteins lack one or several of the transmembrane segments, what is expected to have deleterious effects on protein folding, oligomerization, trafficking and localisation, and ultimately result in a complete loss of function.

In contrast, it is difficult to establish the pathogenic potential of *GJB2* sequence variants not producing truncated proteins. Diverse assays have been developed to probe their effects on different aspects of gene or protein functions, such as transcription, translation, membrane insertion, oligomerization, intracellular traffic and plasma membrane targeting, and connexin-dependent intercellular communication (43, 44). These assays are robust and provide very helpful insights into Cx26 function, but they have two crucial shortcomings. The first one is that most of the techniques used are performed on cultured cells transiently or stably transfected with plasmids encoding the mutant Cx26 of interest. Thus, the *in vitro* or *ex vivo* conditions of the assay may not completely mimic the situation in an affected subject. The second shortcoming is specific to the assays for Cx26-dependent intercellular communication. They only probe some of the transport processes of Cx26 (e.g. transfer of fluorescent dyes, ion conductance) and, as Cx26 channels are permeable to many different compounds, the effects of some mutations on the transfer of specific substrates that are critical for the mechanism of hearing may be overlooked.

Moreover, experience shows that it is essential to combine genetic data obtained from sufficient numbers of affected subjects and ethnically-matched normal-hearing controls with results from functional assays, in order to conclude about the pathogenic potential of a non-truncating sequence variant. The cases of mutations p.Met34Thr and p.Val84Leu are illustrative. Mutation p.Met34Thr was originally described as a dominant negative DFNA3 allele on the basis of genetic data obtained from very few patients combined with the results of a single type of permeability assay (6, 45). Thereafter, genetic data from analyses of normal-hearing controls and of larger series of patients (46) established that p.Met34Thr was a hypomorphic recessive allele, as it was finally confirmed by more extensive testing with different types of functional assays (47-50). Conversely, results of assaying the wild-type Cx26 and the p.Val84Leu mutant protein in several model systems were indistinguishable (51), though genetic data supported that p.Val84Leu was a loss-of-function mutation (52). Eventually, experiments performed on cochlear organotypic cultures showed that the p.Val84Leu mutation impaired Cx26-dependent transfer of the second messenger IP_3 between supporting cells of the organ of Corti, providing an explanation for its pathogenic effect (32).

Non-truncating sequence variants have been described in nearly all the structural elements of Cx26, with

Table 1. Missense variants affecting residues that are critical for the structure of Cx26

Mutations affecting residues that participate in intra-monomer interactions				
Residue	**Localization**	**Mutation**	**Comments**	**References**
Arg-32	TM1	p.Arg32His		102
		p.Arg32Leu		39
		p.Arg32Cys		55
Ala-40	TM1	p.Ala40Glu		39
		p.Ala40Gly		39
Trp-77	TM2	p.Trp77Arg	Impaired oligomerization, intracellular retention, impaired electrical conductance and dye transfer.	47, 51, 53
Gln-80	TM2	p.Gln80Arg		101
		p.Gln80Leu		113
		p.Gln80Pro		39
Ser-139	TM3	p.Ser139Asn		156
Arg-143	TM3	p.Arg143Trp	Normal electrical conductance and dye transfer in N2A cells. Loss of electrical conductance in *Xenopus* oocytes.	133, 181, 182
Glu-147	TM3	p.Glu147Lys		183
Ser-199	TM4	p.Ser199Phe		68
Asn-206	TM4	p.Asn206Ser	Reduced electrical conductance, altered voltage gating.	104, 182
Mutations affecting residues that participate in inter-monomer interactions				
Residue	**Localization**	**Mutation**	**Comments**	**References**
Glu-47	E1 loop	p.Glu47Lys		55
Arg-184	E2 loop	p.Arg184Pro	Unable to oligomerize, probably quickly degraded	48, 49, 57, 184
		p.Arg184Trp		56
Mutations affecting pore-lining residues				
Residue	**Localization**	**Mutation**	**Comments**	**References**
Gly-12	N-ter	p.Gly12Val	Intracellular retention	48, 58
Asn-14	N-ter	p. Asn14Asp	Impaired plasma membrane targeting	185
Met-34	TM1	p.Met34Thr	Loss of electrical conductance, impaired dye transfer	6, 45, 47-49
Val-37	TM1	p.Val37Ile	Loss of electrical conductance	51, 52
Lys-41	TM1	p.Lys41Arg	Found just once in homozygosity in one deaf subject. Originally reported as a harmless polymorphism.	60
Gly-45	E1 loop	p.Gly45Glu	Reported in compound heterozygosity with several fully inactivating mutations in nonsyndromic deafness. Found as a *de novo* mutation in heterozygosity in lethal cases of keratitis-ichthyosis-deafness syndrome. It forms hemichannels with aberrant gating properties.	64, 129, 130, 186-189
Mutations that kink the TM2 helix				
Residue	**Localization**	**Mutation**	**Comments**	**References**
Leu-79	TM2	p.Leu79Pro		59
Ser-85	TM2	p.Ser85Pro		60
Leu-90	TM2	p.Leu90Pro	Impaired oligomerization, loss of electrical conductance and dye transfer	48, 49, 51, 61

the exception of the very short C-terminus. Many of those that are pathogenic affect residues that are involved in connexon stabilization (through intra- and inter-monomer interactions) or that line the channel pore (Table 1).

