

LETTER TO JMG

Non-syndromic recessive auditory neuropathy is the result of mutations in the otoferlin (*OTOF*) gene

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It is estimated that about 1 in 500 children are born with a significant hearing loss.¹ Non-syndromic recessive hearing loss (NSRHL) represents a major aetiologic factor in childhood hearing loss since it accounts for approximately 40% of all cases.² Many of these genetic forms of hearing loss are indistinguishable with current clinical methods. Even so, more than 12 recessive genes have been identified primarily from large consanguineous pedigrees (see the Hereditary Hearing Loss Homepage <http://www.uia.ac.be/dnalab/hhh> for an overview).

By definition, non-syndromic suggests a “simple” phenotype limited to hearing loss with no other associated symptoms. However, hearing is a complex process. Since a hearing defect might occur at any place along the auditory pathway, it would seem reasonable to expect to be able to differentiate types of NSRHL based on the location where the auditory process is disrupted. Indeed, new audiological testing strategies now give insight into the point where such defects have occurred.

Pure tone audiometry has been the standard method used to measure hearing threshold but, since it subjectively tests the overall integrity of the auditory pathway, it gives only limited information about where that pathway is failing. The auditory brainstem response (ABR) is an objective measure of the overall auditory transduction process. The otoacoustic emissions (OAEs) test is another objective measure of the auditory pathway, which detects responses of the outer hair cells (OHCs) to environmental sound.^{3–6} A good review of auditory tests can be found in Hood.⁷

Some children have a hearing loss based on pure tone audiometry and ABR, but with normal OAEs. This type of hearing loss has been defined as auditory neuropathy (AN).⁸ Subjects with AN can have varying degrees of hearing loss with poor speech reception out of proportion to the degree of hearing loss. In contrast to those with non-AN hearing loss, most subjects with AN are not helped by hearing aids. The results of cochlear implantation (CI) have been mixed. Some cases of AN have been helped by CI,^{9–10} whereas others have not had such good results.^{11–12}

The genetic study of AN is complicated by the fact that the term includes hearing losses with varied aetiologies. AN can be non-genetic, owing to factors such as neonatal hyperbilirubinaemia.¹³ AN is also observed in many syndromic peripheral neuropathies, like Friedreich's ataxia and hereditary sensorimotor neuropathy (HSMN) formerly known as Charcot-Marie-Tooth disease.^{14–15} Hearing loss in some forms of HSMN has been shown to be associated with specific genetic alterations, including 8q23 in HSMN-Lom¹⁶ and a mutation of Thr124Met in the myelin protein zero gene.¹⁷ The children with AN described here have an autosomal recessive disorder and are distinguished by their lack of any other detectable peripheral neuropathy. We have elected to call this non-syndromic recessive auditory neuropathy (NSRAN). Linkage studies and mutation analysis have identified the *OTOF* gene as being responsible for this type of hearing loss in our families.

SUBJECTS AND METHODS

The appropriate institutional review boards approved this study and informed consent was obtained from human subjects. We observed four families with two or more children who had sensorineural hearing loss and normal OHC function (fig 1). The degree of hearing loss was determined by standard pure tone audiometry. The scale used to classify the degree of hearing loss is as follows: 0–20 dB HL is normal, 21–40 dB HL is mild, 41–60 dB HL is moderate, 61–80 dB HL is severe, and above 80 dB HL is profound. The status of the auditory pathway was determined using tympanometry, middle ear muscle reflex (MEMR), and ABR. OHC function was measured using distortion product OAE (DPOAE),³ transient evoked OAE (TEOAE),¹⁸ and/or cochlear microphonics (CM).¹⁹ All subjects diagnosed with hearing loss were examined by an otolaryngologist, a neurologist, and geneticist and underwent testing to rule out syndromic disorders.

