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ORIGINAL ARTICLE

OTOF mutations revealed by genetic analysis of hearing loss families including a potential temperature sensitive auditory neuropathy allele

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Introduction: The majority of hearing loss in children can be accounted for by genetic causes. Non-syndromic hearing loss accounts for 80% of genetic hearing loss in children, with mutations in *DFNB1/GJB2* being by far the most common cause. Among the second tier genetic causes of hearing loss in children are mutations in the *DFNB9/OTOF* gene.
Methods: In total, 65 recessive non-syndromic hearing loss families were screened by genotyping for association with the *DFNB9/OTOF* gene. Families with genotypes consistent with linkage or uninformative for linkage to this gene region were further screened for mutations in the 48 known coding exons of otoferlin.
Results: Eight OTOF pathological variants were discovered in six families. Of these, Q829X was found in two families. We also noted 23 other coding variant, believed to have no pathology. A previously published missense allele I515T was found in the heterozygous state in an individual who was observed to be temperature sensitive for the auditory neuropathy phenotype.
Conclusions: Mutations in *OTOF* cause both profound hearing loss and a type of hearing loss where otoacoustic emissions are spared called auditory neuropathy.

The majority of hearing loss in children can be accounted for by genetic causes. Non-syndromic hearing loss accounts for 80% of genetic hearing loss in children, with mutations in *DFNB1/GJB2* being by far the most common cause.[1] Among the second tier genetic causes of hearing loss in children are mutations in *DFNB9/OTOF* (OMIM: 603681). This type of hearing loss is further complicated in that at an early stage it may present itself as an auditory neuropathy and thus evade detection by newborn hearing screening based on otoacoustic emissions testing.[2–4]

Auditory neuropathy/auditory dys-synchrony (AN/AD) is a unique type of hearing loss diagnosed when tympanographs are normal, and acoustic reflexes (AR) and auditory brainstem response (ABR) are absent or severely abnormal, but outer hair cell (OHC) function is normal as indicated by the presence of otoacoustic emissions (OAE) and/or cochlear microphonics (CM). These test results indicate that the auditory pathway up to and including the OHC is functioning but the auditory signal is not transmitted to the brainstem, suggesting that the lesion lies at the level of the inner hair cells (IHC), the IHC synapse to the afferent nerve fibres, or the auditory nerve itself. Individuals with AN/AD can have various degrees of hearing loss as measured by pure tone audiometry. However, they generally have disproportionately poor speech understanding. In contrast to persons with non-AN/AD hearing loss, hearing aids may provide little help in speech understanding in most individuals with AN/AD. Cochlear implantation has been shown to help with speech understanding in some cases of AN/AD,[5–10] but others have not had favourable results.[11 12]

The term ''auditory neuropathy'' was first coined in 1996.[13] However, many early reports describe cases now thought to have been AN/AD.[14–16] At the time, such cases were considered paradoxical because test findings were inconclusive or contradictory. However, advances in the measurement of OHC function allowed further characterisation of this condition.

Approximately 50% of AN/AD patients have no defined aetiology.[17] These patients are generally non-syndromic, and some pedigrees suggest a recessive pattern of inheritance based on the presence of at least two affected siblings and unaffected parents. Recently mutations in *OTOF*, the gene encoding otoferlin were found to cause a non-syndromic recessive AN/AD.[2–4] There have been several reports of mutations in *OTOF* associated with non-syndromic recessive hearing loss (NSRHL).[18–23] None of the NSRHL reports defined the status of the OHC in affected individuals, so it could not be determined whether these individuals had AN/AD.

In adult mouse cochlea, otoferlin mRNA is detectable in the IHCs but is not evident in OHCs.[23] Several isoforms of otoferlin exist, owing to variable start sites and alternative splicing. At least two other members of this gene family have been found in mammals: *DYSF,* encoding dysferlin, and *MYOF*, encoding myoferlin.[24] Dysferlin was recently found to be involved in membrane repair.[25] Otoferlin, dysferlin, and myoferlin are predicted to be signal anchor membrane proteins with the greater part of the protein, including the N-terminus, facing the cytoplasm anchored by the C-terminal transmembrane domain.[26 27] They contain multiple C2 domains, and all bear homology to the synaptotagmins, proteins involved in synaptic vesicle fusion.[28]

We report here the genetic screen of 65 hearing loss families, the discovery of novel otoferlin mutations, and