There are four groups of residues that participate in intra-monomer interactions to stabilize the Cx26 four-helix bundle (10). Two of these groups are hydrophobic cores clustered around residues Trp-44 (with Ala-39, Ala-40, Val-43 and Ile-74) and Trp-77 (with Phe-154 and Met-195). Known variants affecting these residues are pathogenic (Table 1). The two other groups are clustered around arginine residues Arg-32 (which forms bonds with Gln-80, Glu-147 and Ser-199) and Arg-143 (which forms bonds with Ser-139 and Asn-206). Pathogenic mutations affecting every residue in these two groups have already been reported (Table 1). It is considered that mutations affecting residues in any of these four groups disrupt intra-monomer stabilizing interactions, which may result in impaired folding and/or oligomerization, in turn leading to the intracellular retention of the mutant proteins. This has been demonstrated by functional assays of the p.Trp77Arg mutation (47, 51, 53).

Residues involved in inter-monomer contacts are located in extracellular loops E1 (Asp-46, Glu-47, Gln-48, Asp-50, Asn-62, Tyr-65, Asp-66, Ser-72) or E2 (Arg-184), with the exceptions of Arg-75 (in TM2), Thr-186 and Glu-187 (both in TM4). Most of the *GJB2* mutations causing dominantly inherited nonsyndromic deafness and/or skin disorders affect such residues (54), as could be expected because of their role in interactions between monomers. However, some mutations causing ARNSHI also affect residues in this category, such as p.Glu47Lys (55), p.Arg184Trp (56) and p.Arg184Pro (57). It is considered that these mutations would also impair folding and/or oligomerization. Indeed, p.Arg184Pro-Cx26 is unable to oligomerize (49) and it is never detected by immunochemical methods when expressed in HeLa cells (48, 49), suggesting that it is quickly degraded, perhaps due to misfolding.

Mutations in pore-lining residues at TM1 helix or E1 loop affect the permeability properties of the channel, altering its electrical conductance, charge selectivity or gating properties (Table 1). Two hypomorphic recessive mutations affect pore-lining residues in TM1: p.Met34Thr and p.Val37Ile (52). Both of them cause the loss of electrical conductance of the Cx26 gap junction channel when assayed in *Xenopus* oocytes (45, 51). In addition, p.Met34Thr blocks (47, 48) or significantly reduces (49) the ability of the Cx26 gap junction channel to transfer

dyes. Cx26 channel permeability should also be altered by mutations targeting pore-lining residues in the N-terminus, such as p.Gly12Val (58). This does not happen, however, because p.Gly12Val-Cx26 is retained intracellularly (48), probably due to a membrane insertion defect that is also apparent in other mutations that affect N-terminal residues, like p.Ser19Thr (48, 58).

Some residues not participating in the interactions described above are also affected by pathogenic changes. Three mutations that substitute proline for different residues in TM2, p.Leu79Pro (59), p.Ser85Pro (60) and p. Leu90Pro (61), kink the helix and presumably evoke structural changes in the cytoplasmic domains of the Cx26 monomer (10), which may interfere with oligomerization or with the permeability properties of the channel. This has been confirmed in the case of p.Leu90Pro, which slightly impairs oligomerization, causes total loss of electrical conductance and blocks transfer of dyes when tested in functional assays (48, 49, 51). Also, several non-truncating variants affect residues in the cytoplasmic loop. Since the structure of this domain could not be determined, it is difficult to identify residues that are critical for the function of Cx26, and thus classify the sequence variants that are found in this region. However, at least two of them, p.Ser113Arg (52) and p.Glu120del (62), cause total loss of electrical conductance of the Cx26 channel (51) and they are clearly pathogenic.

In contrast, other missense variants do not affect residues critical for the structure of Cx26, and the currently available genetic data indicate that they are harmless polymorphisms. This group includes p.Val27Ile (52), p.Phe83Leu (63), p.Glu114Gly (64), p.Arg127His (65), p.Val153Ile (66), p.Gly160Ser (63) and p.Ile203Thr (67). Some of these sequence variants are very common in specific populations.

5. MUTATIONS AFFECTING NON-CODING PARTS OF THE GENE OR REGULATORY ELEMENTS

As mutation screening of the *GJB2* gene in subjects with ARNSHI became a general practice, it was noticed that in a significant fraction (10-42 %) of affected subjects with mutations in the *GJB2* coding region, only one mutant allele could be found, the expected accompanying mutation remaining unidentified. Part of these unexplained cases may be attributable to intrinsic drawbacks of the techniques for mutation detection, or they could represent just coincidental carriers of the most frequent *GJB2* mutations, their HI having a different cause. However, it was long suspected that the missing mutations might be located in the non-coding parts of the gene, in regulatory elements acting at a distance, or in other genes.