Key points

- Approximately 1 in 500 children has hearing loss, with non-syndromic recessive hearing loss (NSRHL) being the most common. We studied four families with a unique type of NSRHL called non-syndromic recessive auditory neuropathy (NSRAN). Contrary to most hearing losses, the auditory pathway up to and including the cochlear outer hair cells functions normally in these NSRAN subjects, suggesting that the lesion is in the inner hair cells or more central. Most subjects with NSRAN and other types of auditory neuropathy (AN), including syndromic and non-genetic cases, have speech perception disproportionately poorer than the degree of hearing loss and disappointing results with hearing aids. To elucidate the type of hearing loss, we searched for the causative gene in four families.
- Audiological studies confirmed the presence of AN in four NSRHL families. A genome wide linkage study was carried out and the otoferlin gene (*OTOF*) was examined for disease causing mutations.
- A critical region on 2p23 was identified that contained the *OTOF* locus (maximal lod score 3.5, $\theta=0$). Out of eight alleles, four *OTOF* mutations were observed in the NSRAN families: a frameshift, a splice site, and two missense mutations. Four NSRAN subjects have received cochlear implants and reported great benefit.
- *OTOF* mutations cause a type of NSRAN. Subjects with this type of NSRAN are not helped by hearing aids but are helped by cochlear implants. Since approaches to treatment and remediation differ between AN and non-AN groups, finding the genetic cause of this NSRAN has important implications for diagnosis, newborn screening, and prognosis.