Abbreviations: ABR, auditory brainstem responses; AD, auditory dys-synchrony; AN, auditory neuropathy; AR, acoustic reflexes; CM, cochlear microphonics; DHPLC, denaturing high performance liquid chromatography; HL, hearing loss; IHC, inner hair cells; MDE, mutation detection enhancement; MOC, medial olivocochlear; NSRAN, non-syndromic recessive auditory neuropathy; NSRHL, non-syndromic recessive hearing loss; OAE, otoacoustic emissions; OHC, outer hair cell; SAT, speech awareness threshold; SSCP, single strand conformational polymorphism; SRT, speech reception threshold

corresponding clinical data on hearing loss in families with pathological mutations. One of these mutant alleles is of particular interest because the phenotype associated with this allele is temperature sensitive.

MATERIALS AND METHODS

Patient sample and medical information

DNA samples and medical information were collected under institutional review board protocols that have been approved by the respective institutions. Sampling included the proband and, if possible, all full biological siblings, both biological parents, and all biological grandparents. In some cases, research participants were asked to undergo complete physical examinations and other appropriate testing, including audiological, vestibular, neurological, and ophthalmological testing. Audiological testing included air and bone conduction puretone audiometry, ABR, tympanometry, AR thresholds, OAE, OAE suppression, speech awareness threshold (SAT) and/or speech reception threshold (SRT) testing. The following scale was used to classify the degree of hearing loss (HL) using pure tone audiometry: 0–20 dB HL as normal, 21–40 dB HL as mild, 41–60 dB HL as moderate, 61–80 dB HL as severe, and >80 dB HL as profound.

Genotyping and mutation screening

All families in this study were previously screened for DFNB1/GJB2 mutations. In the Nebraska group of 47 NSRHL families (recruited at BTNRH or LSU), genotyping of the chromosomal region around the *OTOF* locus was performed as previously described.[2]

To determine if a difference exists between patient DNA and the control *OTOF* BAC DNA (RP11-638P8; Research Genetics, Inc.), mutation detection enhancement (MDE) heteroduplex analysis was performed as previously described, with slight modifications.[29] PCR products were amplified from both genomic sample DNA and *OTOF* BAC control DNA, mixed, heated at 95℃ for 3 minutes, and then cooled to 25℃ over a 45 minute period. The re-annealed reaction products were then run on an electrophoresis gel at 850 V for 18–24 hours, depending on the size of the fragment. Following electrophoresis, the bands were visually assessed using ethidium bromide staining. The PCR primers used to amplify *OTOF* exons and flanking sequence have been described previously.[2]

Denaturing high performance liquid chromatography (DHPLC, using the Transgenomic WAVE DNA Fragment Analysis System; Transgenomic, Inc.) was also used to identify mutations according to the manufacturer's directions.

In the Iowa group (families recruited at the University of Iowa), 18 DFNB1 negative NSRHL families were analysed for allele status with STRP markers (D2S405, D2S158, and D2S1360). Three families with markers segregating consistent with linkage or uninformative for linkage were further screened using single strand conformational polymorphism (SSCP) for all 48 known *OTOF* exons. Mutations recognised by SSCP were then identified by sequencing. Families where only one mutation was detected were screened by sequencing all 48 known *OTOF* exons.

DNA samples showing a positive heteroduplex pattern by either MDE gel, DHPLC, or SSCP were sequenced using one of two kits (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) or CEQ Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter)). Analysis of the sequence data was performed using Sequence Analysis software (version 3.4), the Lasergene suite (DNASTAR, Inc), and the Wisconsin package (Pharmacopeia, Inc). Mutations were checked in unrelated normal hearing control individuals using restriction enzyme digestion or heteroduplex gel/WAVE DHPLC and DNA sequencing if no restriction enzyme was available. The control population consisted of Americans of white European descent with no report of hearing problems. In this study all families with individuals affected with hearing loss were also American of white European descent except for one family from the UK. Mutations corresponding to the coding region are numbered as in the human brain otoferlin long form AF183185.1, beginning with the starting methionine, AUG (A as number 1). Splice site mutations are designated by their adjacent exon.[23]

RESULTS

Mutations were revealed during two independent screens of NSRHL families. In the Nebraska group, STRP genotyping of the *OTOF* locus was performed on 47 families (38 NSRHL families and nine families with non-syndromic recessive auditory neuropathy (NSRAN) families), 17 (14 NSRHL and three NSRAN) of whom were consistent with linkage to the *OTOF* locus by haplotyping and were therefore included in the genetic screening. Sixteen families were uninformative for linkage (12 NSRHL and four NSRAN). Fourteen families (12 NSRHL and two NSRAN) were informative and not consistent with linkage. The 17 families consistent for linkage and the 16 uninformative for linkage were included in the mutation screen. Seven mutations believed to be pathogenic were found in five families in this group (table 1, fig 1).