5.1. Splice-site mutations

Only one splice-site mutation has been reported to date in *GJB2* (61, 68). It affects the donor splice site of intron 1 (c.-23+1G>A or IVS1+1G>A, sometimes incorrectly referred to as -3170G>A). Since the coding region of *GJB2* is fully contained in exon 2, this splice-site mutation has no effect on protein coding. On the contrary, sequencing of cDNA from a lymphoblastoid cell line that was generated from a heterozygous subject detected no transcript from the c.-23+1G>A allele, suggesting that it was not transcribed or that the transcript was extremely unstable (69). This mutation is the most frequent *GJB2* pathogenic sequence variant in Mongolia (it accounts for about 50% of the pathogenic changes) (117), and it is relatively frequent in the Czech, Polish, Turkish, Kurdish and Chinese populations, being found in a significant fraction of cases with a single mutation in the coding region, and accounting for about 2-9.4 % of all DFNB1 alleles (70-73, 191).

5.2. Promoter mutations

A mutation affecting the *GJB2* basal promoter was reported in a Portuguese subject with non-syndromic HI, who was a compound heterozygote, the accompanying mutation being p.Val84Met (74). The mutation was originally named -3438C>T, although according to the standard rules it should be renamed as g.-77C>T. It alters the fifth nucleotide of the GC box located at position -81 (CCGCCC > CCGCTC) (11), and so it is expected to impair the binding of the Sp1 and Sp3 transcription factors. Reporter-gene analysis performed in different cell lines demonstrated that the activity of the mutant promoter activity decreased dramatically or was abolished (74).

No further subjects with the g.-77C>T mutation have been reported to date, but data from extensive screenings are still scarce (75).

5.3. Large deletions

Four different large deletions have been reported to date in the DFNB1 locus (Table 2, Figure 3). They can be classified into three classes according to the genes that are involved.

The first class is constituted by large deletions encompassing the *GJB2* gene. Only one deletion belonging to this class has been reported to date (76). It was found in the compound heterozygous state with a p.V84M mutant alelle of *GJB2*, in a French subject with non-syndromic, prelingual profound HI. The proximal limit of the deletion was not established, but the deleted segment spans at least 920 kb and removes many genes of the 13q11-q12 chromosomal region, including *GJB2* and *GJB6* (encoding connexin-30). The distal breakpoint is located in intron 2 of the *CRYL1* gene, which codes for lambda-crystallin (76) (Figure 3). This deletion seems to be a private mutation, as it has not been reported in any other unrelated subject. Detection of this class of deletions is facilitated by the fact that they result in false homozygosity for the accompanying *GJB2* point mutation in the affected subject, what can be noticed by studying its segregation in the pedigree.

The second class of deletions is constituted by those truncating *GJB6*, but not encompassing the *GJB2* gene. The *GJB6* gene has six exons (77), the coding region being fully contained in the last one. Several research groups reported large deletions affecting the *GJB6* coding region in deaf subjects carrying only one mutant *GJB2* allele (78-80). In one of these studies (79), the deletion

Table 2. Large deletions at the DFNB1 locus on 13q12

Abbreviated name	Name including exact coordinates[1]	Size of deleted interval	Reference
del(*GJB6*-D13S1830)	Chr13:g.(20,797,177_21,105,945)del (NCBI Build 37.2)	309 kb	79
del(*GJB6*-D13S1854)	Chr13:g.(20,802,727_21,034,768)del (NCBI Build 37.2)	232 kb	82
del(131-kb)	Chr13:g.(20,939,344_21,070,698)del (NCBI Build 37.2)	131 kb	90
del(920-kb)	Not applicable	>920 kb	76