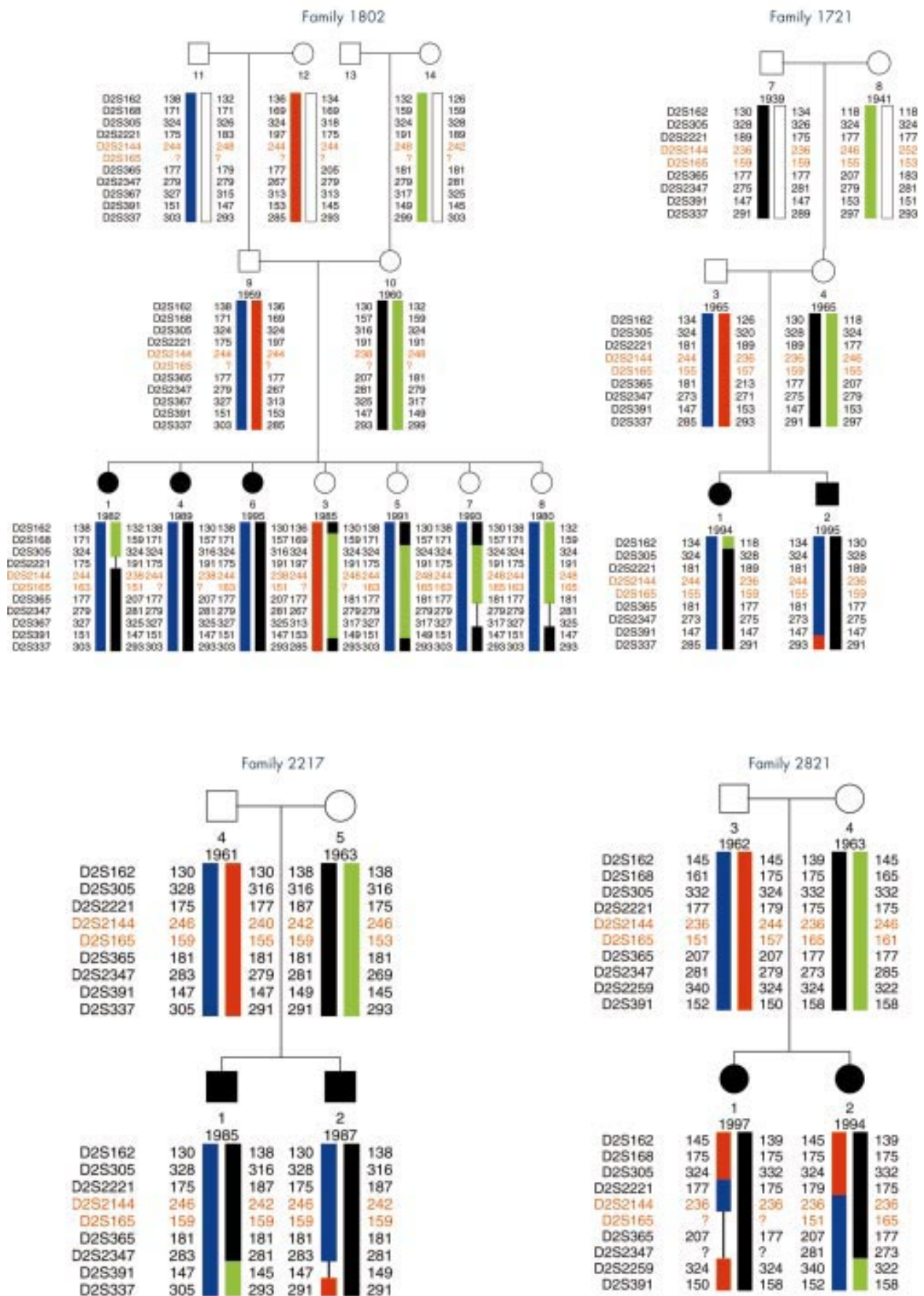


Figure 1 Pedigrees of non-syndromic recessive auditory neuropathy (NSRAN) families and their haplotype data for chromosome 2. The *OTOF* locus is flanked by markers D2S2144 and D2S165. Black symbols are subjects with NSRAN and white symbols are subjects with normal hearing levels. The number directly under the symbol is an identification number. The four digit number below the ID number is that person's year of birth

Table 1 Additional primers for PCR amplification of human *OTOF* exons

Exon	Forward primer	Reverse primer	Product size (bp)
20	5' AGGATGGCCCTAGCGAGAG 3'	5' CGAGCCGGGGTTAGC 3'	343
21	5' GAAGGCCTGAACGACATAC 3'	5' GGAGGGGGTGTCACTG 3'	435
22	5' GAGGGCCTGGTTGTGAGAAG 3'	5' GAGCCCCCTGATCCTGAG 3'	266
23	5' CAGGGGGCTCAATCAGGAG 3'	5' AAGGCCCCACCCTGTAC 3'	297
34	5' CAGATGCATAAACCCCTAAGT 3'	5' TGCTCCCTGGCTACTAAG 3'	391
35	5' GCACATGGACATCCGAGAGT 3'	5' AGACAGACCAGGCTGTAGGG 3'	362
36	5' AACCCAAGATTGATGAGCTTA 3'	5' GGTGGCATTCAAATAGCTAA 3'	470
48	5' TTGGGGTCCAGAAGGACAGA 3'	5' TCGGCCAAGGCATGAAGA 3'	374

DNA extraction and linkage analysis

Blood was drawn from affected children, their sibs, parents and, if possible, their grandparents. DNA was extracted using Puregene DNA isolation kits (Gentra Systems Inc). Genomic DNA was PCR amplified and genotyped using ABI Prism set 1 on an ABI Prism 377 DNA Sequencer according to standard protocol.²⁰ All other primers used in this study, including locus specific primers, were purchased from Integrated DNA Technologies Inc (IDT). Linkage analysis was performed using LINKAGE program version 5.1.²¹ Lod scores were generated using MLINK for two point analyses and LINKMAP for multipoint analyses.

Mutation detection

OTOF (otofelin) intronic and exonic primers^{22, 23} (tables 1 and 2) were used to PCR amplify genomic DNA or cDNA (derived from transformed peripheral blood lymphocytes). Sequence data analysis was performed using ABI Sequence Analysis 3.4, Fractura 2.2.0, Navigator 1.1 software, GCG suite (Oxford Molecular Group Inc), and the Lasergene suite (DNASTAR Inc). Mutations were confirmed and controls were screened by restriction enzyme digestion of PCR products.

RESULTS

Clinical features of non-syndromic recessive auditory neuropathy (NSRAN)

Nine children from four families with NSRAN had sensorineural hearing losses ranging from moderate to profound with functioning OHCs. Neurological examinations and nerve conduction studies indicated that none of the children had peripheral neuropathy. There was no evidence that the hearing loss was caused by a recognised syndrome or any known non-genetic factors. MEMRs were absent and tympanometry was normal. All the children reported receiving no benefit from hearing aids and had poorer speech comprehension than expected based on their pure tone audiograms. The clinical details of the affected children are summarised in table 3.