In the Iowa group, 18 NSRHL families were screened with markers linked to *OTOF*. None of the Iowa families had been previously diagnosed with auditory neuropathy. Of these 18 families, all were informative for linkage but only three were consistent for linkage to *OTOF*. These families were screened by SSCP. A single missense mutation deemed pathogenic was identified in family 53510 in this group (table 1, fig 1). An audiological summary is given for each family (table 2).

We found a high degree of neutral variation within the subject population during the mutation screen; 23 coding polymorphic changes were noted (*denotes alleles found in the Spanish population).[20] Eleven of these were missense alleles (158C→T, A53V; 244C→T*, R82C*; 1723G→A, V575M; 2317C→T*, R773S*; 2464C→T, R822W; 3247G→C, A1083P; 3470G→A, R1157Q; 3966C→G, D1322E; 4874G→A, V1625M; 4936C→T, P1646S; and 5663G→A, G1888D), and 12 were silent alleles (372A→G*, T124T*; 945G→A*, K315K*; 1926C→T, N642N; 2022CvT, D674D; 2025G→A, E675E; 2580C→G*, V860V*; 2736G→C*, L919L*; 3189G→A, A1063A; 4677G→A, V1559V; 4767C→T, R1589R; 5391C→T*, F1797F*; and 5655C→T*, R1885R*).

Probably the most unusual family of the group is 3467, a family with temperature sensitive AN/AD, with two affected siblings. The variant 1544T→C, I515T was found heterozygously in the father and the two affected children of this temperature sensitive NSRAN family. The maternal mutation is still unknown.

The audiogram for individual 3467-1, when she was afebrile, showed a mild low frequency hearing loss and speech comprehension was below the 10th percentile for both quiet and noise. Tympanometry was normal and AR were absent. ABR was abnormal, but CM were present. On two occasions, testing was performed during febrile illness. Her core temperature was defined approximately 2 hours before testing. At a temperature of 38.1℃, her pure tone thresholds decreased to profound deafness in the low frequencies, rising to severe hearing loss in the high frequencies. SAT was 80 dB HL, but she was unable to repeat any of the test spondee words. Tympanometry and OAE were normal, but AR and ABR were absent. With a temperature of 37.8℃, she was tested again and showed a mild to moderate hearing loss and zero speech comprehension. ABR and OAE were not tested.

Table 1 *OTOF* mutations including mutations from genetic screening of 65 NSRAN and NSRHL families (in **bold**)

Exon or IVS	Mutation	Codon	Controls, no. of chromosomes	Family	Diagnosis	Origin	Reference
8	709C→T	R237X	0/36		NSRHL	UAE	19
8 IVS	IVS8−2A→G		0/218		NSRHL	India	23
15	1469C→A	P490Q	0/220		NSRHL	Turkey	21
15	1544T→C	I515T	0/178, 0/220	3467	TS-NSRAN, NSRHL	USA, Turkey	21
16	1651delG		0/188		NSRAN	USA	2
17	1886_1887insA	K629fs	0/188	3466	NSRAN	USA	
18 IVS	IVS18 +1G→T		0/188	3539	NSRAN	USA	
19	2122C→T	R708X	0/100		NSRHL	Spain	3
21	2348delG	G783fs	0/184	3466	NSRAN	USA	
21	2381G→A	R794H	0/160	53510	NSRHL	USA	
22	2485C→T	Q829X	0/172, 0/400	3456, 3540	NSRAN, NSRHL	UK, USA, Spain, Cuba	3 20
24 IVS	IVS24 +1G→A		0/240		NSRHL	Druze/Israel	18
26	3032T→C	L1011P	0/100		NSRAN	Turkey	4
28 IVS	IVS28 −2A→C		0/184	3456	NSRAN	UK	
36	4275G→A	W1425X	0/100		NSRAN	Spain	3
36 IVS	IVS36 +2T→G		0/100		NSRAN	Spain	3
37	4491T→A	Y1497X	0/212		NSRHL	Lebanon	22
39 IVS	IVS39 +1G→C		0/188		NSRAN	USA	2
44	5473C→G	P1825A	0/200		NSRHL	Spain	20
48	5860_5862delATC	I1954del	0/100		NSRAN	Spain	3
48	6014G→A	R1939Q	0/188		NSRAN	USA	2
48	6158C→G	P1987R	0/188		NSRAN	USA	2

IVS, intervening sequences or intron.