[1] First and last nucleotide that are eliminated by the deletion

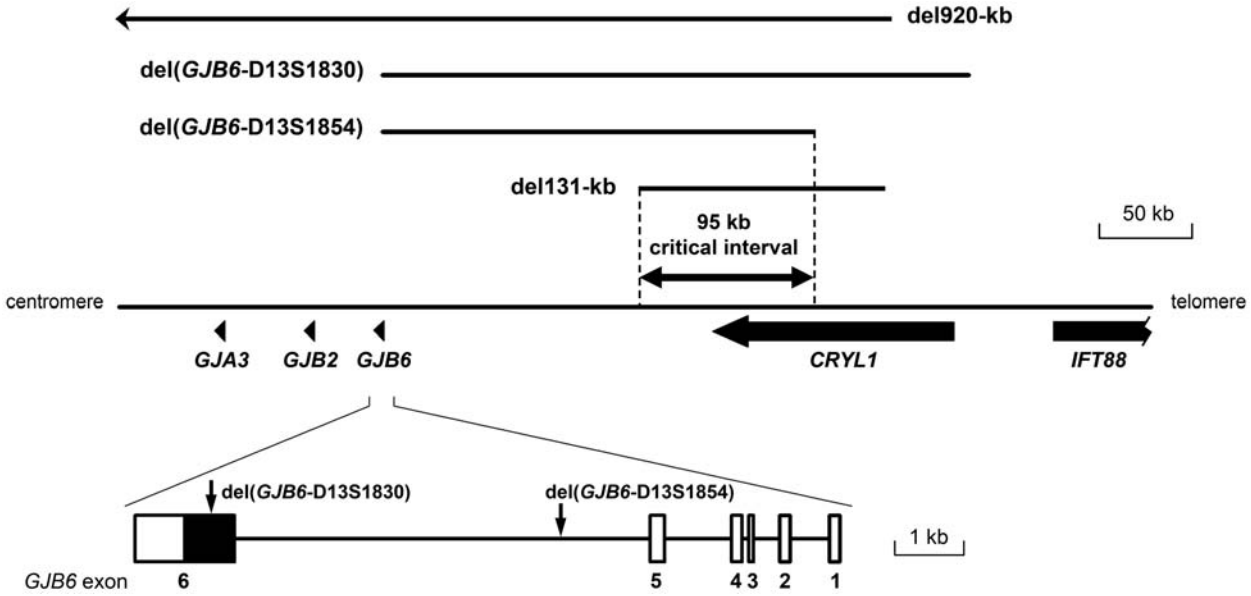

Figure 3. Physical map of the region on chromosome 13q12 around the *GJB2* gene, showing the sequences that are lost in each of the four deletions responsible for DFNB1 HI. The centromeric breakpoint of del920-kb lies outside the region shown in the map. The limits of the 95.4-kb interval that must contain the *cis*-acting regulatory element controlling the expression of *GJB2* are the centromeric breakpoint of del131-kb and the telomeric breakpoint of del(*GJB6*-D13S1854). Note that the centromeric breakpoints of deletions del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) are located inside the *GJB6* gene (vertical arrows, bottom).

breakpoint junction was isolated and sequenced. The proximal breakpoint lies within the coding region in *GJB6* exon 6 and the distal breakpoint lies within the intergenic region between *CRYL1* and *IFT88* (intraflagellar transport 88 homolog), so truncating the *GJB6* gene (the first five exons are eliminated) and removing the whole *CRYL1* gene (Table 2, Figure 3). The analysis of the breakpoint junction suggested that this deletion most likely arose through a mechanism of illegitimate recombination. The size of the deletion interval was initially estimated to be 342 kb, according to the contemporary human sequence draft (79). Further investigation revealed that the deletions found by the three research groups (78-80) were the same one (81), which was named del(*GJB6*-D13S1830). Subsequent corrections of the human sequence draft established definitely its size in 309 kb (Table 2). The del(*GJB6*-D13S1830) mutation is a common allele in many European countries, its frequency peaking in Western Europe (Spain, France, United Kingdom) and decreasing towards the east. It has been reported in populations of European descent in America (United States, Brazil) and in Australia, and also in Ashkenazi Jews. On the contrary, it has not been reported to date in Asian or African populations. Although the finding of the del(*GJB6*-D13S1830) mutation provided an explanation for the hearing impairment in as many as 30%-70% of unelucidated *GJB2* heterozygotes in some populations, it became evident that other DFNB1 mutations remained to be identified. Few years later, another deletion of the same class, del(*GJB6*-D13S1854), was detected, and its breakpoint junction was isolated and characterized (82). This deletion spans 232 kb and it was originated by homologous recombination between two Alu sequences, the proximal repeat being located in *GJB6* intron 5 (so removing the first five exons), and the distal repeat in *CRYL1* intron 4 (Table 2, Figure 3). It is a relatively common DFNB1 allele in some populations (Spain, United Kingdom, Brazil), but rare or absent in most populations. The detection of the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) mutations for diagnostic purposes is facilitated by a multiplex PCR assay that amplifies the DNA segments containing the breakpoint junctions (82).

These findings could be interpreted on the basis of a digenic pattern of inheritance of mutations in *GJB2* and *GJB6*. This hypothesis was supported by several facts: i) Both Cx26 and Cx30 are expressed in the same cochlear structures and co-localize (14, 16); ii) Connexons composed of Cx26 can bind connexons composed of Cx30 to form heterotypic gap-junction channels (83); iii) A mutation in *GJB6* was reported in a case of autosomal

dominant hearing impairment (84); iv) Cx30-deficient mice lack the endocochlear potential and exhibit a severe constitutive hearing impairment (85). However, the fact that point mutations in *GJB6* have not been found to date in cases of ARNSHI in humans, argues against this hypothesis. In addition, $Cx26^{+/-}/Cx30^{+/-}$ double heterozygous mice have only a moderate hearing impairment (86), in contrast with the phenotype observed in humans, where a majority of double heterozygotes for del(*GJB6*-D13S1830) and a *GJB2* mutation have severe or profound hearing impairment (87). An alternative hypothesis postulated the existence of a *cis*-acting regulatory element that would activate the expression of *GJB2* in the inner ear. This regulatory element would have been removed by the deletions, and its absence would have dramatic effects on the expression of *GJB2*, to the point that an otherwise normal allele would behave as a null allele. In fact, immunohistochemical analysis of a skin biopsy from a subject who was double heterozygous for c.35delG in *GJB2* and del(*GJB6*-D13S1830) revealed that the expression of Cx26 was dramatically reduced in the ductal epithelium of sweat glands (23). Another study, using qualitative allele-specific RT-PCR to assess the relative abundance of the *GJB2* transcript in buccal epithelium cells from three double heterozygous subjects (del(*GJB6*-D13S1830)/*GJB2* mutation), demonstrated no detectable expression from the wild-type *GJB2* allele that was in *cis* with the deletion (88).

