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RESEARCH ARTICLE

A Multicenter Study on the Prevalence and Spectrum of Mutations in the Otoferlin Gene (*OTOF*) in Subjects With Nonsyndromic Hearing Impairment and Auditory Neuropathy

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Autosomal recessive nonsyndromic hearing impairment (NSHI) is a heterogeneous condition, for which 53 genetic loci have been reported, and 29 genes have been identified to date. One of these, OTOF, encodes otoferlin, a membrane-anchored calcium-binding protein that plays a role in the exocytosis of synaptic vesicles at the auditory inner hair cell ribbon synapse. We have investigated the prevalence and spectrum of deafness-causing mutations in the OTOF gene. Cohorts of 708 Spanish, 83 Colombian, and 30 Argentinean unrelated subjects with autosomal recessive NSHI were screened for the common p.Gln829X mutation. In compound heterozygotes, the second mutant allele was identified by DNA sequencing. In total, 23 Spanish, two Colombian

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and two Argentinean subjects were shown to carry two mutant alleles of OTOF. Of these, one Colombian and 13 Spanish subjects presented with auditory neuropathy. In addition, a cohort of 20 unrelated subjects with a diagnosis of auditory neuropathy, from several countries, was screened for mutations in OTOF by DNA sequencing. A total of 11 of these subjects were shown to carry two mutant alleles of OTOF. In total, 18 pathogenic and four neutral novel alleles of the OTOF gene were identified. Haplotype analysis for markers close to OTOF suggests a common founder for the novel c.2905_2923delinsCTCCGAGCGCA mutation, frequently found in Argentina. Our results confirm that mutation of the OTOF gene correlates with a phenotype of prelingual, profound NSHI, and indicate that OTOF mutations are a major cause of inherited auditory neuropathy. Hum Mutat 29(6), 823–831, 2008.

KEY WORDS: hearing impairment; DFNB9; OTOF; otoferlin; auditory neuropathy; genetic epidemiology

INTRODUCTION

Hearing impairment (HI) is a highly heterogeneous group of disorders caused by environmental and genetic factors, with a global incidence of about 1 in every 650–1,000 newborns [Morton, 1991; Mehl and Thomson, 2002]. When the onset of the HI takes place before speech acquisition (prelingual HI), it represents a serious handicap for normal communication and social integration. In developed countries, over 60% of all cases result from a genetic cause [Petit et al., 2001]. Nonsyndromic HI (NSHI) encompasses a variety of disorders, the common feature of which is that the hearing deficit is not associated with any other clinical sign (about 70% of all inherited HI). Different patterns of inheritance are observed in NSHI, but autosomal recessive forms are by far the most frequent.

To date, 53 genetic loci and 29 genes have been involved in autosomal recessive NSHI (Hereditary Hearing Loss home page; http://webh01.ua.ac.be/hhh) [Petersen and Willems, 2006]. Molecular diagnosis is complicated by this extreme genetic heterogeneity. Mutations in the genes encoding connexin-26 (GJB2; MIM# 121011) and connexin-30 (GJB6; MIM# 604418), in the DFNB1 locus (MIM# 220290), are responsible for up to 50% of all cases of autosomal recessive NSHI [Kenneson et al., 2002; del Castillo et al., 2003]. However, the contribution of mutations in other genes is still under investigation, and the study is complicated by the fact that the genetic epidemiology of NSHI is widely variable among populations [Petit et al., 2001; Friedman and Griffith, 2003; Petersen and Willems, 2006].

The OTOF gene (MIM# 603681), encoding otoferlin, is 1 out of the 29 genes known to be involved in autosomal recessive NSHI [Yasunaga et al., 1999]. It is located in the DFNB9 locus (MIM# 601071) on 2p23.1. The gene contains 49 exons (named 1–48 and 5′UTRsf1), and it encodes multiple long and short isoforms of the protein, by alternative splicing combined with the use of several translation initiation sites [Yasunaga et al., 2000]. The first 19 exons are exclusive of the long isoforms. Otoferlin belongs to a family of membrane-anchored cytosolic proteins that contain several repeats of a calcium-binding structural module (the C2 domain) [Rizo and Südhof, 1998], and which are involved in vesicle membrane fusion. Otoferlin long isoforms have six C2 domains, whereas the short isoforms have only three C2 domains. Otoferlin is expressed in cochlea, vestibule and brain [Yasunaga et al., 1999, 2000], and it seems to play a role in the exocytosis of synaptic vesicles at the auditory inner hair cell ribbon synapse [Roux et al., 2006].