The following day her auditory functions returned to normal after her fever abated. She has reported to her parents that her hearing becomes affected ''suddenly'' when she is febrile.

Individual 3467-2 was examined twice when afebrile. He has a mild low frequency hearing loss, normal tympanograms, absent AR, and abnormal ABR. CM and OAE were present bilaterally. We were unable to test 3467-2 when febrile, but his parents report that under those conditions he experienced hearing loss similar to his sister. OAE suppression was tested to determine medial olivocochlear (MOC) neurone integrity. Activation of these neurones by presenting sound to the contralateral cochlea will induce a suppression of ipsilateral OAEs.[30] Patients with AN/AD cannot suppress their OAE during this test.[31] As expected, the OAE of 3467-2 were not suppressed, which indicates that the sound signal reached the OHC, but not the efferent MOC neurones that feed back to suppress the OHC. We have tested the parent carrying the I515T mutation and no abnormality of pure tone threshold, speech comprehension in quiet and noise, ABR, AR, or OAE was identified. The clinical details of this family have been published previously.[32]

Both *OTOF* pathological alleles have been found in family 3456. The maternally inherited mutation is a novel splice site mutation, IVS2 –2A→C. The mutation inherited through their father is a previously published nonsense mutation 2485C→T, Q829X, found in a group of Spanish families and one Cuban family.[3 20] Family 3456 is a white family from England with no known Hispanic ancestry, with two sons

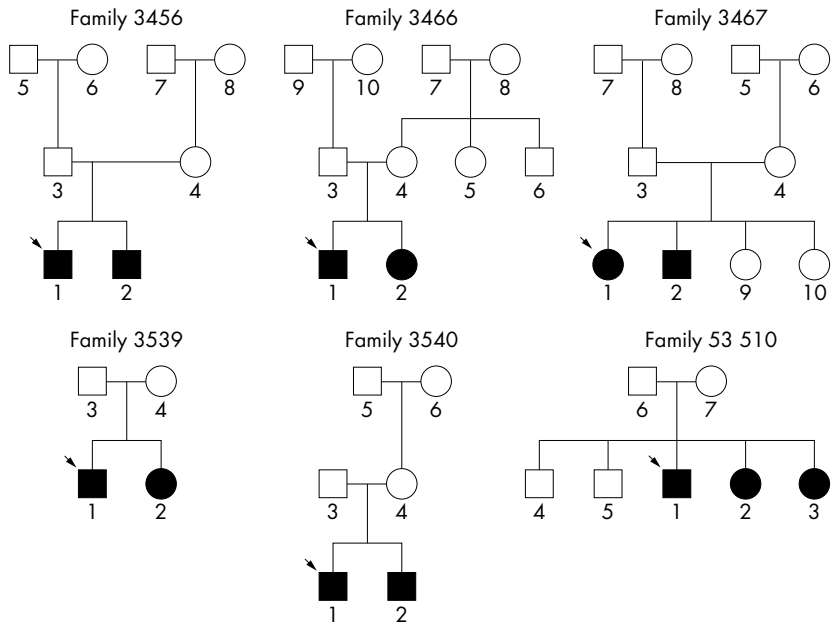

Figure 1 Pedigrees of families with *OTOF* mutations. Proband is identified by arrow.