2p23 is a critical region for NSRAN

A genome screen was performed on three NSRAN families (1802, 1721, 2117) and linkage was found for a 21.7 cM region on chromosome 1 (lod=2.905) and a 16.3 cM region on chromosome 7 (lod=2.908).²⁹ No DFNB loci were located in our critical regions; however, while finishing the genome search a new hearing loss gene called *OTOF* was discovered. The mRNA

Table 2 RT-PCR primers

Primer set	Region amplified	Forward primer	Reverse primer	Product size (bp)
1	635-3206	5' AGAGCCGGGAGGAGCGTGTTC 3'	5' TGACAATGATGGGCGGATCGTC 3'	2593
2	2987-5321	5' TACACCAAGAAGCAGGCGTTC 3'	5' GATGATGACCCGCGACTCGTA 3'	2345
3	4986-6225	5' GCAAAGACGGCAAAGTGGACGG 3'	5' AACAGCGCCAGCAGCATCTTGA 3'	1261
4	193-710	5' AGTGACTTCCGAGGGCAATCC 3'	5' CAGCACCAGCCGGTTCATC 3'	535
5	283-2049	5' GGCCAGCAGCATCGACAG 3'	5' GACAGGCCATCAACTTCGTTC 3'	1788
6	1840-3206	5' GGGCCTGGCTGTGGAGATCGT 3'	5' TGACAATGATGGGCGGATCGTC 3'	1388
7	2654-4206	5' CAGCACAGCATTCCTCGACATC 3'	5' CCCTTCAGGCCCTCGGTATTGT 3'	1574
8	3976-5082	5' GGTGAAGCTGGACGCGACTT 3'	5' GTGGGCTTCCTTGACCGTTC 3'	1128
9	4706-6225	5' CGCGACAAGGAGAATCATCT 3'	5' CGCCAGCACGATCTTGA 3'	1536
10	280-711	5' GGTGGCCAGCAGCATCGACA 3'	5' ATCTCCAGCACCCCGGTTTCAT 3'	431
11	464-711	5' TGCGTGGAGTCCGGTATCAGG 3'	5' CTCCAGCACCCCGGTTTCAT 3'	247
12	634-904	5' GAGAGCCGGGAGGAGCGTGTTC 3'	5' CTGCCGGGCTCGATCACC 3'	270
13	1300-1654	5' GGCCAATGAGACCGACGAAGA 3'	5' GTCAATGAAGTGGGTGCCGATG 3'	354
14	5323-5823	5' CGAGCTGCGGGTTCATCATCT 3'	5' TTGCCGTGAGCTCAAATCAT 3'	522
15	2353-5886	5' CGAGCTGCGGGTTCATCATCT 3'	5' CCAGGCCACTGGGTCTTCT 3'	584
16	4903-5404	5' CGGCATCGCCAGACCTAC 3'	5' GCCACCCCTCACGAAGATG 3'	521
17	4431-4935	5' AGACCGGGGATGATGAGGATG 3'	5' GGGGTCCCGCCAGATATTGT 3'	524
18	4214-4614	5' GAGGCCTGAAGGGTCAAT 3'	5' GTGGCCCGACCAACATAGA 3'	419
19	3893-4412	5' AATGGGGGCTCCTCTCTCA 3'	5' TTGCCCGAAGCAAGTTGAA 3'	539
20	3545-3975	5' TGGGGCCTACGGGACCTAAA 3'	5' AGTCGCTCCAGCTTACCA 3'	450
21	3074-3609	5' CCCTTGCCCGCTCTTCT 3'	5' CACCCCTTCCCTGCACAT 3'	555
22	2826-3264	5' GCTCGGCAGGCTGGACAGT 3'	5' GAAGTCCGGCCCATGAAGT 3'	458
23	2482-2897	5' ATCCCGCACCAGGCTTGAC 3'	5' GCAGGCCGACAGGAATC 3'	434
24	2101-2536	5' GCCGGGGGATGAGGAAGA 3'	5' GCCTGCTGCCCATGTTT 3'	453
25	1550-2140	5' GAGCCCTGTGGAATGAGCA 3'	5' CACCGCCCTCGTCACATT 3'	610
26	1208-1665	5' GACCCCGATGACATCTCCTC 3'	5' CTGCGCAGGTCATGAAGT 3'	477
27	637-1215	5' AGCCGGGAGGAGCGTGT 3'	5' CAGCCCGAGGAGATGTCAT 3'	598
28	30-664	5' AGGCAGCGGACGAGAAGAGAG 3'	5' CCGGTTTTGCGGACTTC 3'	653
29	4684-5927	5' CCGGCTAGGCAAGACTGACAT 3'	5' GGCCGGTGGGTTTCTAG 3'	1263