Definitive evidence supporting the hypothesis of the cis-acting regulatory element has recently been provided by the detection and characterization of the first member of the third class of DFNB1 deletions, i.e. those encompassing neither *GJB2* nor *GJB6* (89, 90). The size of the deletion interval was estimated to be 131 kb, with the proximal breakpoint in the intergenic region between *GJB6* and *CRYL1*, more than 100 kb upstream of the transcriptional start sites of *GJB2* and *GJB6* (90) (Table 2, Figure 3). It was found in several affected subjects of the same family, who were double heterozygous for the deletion and the c.35delG *GJB2* mutation. In buccal epithelium cells from these subjects, qualitative allele-specific RT-PCR revealed that the expression from the wild-type *GJB2* and *GJB6* alleles in *cis* with the deletion was dramatically reduced. These results not only support the existence of the *cis*-acting regulatory element, but they also suggest that it could be involved in *GJB2* and *GJB6* co-regulation. If this were the case, the removal of *GJB6* by the deletions of the second class could contribute to worsen the phenotype of HI of affected subjects, as it has been shown for del(*GJB6*-D13S1830) (see below, section 7.2). Overlapping of all deletion intervals that have been characterized to date defines a 95.4 kb interval that must contain the regulatory element, whose localization could be refined by molecular characterization of other novel DNA rearrangements in the DFNB1 locus leading to hearing impairment.

6. GENETIC EPIDEMIOLOGY OF DFNB1 HEARING IMPAIRMENT

Progress in the genetic epidemiology of ARNSHI is complicated by the large heterogeneity of these disorders and by the variability among different populations as regards the prevalence of each genetic subtype and the diversity and relative frequency of mutations in the different genes. A large part of this variability is due to diverse founder effects for specific mutations and to the abundance of private mutations. Among the many different subtypes of ARNSHI, DFNB1 is remarkable for being highly prevalent in most of the populations that have been tested. Consequently, a plethora of studies have provided epidemiological data for populations all over the world.

It is important to note that there are many differences between the diverse studies as regards issues that are critical for a proper comparison. These include: i) Differences in the clinical criteria for inclusion/exclusion of affected subjects in the cohorts under study. Special attention should be paid to whether results of a given study refer to prelingual non-syndromic HI, childhood non-syndromic HI (including prelingual and postlingual forms), or just ARNSHI. Although it is true that most ARNSHI corresponds to prelingual HI, these are not equivalent categories. Attention should also be paid to possible biases in selection as regards the severity of the HI; ii) Differences in the proportion of multiplex (familial) or simplex (sporadic) cases in the cohorts under study. Given that the frequencies of DFNB1 HI that are observed in multiplex cases are usually significantly higher than those observed in sporadic cases, it is advisable to present these data separately, not combining them in a single value, to facilitate accurate comparison between populations; iii) Differences in the criteria for classifying a case as DFNB1. Only cases with two DFNB1 mutant alleles (the so-called biallelic cases, i.e. confirmed DFNB1 cases) should be counted up. Cases heterozygous for a single pathogenic mutation (so-called monoallelic), who could be just coincidental carriers, their HI being caused by mutations in other genes, should not be added up to the confirmed DFNB1 cases. Since this information is also valuable, it should be provided as a separate number; iv) Differences in the genotyping methods (direct sequencing or previous screening by mutation detection techniques with different detection rates) and in how comprehensive was the screening, i.e. whether only the *GJB2* coding region was examined or whether mutations affecting non-coding parts of the gene and large deletions were also investigated. It should be noted that these differences might have a strong impact on the number of cases with a single pathogenic mutation, being a source of error when estimating the contribution of DFNB1 to ARNSHI; v) Differences in the consideration of specific alleles as pathogenic or harmless. Relative frequencies of DFNB1 pathogenic alleles are consequently biased; vi) Differences in the ethnic composition of the cohorts from a same country; and vii) Differences in the size of the cohorts under study. In spite of all these issues, which make that most of the studies are not strictly comparable, several conclusions have emerged.

The high prevalence of DFNB1 among ARNSHI was early observed (56, 91). Subsequent studies indicated that it is a major cause of ARNSHI in many populations. DFNB1 HI is highly frequent (20-40% of all cases with ARNSHI) in Europe (58, 61, 92-99), in the Middle East (69, 100-102), and in Argentina (103). It is less frequent (12-22%), but still a major cause of ARNSHI, in USA (104-107), Brazil (108, 109), East Asia (110, 111), and Australia

(57, 112). In Central Asia, moderate frequencies (about 15%) are observed in Iran and India (113-115), whereas low frequencies are reported in Pakistan (6%) (116) and Mongolia (4.5 %) (117). It seems to be rare in Indonesia (118) and it was not found in the Omani population (data from a small cohort of affected subjects) (119).