Table 2 Summary of audiological data

NSRAN individual	Age tested	Audiometric loss	Audiogram shape	ABR	CM	OAE	Acoustic reflexes	Diagnosis
3456-1	3 years	Profound	Corner	−*	+†	+	NT‡	NSRAN
3456-2	15 months	Profound	Corner	−	+	+	NT	NSRAN
3466-1	4 years	Profound	Flat	−	NI§	+	NI	NSRAN
3466-2	16 months	Profound	Flat	−	NI	+	NI	NSRAN
3467-1 37°C	6 years	Mild to normal	Rising	Abnormal	+	+	−	TS-NSRAN
3467-1 37.8°C		Mild to moderate	Cookie bite	NT	NT	NT	NT	TS-NSRAN
3467-1 38.1°C		R: profound, L: severe to profound	R: flat, L: rising	−	NT	+	−	TS-NSRAN
3467-2 37°C	2 years	mild to normal	Rising	Abnormal	+	+	−	TS-NSRAN
3539-1	6 years	Moderate to severe	Rising	−	+	+	−	NSRAN
3539-2	3 years	Moderate to severe	Rising	−	+	+	−	NSRAN
3540-1	3 years	NT	NT	−	+	+	−	NSRAN
53510-1	26 years	Profound	Corner	NT	NT	−	NT	NSRHL
53510-2	33 years	Severe to profound	Corner	NT	NT	NT	NT	NSRHL
53510-3	35 years	Profound	Corner	NT	NT	NT	NT	NSRHL

*Absent bilaterally; †present bilaterally; ‡not tested. ABR, auditory brainstem response; CM, cochlear microphonics;OAE, otoacoustic emissions.

both having AN/AD. Pregnancy and birth history were unremarkable for the two affected boys except for slight jaundice in the older boy (individual 3467-1). Motor milestones were met at an appropriate age. As is consistent with AN/AD, ABR was absent and OAE were present. Both boys have profound hearing loss and corner audiograms. OAE suppression was tested in individual 3467-1 and, as expected, his OAE were not suppressed, but were instead increased in amplitude. Vestibular function testing in the older boy indicated that there may be a slight hypofunction on the left side. The younger was not tested for vestibular function. Computed tomography scans were normal in both boys, and magnetic resonance imaging in the older boy. Both boys have had positive experiences with their cochlear implants and consider them to be beneficial.

Both mutations in family 3466 result in a frameshift. The maternally inherited mutation in exon 17 contains an insertion 1886_1887insA (K629fs). The paternally inherited mutation is a deletion in exon 21, 2348delG (G783fs). This family has two affected children, a boy (3466-1) and a girl (3466-2) with normal OAE and absent ABR. Birth histories were uneventful and developmental milestones, except speech, were attained at a normal age. The children underwent neurological examinations at the age of 5 years (3466-1) and 7 years (3466-2), which were normal. However, 3466-1 may also have a vestibular neuropathy or hypoactive vestibular function, as no nystagmus developed after spinning in a chair and his father reported that the child can spin without falling down.

Family 3539 has two affected children with normal tympanometry, OAE and CM, but absent AR and ABR. The daughter was born prematurely at 32 weeks gestation; otherwise, the birth histories were unremarkable. A splice site mutation, IVS18 +1G→T, was found in the maternal allele.

The Hispanic nonsense mutation 2485C→T, Q829X has been found heterozygously in family 3450. This family has Mexican ancestry. Both affected children are ''typical'' of NSRAN, in that OAE and CM are present, but ABR and AR are absent.

In the Iowa group of families, an *OTOF* mutation was found in one family. There were three affected children in family 53510, all heterozygous for 2381G→A, R794H. Two of these individuals had a profound hearing loss while one had a severe to profound hearing loss. All three individuals had a corner audiogram.

DISCUSSION

Seven *OTOF* mutations were discovered and believed to be pathogenic in five NSRAN families in the Nebraska group. One missense mutation was found in the Iowa group of NSRHL families. It is possible that the previously published *OTOF* NSRHL families were actually NSRAN families; however, proving this could be difficult owing to the fact that, in many cases, OHC function has been observed to decline with age. The mutation found in the Iowa group of families reported in this study may also have been NSRAN for the same reason. Recent studies, including this work, indicate that all individuals with *OTOF* pathological variants in both the maternal and paternal alleles will present with auditory neuropathy as young children if comprehensively tested (including OHC function).[2–4] In this report, two families were heterozygous for the nonsense allele 2485C→T, Q829X, found recurrently in Spanish families.[3 20] Two frameshift, two splice site, and two missense mutations have also been found (table 1). One of the missense alleles, 1544T→C, I515T is associated with a temperature sensitive NSRAN phenotype. It was previously described in *cis* with another missense mutation 1469C→A, P490Q not detected in the families reported here.[21]