Table 3 Clinical details of the children with auditory neuropathy

Subjects	Hearing loss	Audiogram shape	ABR	CM	OAE	Vestibular testing
1802-6	Severe-profound	Bowl shaped	-	DNT	+	RC
1802-4	Severe	Flat	-	DNT	R +4-8 kHz, L absent	RC
1802-1	Severe-profound	Bowl shaped	-	+	R +6 kHz, L absent	RC, BC
1721-2	Profound	Sloping	-	+	+	DNT
1721-1	Profound	Sloping	-	+	+	RC
2117-2	Severe-profound	Gradually sloping	-	R + L DNT	+	RC
2117-1	Moderate-severe	Bowl shaped	-	+	+	RC
2821-2	Severe-profound	Bowl shaped	-	R + L DNT	-	DNT
2821-1	Profound	Flat	-	+	+	DNT

ABR=auditory brainstem response; CM=cochlear microphonics; OAE=otoacoustic emissions; DNT=did not test; +=present bilaterally; -=absent bilaterally; R=right; L=left; RC=rotary chair was normal; BC=bithermal calorics was normal.

Table 4 OTOF mutations found in NSRAN families

Family	Exon	Mutation	Consequence	Segregates	R enzyme verification	Control chrom
1802	16	1778delG	Frameshift	Yes	<i>BsaBI</i>	0/188
1802	48	6141G>A	Arg>Gln	Yes	<i>Sau96I</i>	0/188
1721	39	IVS39+1 G>C	Splice site	Yes	<i>BsiI</i>	0/188
2117	48	6285C>G	Pro>Arg	Yes	<i>BtgI</i>	0/188

hOTOF e48 NRPDTA~~F~~VWFLNPLKSIKYLICTRYKW LIIKIVLALLGLLMLGLFL YSLPGYMKKLLGA~~
mOTOF e48 NRPDTA~~F~~VWFLNPLKSIKYLICTRYKW LIIKIVLALLGLLMLALFL YSLPGYMKKLLGA~~
hOTOF e47 NRPDTSFIWFLNPLKSARYFLWHTYRW LLLKLLLLLLLLLLLLLALFL YSVPGYLKVKILGA~~
mOTOF e47 NRPDTSFIWFLNPLKSARYFLWHTYRW LLLKFLLFLLLLLLALFL YSLPGYLAKKILGA~~
hDYSF e48 ~RPDTSFLWFTSPYKTMKFIWRRFRW AIILFIIILFIIILFLAIFI YAFPNYAAMKLVKPFPS
hMYOF e48 NRPETSFLWFTNPCKTMKFIWRRFRW VII~~G~~LLFLLIILLLFVAVLL YSLPNYLSMKIVKPNV

Figure 2 Comparison of the carboxy-terminus amino acid sequence between ferlin-like proteins. Comparison between the deduced amino acid sequences encoded by exon 47 of the brain isoform, exon 48 of the cochlear isoform in human and mouse, and the corresponding predicted amino acid sequence from human dysferlin and human myoferlin cDNA. The transmembrane region is underlined. The amino acids in bold are those changed in human exon 48 by the missense mutations described in table 4.

expression pattern and proposed function made it an ideal candidate for NSRAN. Linkage data were re-evaluated and found to be incomplete for the *OTOF* locus, so more markers were purchased. During this time, a fourth NSRAN family was obtained, so all four families were genotyped using the five dinucleotide markers from the *OTOF* locus, D2S2221, D2S2144, D2S165, D2S365, and D2S2347. Haplotype analysis was consistent with linkage for all four families (fig 1). The otoferlin gene (*OTOF*) was noted to be located between markers D2S2144 and D2S165.²² Two point linkage analysis gave a maximum lod score of 2.908 for the four families at marker D2S2144 and a multipoint analysis produced a maximum lod score of 3.47.