The spectra of DFNB1 mutations also vary across the different populations, as regards the list of the sequence variants and their relative frequencies. Many different mutations in the DFNB1 locus have been found repeatedly in diverse populations (39), but few of them predominate, as indicated below.

The c.35delG mutation was early shown to be highly frequent in Caucasians (56, 91). It accounts for 42-88% of mutant *GJB2* alleles found in subjects with DFNB1 HI in Caucasian populations from Europe, America, and Australia (57, 58, 61, 92-99, 103-109, 112). Its carrier frequency in the general population ranges from about 1% in Northern and Central Europe to 2-3% in the European Mediterranean countries (120). It was initially hypothesized that the stretch of six guanosine nucleotides that is affected by c.35delG might represent a hot spot for mutation, what would imply recurrency and multiple origins (52, 121). However, further studies revealed a common founder (122-126). The mutation seems to trace its origins to the Eastern Mediterranean region or to the Middle East. A Greek origin has been postulated (127, 128).

The c.235delC mutation was first identified in a cohort of Japanese affected subjects (64). Later on it was shown to be frequent in East Asian populations, accounting for 34-70 % of mutant *GJB2* alleles found in subjects with DFNB1 HI (60, 110, 111, 129, 130), carrier frequencies in the general population being 1-2% (60, 129-131). A common founder is responsible for this high frequency (130, 132).

The p.Arg143Trp mutation was first reported in families from a small village in Ghana, West Africa (133), and later on it was shown to be highly frequent (91% of *GJB2* pathogenic alleles) in a cohort of affected subjects from all the country (59). This high frequency might be related to a putative selective advantage provided by the mutation. A study on skin biopsies from homozygotes, heterozygotes and wild-type subjects from the same family, showed that the epidermis was significantly thicker in homozygotes and heterozygotes for p.Arg143Trp. Moreover, sodium and chloride concentrations in sweat were higher in homozygotes (134). These findings were postulated to provide a more robust mechanical skin barrier to trauma and insect bites, and an unfavourable osmotic milieu for microbial colonization, as demonstrated for the enteric pathogen *Shigella flexneri* (134, 135). However, this high frequency is likely not to be extrapolatable to all Subsaharan Africa, since the mutation was not found in a combined cohort of subjects from Kenya and Sudan (136).

The c.167delT mutation was early identified (91), and subsequently it was shown to be highly frequent in Ashkenazi Jews because of sharing a common founder (121, 137). It accounts for 76% of *GJB2* pathogenic alleles (138), and its carrier rate in the general population has been estimated to be 2.8-7.5% (121, 137-139).

The p.Trp24X mutation was first described in a Pakistani family (6). Subsequent studies demonstrated that it is the predominating *GJB2* allele in India (83-91% of the pathogenic DFNB1 alleles) (114, 115) and also in the European Romani (Gypsy) populations (64-93% of the pathogenic DFNB1 alleles) (96, 140, 141). Its carrier rate in India was estimated to be 2.4% (115), and it is consistently found in the 4%-5% range in the Romani populations, although it is highly variable among cohorts (140-142). These results place p.Trp24X among the three most common founder mutations in the Gypsies, and classify them as one of the high-risk populations for prelingual deafness (142). Common founders have been demonstrated separately for the Indian and Romani populations (115, 141), but it is very likely that the origin of the mutation took place in the Indian subcontinent, where the Romani people was originally settled before moving in successive migrations towards the European countries.

7. GENOTYPE-PHENOTYPE CORRELATIONS

Early studies on genotype-phenotype correlations in the DFNB1 subtype of ARNSHI concluded that it was prelingual, with a great variability in severity, which ranged from mild to profound even for equal mutant genotypes (61, 143). Audiogram shapes were generally flat or sloping, but they were not pathognomonic (61). The HI was stable or slightly progressive, and no radiologically detectable inner ear malformations were observed (61, 143). Further studies have essentially confirmed these conclusions (87, 97-99, 105, 107, 144-151), but they have also provided insight into some specific issues.

7.1. Age of onset and evolution of the hearing impairment

DFNB1 HI is mostly prelingual (only a few specific mutations may result in later onset; see below), but evidence is accumulating against being mostly congenital. A study reported nine children with two DFNB1 mutant alleles who passed the newborn audiologic hearing screening and were diagnosed with HI later on (152). In another study on 47 children with profound HI and two *GJB2* mutations, 55% of them had good auditory capacity at 3 months of age, which worsened suddenly later on (153). In fact, it has been postulated that the onset of DFNB1 prelingual non-congenital HI could be followed by a rapidly progressive hearing loss (154). A sudden late onset followed by rapid progression of HI was also documented in an 8-year-old boy with a c.35delG/c.299-300delAT genotype (151). Furthermore, another boy who was homozygous for c.35delG and had postlingual HI (onset at age 8 years), experienced a sudden worsening of his HI at age 23 years (155). Given the high prevalence of DFNB1 HI, these issues are of concern to the protocols that try to anticipate the clinical diagnosis of HI.