To date, 24 different pathogenic mutant alleles of OTOF have been reported in subjects with autosomal recessive NSHI [Yasunaga et al., 1999, 2000; Adato et al., 2000; Houseman et al., 2001; Migliosi et al., 2002; Mirghomizadeh et al., 2002; Rodríguez-Ballesteros et al., 2003; Varga et al., 2003, 2006; Hutchin et al., 2005; Tekin et al., 2005; Rouillon et al., 2006]. Most of these mutations are private, each one being reported in only one family. A remarkable exception is c.2485C>T (p.Gln829X), found in about 3% of cases of autosomal recessive NSHI in the Spanish population, this high frequency being due to a common founder [Migliosi et al., 2002; Rodríguez-Ballesteros et al., 2003]. Clinical study of subjects carrying two mutant alleles of OTOF revealed a very homogeneous phenotype of profound prelingual HI, younger individuals presenting with signs of auditory neuropathy, i.e., preserved otoacoustic emissions (OAEs) but absent or grossly abnormal auditory brainstem responses (ABRs) [Starr et al., 1996; Rodríguez-Ballesteros et al., 2003; Varga et al., 2003]. Here we report on the results of an international multicenter study on the prevalence and spectrum of mutations in OTOF, as well as on the genotype–phenotype correlations in subjects with this subtype of NSHI.

SUBJECTS AND METHODS

Subjects

We enrolled 821 unrelated subjects with bilateral sensorineural NSHI, from pedigrees in which the mode of inheritance was compatible with an autosomal recessive pattern. These included 708 probands from Spain (283 multiplex cases and 425 simplex cases), 83 from Colombia (30 multiplex, 53 simplex), and 30 from Argentina (10 multiplex, 20 simplex) (Table 1). The Spanish cohort included 15 subjects with a clinical diagnosis of auditory neuropathy (14 with profound HI, one with severe HI).

In addition, we enrolled a cohort of 20 unrelated subjects specifically selected because of having autosomal recessive NSHI and auditory neuropathy, from several countries, as indicated: three Argentinean, one Austrian, one French, six German, seven Italian, one Lebanese, and one Libyan. A total of 16 of these subjects had profound HI, three had severe HI (one Italian and two German cases), and one had moderate HI (one Italian case). A total of five cases were multiplex (one Argentinean, one German, one Italian, one Lebanese, and one Libyan), and the other 15 were simplex.

Written informed consent was obtained from all the subjects included in the study. None of the hearing-impaired subjects had

TABLE 1. **Composition of the Cohorts of Subjects With NSHI Included in this Study**

			Severity of the hearing impairment (%)			
Origin	N	Age of onset	Mild	Moderate	Severe	Profound
Spain	708	71% prelingual	1	17	14	39
		29% postlingual	4	12	6	7
Colombia	83	87% prelingual	0	4	17	66
		13% postlingual	1	6	4	2
Argentina	30	100% prelingual	0	15	8	77

syndromic features, according to their history and findings on clinical examination. Subjects whose HI might have an environmental cause were not included in the study. Special attention was paid to exclude environmental causes of auditory neuropathy, such as severe neonatal hyperbilirubinemia (kernicterus), neonatal hypoxia, and prematurity with low birth weight [Starr et al., 1996; Cone-Wesson and Rance, 2000; Rapin and Gravel, 2003].

Clinical Tests

HI was evaluated by at least one of these techniques, depending on suitability and availability: 1) pure-tone audiometry, testing for air conduction (frequencies 125–8,000 Hz) and for bone conduction (frequencies 250–4,000 Hz); 2) ABRs; 3) transient-evoked OAEs (TEOAEs). The degree of the HI was determined by calculating the binaural mean of the hearing thresholds for air conduction at frequencies 0.5, 1, and 2 kHz, and it was classified as mild (average thresholds in the range of 21–40 dB), moderate (41–70 dB), severe (71–90 dB), or profound (>90 dB). Auditory neuropathy was diagnosed on the basis of absent or grossly abnormal ABRs and preserved TEOAEs [Starr et al., 1996].