NSRAN mutations in *OTOF*

All of the known *OTOF* exons and approximately 100 bases of the introns flanking these exons from each of the proband's DNA from the four families was sequenced and examined for mutations. All mutations are identified by their position in GenBank file AF183185. Once a mutation was identified, DNA from the rest of the family was sequenced to determine mutation segregation. A control sample of 94 normal hearing,

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78905  tccacctatg tggggcatgg ggcagagggc gggggcagtg tgctgcttt
      S T Y V G H G A E G G G S V P A L
78955  gctccactc taaaatgaga ttcagatcct ggtgatttga gagGTtagac
      P P L X
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Figure 3 cDNA sequence from family 1721 of the allele with the splice site mutation IVS39+1G>C (bold). The coding region is underlined and translated.

unrelated persons was tested using restriction enzyme digestion. The results are summarised in table 4. In family 1802, the affected subjects were observed to be compound heterozygous for a frameshift mutation and a missense mutation. The frameshift mutation is the result of deletion of a guanine at position 1778 (exon 16), which causes a stop codon after four additional amino acids. The missense mutation, 6141G>A, substitutes a glutamine for an arginine at the second codon in exon 48 (fig 2). In family 1721, both children and their father were observed to be heterozygous for a conserved G to C transversion in the donor splice-site (GT) of intron 39 (IVS39+1G>C). Using RT-PCR, it was found that this leads to an mRNA with 30 new codons, the twentieth of which codes for a stop codon and is predicted to result in a truncated protein (fig 3). Family 2117 was heterozygous for the missense mutation in exon 48, 6285C>G, which changes the amino acid proline to an arginine in codon 50 of exon 48. These missense mutations are believed to be pathological because, as seen in fig 2, these amino acids are highly conserved in mouse and man for otoferlin, as well as the myoferlin and dysferlin genes. None of these mutations was observed in a set of 94 unrelated controls (188 chromosomes). Mutation segregation was consistent with autosomal recessive inheritance in all cases.

DISCUSSION

Mutations in the gene encoding otoferlin cause a non-syndromic recessive auditory neuropathy. The evidence for this assertion is threefold. First, NSRAN families show linkage to a small region on chromosome 2 that contains the otoferlin gene. Second, the gene encoding otoferlin is expressed in the

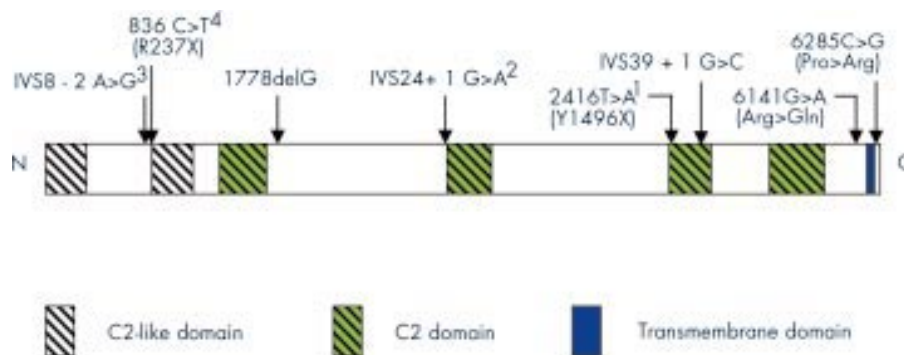


Figure 4 Otoferlin mutations and functional domains. A diagram of otoferlin's structure showing the location of all mutations found to date. (1) Yasunaga *et al.*²² (2) Adato *et al.*²⁸ (3) Yasunaga *et al.*²³ and (4) Houseman *et al.*²⁷

cochlea in the appropriate cell type, the cochlear inner hair cells (IHCs). Third, mutations have been found in the otoferlin gene in three of four families with NSRAN. Our conclusion not only raises several interesting questions but also has important implications with regard to the diagnosis and treatment of children with hearing loss.

Knowledge of the tissue specific expression of otoferlin is currently limited to results, primarily from mouse, based on *in situ* hybridisation, northern analysis, and RT-PCR studies.²³ *In situ* data indicate that otoferlin mRNA from mouse embryos is expressed in IHCs, OHCs, and spiral ganglion cells. In the cochlea, it persisted in only IHCs for the adult mouse. *Otof* was not expressed in the supporting cells of the organ of Corti. In the mouse vestibule, *Otof* is expressed in the neuroepithelia of the saccule, utricle, and semicircular canals; however, no vestibular abnormality was observed in our families. The mRNA was detected in other tissues by RT-PCR, primarily in the brain but also in heart, kidney, and liver.