The nonsense mutation 2485C→T, Q829X appears to be the most common otoferlin mutant allele discovered to date (Rodriguez-Ballesteros *et al*,[3] Migliosi *et al*,[20] and this report). In the Spanish population, it is the third most common cause of genetic hearing loss in children.[3] Two splice site mutations, IVS28 −2A→C and IVS18 +1G→T, were discovered, but their consequences have not been investigated. A single base insertion 1886_1887 insA, K629fs and single base deletion 2348delG, G783fs have been detected. Both of these mutations are predicted to cause a frameshift with premature termination, which should result in mRNA destabilisation via nonsense mediated mRNA decay.[33]

The two missense mutations occur at amino acids that are conserved by identity in human, mouse, chicken, and zebrafish otoferlin. Missense mutations were considered pathological if: (*a*) their segregation was consistent with affected status, (*b*) if no such variant was found in the control population, and (*c*) the reference amino acid was conserved across vertebrate lines. The I515T mutation is discussed in detail below. The arginine to histidine change, R794H, seen in family 53510 occurs as part of a coiled coil region identified by the SMART/COILS algorithm,[34 35] which extends across otoferlin residues 792–820. This is a conservative change as both arginine and histidine are polar basic amino acids. However, arginine is more hydrophilic than histidine and differs significantly structurally. Coiled coil domains are characterised by alpha helixes that interact with one another, potentially forming a ''peptide Velcro'' interaction with other proteins.[36] Weakening the hydrophilic

interaction in such a structure could affect an important protein protein interaction disrupting otoferlin function. Marker analysis for the 53510 family was consistent and informative for linkage at DFNB9. None of the mutations considered pathological was detected in a hearing control population of at least 80 individuals.

This is the second study to discover a high degree of polymorphic variation at the *OTOF* locus.[3] All the polymorphisms found in the Spanish population were also found in the population described here, and 15 additional neutral variants were discovered. The primary criterion for describing a variant as neutral was the detection of that mutation in a set of normal hearing controls.

The proband in family 3467 has abnormal ABR, present OAE, and relatively normal hearing until she becomes febrile, when OAE remain normal but hearing degrades, and ABR worsens from abnormal with unidentified waves I–III and delayed latency of the wave IV–V complex to being totally absent. The amount of decline in hearing in the proband is dependent on the degree of fever. A mild to moderate hearing loss was present when she had a temperature of 37.8°C and a profound hearing loss was present at 38.1°C.

Only one mutation has been found in family 3467 thus far, 1544T→C, I515T. This amino acid is conserved in human, mouse, chicken, and zebrafish otoferlin. It is a missense mutation occurring in the otoferlin C2C domain, which was previously found in a consanguineous family from Eastern Turkey with profound prelingual hearing loss.[21] No mention was made of a temperature associated phenotype in this family. The affected members of the family from Eastern Turkey are homozygous for the I515T missense mutation and another missense mutation, 1469C→A, P490Q inherited in *cis*.

Both the P490Q and I515T mutations in the Turkish kindred were found in the C2C domain (third C2 domain), which is predicted to bind calcium.[21] The alignment of otoferlin and otoferlin related proteins revealed remarkable conservation of amino acids within the human and mouse C2C domains.[21] Mirghomizadeh *et al* predicted that either of these two mutations would severely disrupt the structure of the C2C domain, with the I515T mutation resulting in the creation of a new myristylation site.[21] Temperature sensitive mutations have been previously recognised in several human genetic diseases.[37–39]

Eight *OTOF* mutations were found in six families in this study. In previous studies, four mutations were found in three families[2] and 10 mutations were found in six families.[3] All other *OTOF* mutations described are in consanguineous families or in Spanish families homozygous for Q829X.[3 20] *OTOF* is a large gene with multiple isoforms coded by at least 48 known exons. The screening of this gene focused on the transcribed part of the gene that is predicted to code for protein and the intron sequence flanking the splice sites of individual exons. The screens employed in this study depended on PCR amplification of genomic DNA and assumes that both alleles are represented equally in the amplified product. Heterogeneity at this locus may interfere with specific PCR primers annealing to specific alleles, preventing the amplification of one allele but not the other.

This report summarises the clinical findings associated with eight *OTOF* mutations, five of them novel, in 65 NSRHL and NSRAN families. One of these families has an allele associated with a temperature sensitive AN/AD phenotype. A temperature sensitive allele for otoferlin should provide a valuable tool to understand the function of otoferlin and its role in the auditory process.

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