Genetic Techniques

DNA was extracted from peripheral blood samples by standard procedures. Genetic tests for mutations in the GJB2 gene and for the large deletions affecting the GJB6 gene at the DFNB1 locus were performed as published [Álvarez et al., 2005; del Castillo et al., 2005]. The specific test for detection of the p.Gln829X mutation was performed as described [Migliosi et al., 2002]. Primers and conditions for polymerase chain reaction (PCR) amplification and DNA sequencing of each exon of the OTOF gene have been reported [Migliosi et al., 2002; Rodríguez-Ballesteros et al., 2003]. Genotyping of microsatellite markers D2S158, D2S2223, D2S2350 and D2S174 was performed as previously described [Dib et al., 1996; Migliosi et al., 2002].

Specific tests were developed for the detection of the OTOF sequence variants that were found during this study. They included restriction fragment length polymorphism (RFLP) assays and genotyping of single-nucleotide polymorphisms (SNPs) by the SNaPshot methodology in an ABI 3130 Genetic Analyzer, as recommended (Applied Biosystems, Foster City, CA). Conditions and sequences of the primers used in each test are available upon request.

Nomenclature of mutations is based on cDNA sequence (GenBank accession number AF183185.1), the A of the translation initiation codon being considered as +1.

RESULTS

We screened a group of 821 unrelated probands with NSHI, composed of 708 Spanish subjects (including 15 subjects with a clinical diagnosis of auditory neuropathy), 83 Colombian subjects, and 30 Argentinean subjects, for mutation c.2485C>T (p.Gln829X) in the OTOF gene. In the Spanish cohort we found 15 homozygotes and 11 heterozygotes for p.Gln829X. Sequencing of the entire coding region and all the exon–intron junctions of the OTOF gene revealed the accompanying mutation in 8 of these 11 heterozygotes (Table 2). In total, 13 of the 15 Spanish cases with a diagnosis of auditory neuropathy carried two mutant alleles of

TABLE 2. Hearing-Impaired Subjects Carrying Two Mutant Alleles of OTOF.

Case	Type	Age (years)[a]	Genotype	Onset	Severity	TEOAEs	Origin
S837	Multiplex	23	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Not tested	Spain
S907	Multiplex	11	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Absent	Spain
S973	Multiplex	6	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Not tested	Spain
S1248	Multiplex	3	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Not tested	Spain
S1311	Multiplex	4	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Present	Spain
S1332	Multiplex	5	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Present	Spain
S1351	Multiplex	25	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Not tested	Spain
S1356	Multiplex	32	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Not tested	Spain
E394	Multiplex	1	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Present	Spain
S917	Simplex	10	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Present	Spain
S1379	Simplex	5	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Present	Spain
E425	Simplex	3	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Present	Spain
E440	Simplex	2	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Present	Spain
E442	Simplex	2	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Present	Spain
E487	Simplex	1	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Present	Spain
S1285	Simplex	25	[p.Gln829X] + [c.2348delG]	Prelingual	Profound	Not tested	Spain
E411	Simplex	5	[p.Gln829X] + [c.4227+1G>T]	Prelingual	Profound	Present	Spain
E423	Simplex	3	[p.Gln829X] + [c.2684_2685delGG]	Prelingual	Profound	Present	Spain
E447	Simplex	1	[p.Gln829X] + [c.1236delC]	Prelingual	Profound	Absent	Spain
E528	Simplex	2	[p.Gln829X] + [p.Cys883X]	Prelingual	Profound	Absent	Spain
E601	Simplex	3	[p.Gln829X] + [c.1180dupG]	Prelingual	Profound	Present	Spain
E620	Simplex	2	[p.Gln829X] + [c.5800dupC]	Prelingual	Profound	Absent	Spain
E722	Simplex	2	[p.Gln829X] + [c.5011dupT]	Prelingual	Profound	Present	Spain
13NS	Simplex	17	[p.Gln829X] + [p.Gln829X]	Prelingual	Profound	Absent	Colombia
32NS	Multiplex	7	[p.Gln829X] + [p.Arg708X]	Prelingual	Profound	Present	Colombia
AEF05	Simplex	13	[p.Gln829X] + [c.2905_2923delinsCTCCGAGCGCA]	Prelingual	Profound	Absent	Argentina
AEF27	Simplex	12	[p.Gln829X] + [c.2905_2923delinsCTCCGAGCGCA]	Prelingual	Profound	Not tested	Argentina
NASF12	Multiplex	2	[c.4227+1G>T] + [c.4227+1G>T]	Prelingual	Profound	Present	Argentina
NAEF22	Simplex	4	[c.4227+1G>T] + [c.2905_2923delinsCTCCGAGCGCA]	Prelingual	Profound	Present	Argentina
E552	Simplex	4	[c.1601delC] + [c.2905_2923delinsCTCCGAGCGCA]	Prelingual	Profound	Present	Argentina
AU-231	Simplex	6	[c.1601delC] + [p.Leu1138Pro]	Prelingual	Profound	Present	Austria
AL-4	Simplex	1	[p.Gln829X] + [p.Arg1495X]	Prelingual	Profound	Present	Germany
AL-6	Simplex	6	[p.Leu1138Pro]+ [p.Gly1451X]	Prelingual	Profound	Present	Germany
IT-1	Simplex	2	[p.Arg1134X] + [p.Gln255His]	Prelingual	Profound	Present	Italy
E815	Simplex	2	[p.Phe1795Cys] + [p.Phe1795Cys]	Prelingual	Profound	Present	Italy
S1398	Multiplex	1	[c.2732_2735dupAGCT] + [p.Ala964Glu]	Prelingual	Profound	Present	Italy
S1416	Multiplex	4	[p.Tyr1497X] + [p.Tyr1497X]	Prelingual	Profound	Present	Lebanon
S1216	Multiplex	2	[p.Glu747X] + [p.Glu747X]	Prelingual	Profound	Present	Libya