Analysis of its primary structure identifies two domain types that offer an indication of what otoferlin might be doing and what other molecules might interact with it. Otoferlin has six C2 motifs (Ca⁺⁺ binding domains) and a transmembrane domain at its C terminus (fig 4). Because of the presumed role of otoferlin's orthologue, the *Caenorhabditis elegans fer-1* gene in membrane fusion,²⁴ and the sequence similarity of its C2 domains to synaptotagmin I, it is likely that otoferlin plays a role in membrane trafficking activated by increased local Ca⁺⁺ concentration. It has been suggested that otoferlin may play a synaptotagmin-like role in IHC synaptic vesicle fusion.²² However, the otoferlin missense mutations found in the NSRAN families do not involve the C2 domains but are adjacent to the transmembrane domain (fig 4).

Alternatively spliced forms of otoferlin have been found in human and mouse.²³ In humans, spliced forms were seen in the cochlea but not the brain and other isoforms were found both in the brain and cochlea. Exon 47 is skipped in cochlea, heart, kidney, and total fetus leading to a different carboxy-terminal peptide sequence from that of the brain isoform, which expresses exon 47. In mRNA with exon 47, translation terminates in that exon, leaving exon 48 untranslated. In mRNA without exon 47, translation continues into exon 48 where it is terminated.

Exon 47 of the brain isoform and exon 48 of the cochlear isoform specify the C-terminal 60 amino acids of otoferlin. The amino acid sequences are 63% identical, differing in 22 amino acids (fig 2). This transmembrane domain and the flanking sequence may play an important role in targeting this protein to a specific location in the cell or specify the interaction of otoferlin with specific ligands.

The hearing loss described in previous DFNB9 reports was characterised by pure tone audiometry or ABR,^{22 23 25-28} but there was no report of testing for OHC function. The hearing

loss families with *OTOF* mutations that we report here are distinguishable by the relative preservation of OHC function. It is not clear whether the preservation of OHC function is a consistent and stable phenotype for hearing loss caused by *OTOF* mutations. In family 1802, there is clear evidence that OAEs diminish in older children.²⁹ While this may be the result of damage from hearing aid use, it is also possible that it represents an effect of the *OTOF* mutation on the OHCs. In either case, such a trend would make the phenotype of older subjects with NSRAN indistinguishable from other forms of NSRHL. If OAEs were extinguished progressively in NSRAN patients, it would suggest that this form of hearing loss is more common than previously estimated.

It is possible that the preservation of OHC function in the hearing loss families reported here is the result of a specific class of *OTOF* mutations. All previously reported *OTOF* mutations have been found in consanguineous families and are present in the homozygous state. Four *OTOF* mutations were found, two nonsense^{22 27} and two splice site mutations.^{23 28} These mutations would be expected to completely "knock out" the otoferlin function in all tissues, and we would predict a profound pan-cochlear hearing loss.

Affected subjects in NSRAN family 1802 are heterozygous for a frameshift (1778delG) and a missense mutation (6141G>A). The deletion would be expected to knock out this otoferlin allele in all tissues since a stop codon occurs after four additional amino acids in exon 16. However, its companion is a missense mutation in the cochlear specific, alternatively spliced coding region of exon 48. This otoferlin allele would be predicted to have no effect on the primary structure of the brain isoform. In tissues expressing the cochlear isoform, such as the IHCs, a dysfunctional protein would be created which could disrupt the protein's subcellular localisation or otoferlin's interaction with other proteins critical to IHC function and, thus, an AN phenotype might be observed.

The mutations found in families 1721 and 2117 also support this hypothesis, but their companion mutations have yet to be found. While every known exon has been sequenced in these two families, it is possible that the other mutations are in the promoter, introns, or enhancers or hidden because of the lack of amplification of the mutated allele. Despite the suggestive linkage of family 2821, hearing loss in this family could be caused by mutations in a different NSRAN gene.

The discovery of a cause for NSRAN will allow a rapid genetic diagnosis of this form of hearing loss and will probably improve our ability to provide appropriate communication skills and tools in a timely manner to those affected. Understanding the mechanism by which mutations in otoferlin can cause NSRAN will further define the mechanism of hearing and the specific roles IHCs and OHCs play in this phenomenon.

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