7.2. Severity of the hearing impairment

In a cross-sectional analysis of DFNB1 genotype and audiometric data from over 1,500 affected subjects

from 16 countries, mutations were classified as truncating (those resulting in premature stop codons, and also del(*GJB6*-D13S1830) and c.-23+1G>A) or non-truncating (missense mutations and in-frame deletions). As expected, variability in the severity of the HI was observed even for the same genotype (e.g. homozygous c.35delG). But interestingly, the degree of HI associated with the presence of two truncating mutations was shown to be significantly more severe than that of the HI associated with two non-truncating mutations (87). The severity of HI associated with truncating/non-truncating genotypes was intermediate between those two groups. Furthermore, some genotypes were consistently associated with specific degrees of severity. Genotypes c.35delG/p.Arg143Trp and c.35delG/del(*GJB6*-D13S1830) usually resulted in profound HI (87). In contrast, genotypes including p.Leu90Pro, p.Met34Thr, or p.Val37Ile with a truncating mutation in *trans* were associated with mild or moderate HI (86, 144). Further studies in smaller cohorts have confirmed these conclusions (97, 99, 107, 145, 150, 151).

The pathogenic potential of p.Met34Thr and p.Val37Ile has been a controversial issue for years (46, 52, 67, 156, 157). Both affect pore-lining residues in TM1, both have high carrier rates in specific populations, and both have been reported in subjects with HI and in subjects with normal hearing, either in the homozygous state or in the compound heterozygous state with clearly pathogenic mutations. Taking all available data together, these two alleles must be classified as hypomorphic, i.e. with low penetrance and weak pathogenic potential. The associated phenotype is expected to range from normal hearing to late onset, progressive, mild to moderate hearing loss (71, 111, 158-162).

7.3. Modifier genes

The wide range in the degree of severity that is observed in DFNB1 HI has been attributed at least partly to the influence of modifier genes. To investigate this issue, a whole-genome association study was performed on c.35delG homozygotes, by comparing the genotypes of mild/moderate cases and profound cases. The first analysis consisted in a pooling-based whole-genome association study of 255 samples. The top 250 most significantly associated SNPs were genotyped individually in the same sample set. A total of 192 SNPs still had significant P-values. These were then genotyped in a second independent set of 297 samples for replication. Significant P-values were replicated in nine SNPs. These results suggest that the variability in the degree of severity observed in c.35delG homozygotes cannot be explained by the effect of one major modifier gene. Each one of those nine SNPs might contribute with just a small modifying effect on the phenotype (163).

7.4. Inner ear malformations

The existence of putative inner ear malformations associated with DFNB1 HI has been investigated by means of high-resolution computed tomography (CT) scans of temporal bones of affected subjects. In an early study, no temporal bone anomalies were found in a series of 23 subjects with biallelic *GJB2* mutations (61). Most of the subsequent studies coincide in establishing that the prevalence of temporal bone malformations in DFNB1 HI subjects would be typically lower than 10% (104, 164-166). Only one study contradicts this view, by reporting a prevalence of temporal bone anomalies of 72% in a cohort of subjects with DFNB1 HI. These anomalies included dilated endolymphatic fossa, hypoplastic modiolus, enlarged vestibular aqueduct, or hypoplastic cochlea (167). This discrepancy might be due to differences in methodology (thickness of the CT slices, measurement techniques) and in the composition of the cohorts of subjects as regards the severity of the HI (168). Further research is needed to determine whether subtle anomalies are being missed by conventional methods or whether a too high rate of detection is attributable to a technical artifact.

7.5. Temporal bone histopathology

In contrast to the wealth of audiological data obtained from subjects with *GJB2* mutations, very little is known on the histopathological processes that occur in DFNB1 HI. In the single report published to date (169), the archives of a repository of temporal bones were examined in search of samples obtained from subjects with congenital, severe-to-profound or profound HI. Five temporal bones of subjects meeting those characteristics were screened for *GJB2* mutations and subjected to histological analysis. Only one of the subjects, a compound heterozygote for c.35delG and p.Glu101Gly, had DFNB1 HI. Microscopic analysis of cochlear sections from this subject showed almost total degeneration of hair cells in the organ of Corti, a detached tectorial membrane, and agenesis of the stria vascularis with formation of a large cyst in the region where the stria should have been. Notably, neural degeneration was not observed, as both the spiral ganglion cell population and the eighth cranial nerve were normal. These alterations are in good agreement with the known sites of *GJB2* expression within the cochlea, but the subject had suffered from other pathologies (diabetes mellitus, hypertension, chronic renal failure and coronary atherosclerosis) that might have influenced the degenerative process.

7.6. Vestibular function

Vestibular function of subjects with DFNB1 HI is not routinely explored, because they do not complain of vertigo or dizziness. However, a study performed on two subjects with two pathogenic *GJB2* mutations revealed pathological recordings of vestibular-evoked myogenic potentials, which would suggest a saccular dysfunction. No abnormalities in the function of utricle and semicircular canals were observed. These subjects did not complain of vertigo or dizziness, possibly because of central compensation (170). In another study performed on seven subjects with two pathogenic *GJB2* mutations, vestibular-evoked myogenic potentials revealed unilaterally abnormal responses in three subjects, the four other showing normal responses (171). Investigation of larger series of affected subjects is clearly needed to reach conclusions on this issue.

7.7. Audiologic phenotype of carriers

As expected for a recessive trait, heterozygous carriers of DFNB1 mutations have apparently normal

hearing. However, subtle audiological alterations might be present. In studies performed on heterozygous carriers for c.35delG, p.Trp77Arg or p.Val37Ile from the same Israeli Arab village in Galilee, audiograms and auditory brainstem responses were normal. However, when testing distortion-product oto-acoustic emissions, responses from carriers had significantly lower amplitudes than those of non-carrier controls, a result that would suggest that *GJB2* mutations affect primarily the outer hair cells (172, 173). In another study, audiograms from c.35delG heterozygous carriers showed mild hearing losses at the high frequencies (6 and 8 kHz) (174). Confirmation of these conclusions requires further research of larger series of carriers and more mutations.

7.8. Outcome of cochlear implantation

The outcome of cochlear implantation has been examined in subjects with DFNB1 HI in comparison with subjects having non-DFNB1 HI. Several studies showed a clear improvement in speech perception skills after implantation for the two groups, without significant differences between them (175-178). On the contrary, reading performance of subjects with DFNB1 HI was consistently better (175, 176).

8. ANIMAL MODELS

To fill the gap in our understanding of the histopathological processes that occur in DFNB1 HI, researchers resorted to the generation of a mouse model of the disease. Unfortunately, mice homozygous for a targeted inactivation of *Gjb2* die in mid-gestation due to a defect in placental transport of nutrients, what precluded any analysis of cochlear structure or auditory function (179). Conditional knockouts have been thus used in an attempt to replicate the consequences of Cx26 deficiency on the inner ear. However, it has been technically difficult so far to obtain a conditional model in which Cx26 is homogeneously ablated in all the cochlear structures that express *GJB2*.

The available models use Cre-*loxP* conditional technology to achieve deletion by homologous recombination of the complete coding region of *Gjb2* in the *Gjb2*$^{loxP/loxP}$ mouse line (29). This line has been used in studies that followed different approaches to achieve *Gjb2* deletion: either cell-type specific (*Otog-Cre*, *Foxg1-Cre*, *Pax2-Cre*) (29, 180) or time-specific (R26Cre-ER^{T}; deletion is activated by a dose of tamoxifen) (30). In *Otog-Cre Gjb2*$^{loxP/loxP}$ mice, *Gjb2* deletion was restricted to the organ of Corti (the epithelial gap-junction network) (29). In those mice, the organ of Corti developed normally, but it suddenly degenerated just after the onset of hearing (which takes place on postnatal day 14 in mice), starting with death of the supporting cells that eventually extended to encompass the hair cells. The resulting loss of integrity of the endolymph-perilymph barrier at the organ of Corti caused significant decreases of the endocochlear potential and of the endolymphatic potassium concentration, leading to hearing impairment across all frequencies.

In R26Cre-ER^{T} *Gjb2*$^{loxP/loxP}$, *Foxg1-Cre Gjb2*$^{loxP/loxP}$, and *Pax2-Cre Gjb2*$^{loxP/loxP}$ mice, complete deletion of *Gjb2* was only achieved in the organ of Corti, while deletion was just partial in other cochlear structures, including the stria vascularis (30, 180). The organ of Corti of these three murine lines developed normally at first, although the spaces of Nuel and the tunnel of Corti failed to open at postnatal day 9, suggesting a role for Cx26 in the final development of the organ of Corti (180). Hair cell degeneration started just after the onset of hearing, although cell death occurred more gradually than in *Otog-Cre Gjb2*$^{loxP/loxP}$ mice. Many spiral ganglion neurons subsequently disappeared. Notably, the most severe degeneration occurred in the middle turn of the cochlea, i.e. the region most sensitive to sound, although hearing impairment extended across all frequencies (30, 180).

Altogether, the results obtained with the various *Gjb2* conditional inactivation approaches supports the concept that hair cell death due to lack of Cx26 is triggered by sound-driven activities, whether because of a loss of spatial buffering of excitotoxic substances (29) or because of impaired transport of energy metabolites that are actively being consumed (30). Unfortunately, all these conditional mice, while very valuable, do not mimic the situation in most human DFNB1 patients, who completely lack Cx26. Thus, a model with complete inactivation of Cx26 in the cochlea is necessary to fully understand the pathogenesis of DFNB1 and, eventually, to test prospective therapies.

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Abbreviations: ARNSHI: autosomal recessive non-syndromic hearing impairment; HI: hearing impairment; Hz, hertzs; RT-PCR: reverse transcriptase PCR.

Key Words Hearing impairment, DFNB1, *GJB2*, *GJB6*, Connexin-26, Connexin-30, Genetic epidemiology, Genotype-phenotype correlations, Animal models, Review

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