[a]Age at which the genetic study and TEOAE testing were performed. Nomenclature of mutations is based on cDNA sequence (GenBank accession number AF183185.1), the A of the translation initiation codon being considered as +1.

OTOF. In the Colombian cohort, we found one homozygote and two heterozygotes for p.Gln829X. DNA sequencing revealed the second mutant allele in 1 of these 2 heterozygotes. Subsequently, the clinical reevaluation of the two Colombian cases with two mutant alleles of OTOF showed the presence of normal TEOAEs in one of them (Table 2). Finally, in the Argentinean cohort, we found two heterozygotes for p.Gln829X, and the second mutant allele was identified in both cases by DNA sequencing (Table 2). None of the subjects with two mutant alleles of OTOF had any pathogenic mutation in the DFNB1 locus. One Spanish subject heterozygous for p.Gln829X, but without second mutant allele of OTOF, was shown to carry two mutant alleles of GJB2.

We investigated the two unelucidated Spanish cases of auditory neuropathy as well as a cohort of 20 unrelated subjects with auditory neuropathy from other countries, by sequencing the coding region and exon–intron junctions of the OTOF gene. No pathogenic mutation was found in the two Spanish cases. On the contrary, this study did reveal that 11 of the 20 subjects from the cohort with auditory neuropathy from other countries carried two mutant alleles of OTOF (Table 2; last 11 rows).

In all cases, segregation analysis in the pedigree confirmed that the two mutations reported in each affected subject were carried in *trans*. Besides p.Gln829X, we found the previously reported mutations p.Arg708X [Rodríguez-Ballesteros et al., 2003], c.2348delG [Varga et al., 2006], and p.Tyr1497X [Yasunaga et al., 1999]. In addition, we identified 18 novel pathogenic sequence variants in the OTOF gene (Table 3). They included eight mutations leading to frameshifts, seven of them resulting in premature stop codons (three deletions, c.1236delC, c.1601delC, and c.2684_2685delGG; one indel mutation, c.2905_2923delinsCTCCGAGCGCA (Supplementary Fig. S1, available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat); and three duplications, c.1180dupG, c.2732_2735dupAGCT, and c.5011dupT). The other frameshift mutation (c.5800dupC, in exon 46) results in the bypass of the normal stop codon, which is predicted to add abnormal C-terminal tails of 251 residues to protein isoforms synthesized from transcripts with exon 47, or tails of 185 residues to isoforms from transcripts without exon 47. In both types of isoforms, it eliminates the stretch of leucine residues in the hydrophobic C-terminus that is thought to form the

TABLE 3. **Pathogenic Sequence Variants Reported to Date in the OTOF Gene***

Location	DNA level	Protein level	Reference
Mutations affecting only the long isoforms			
Exon 8	c.709C>T	p.Arg237X	Houseman et al. [2001]
Intron 8	c.711-2A>G		Yasunaga et al. [2000]
Exon 9	c.765G>C	p.Gln255His	This work
Exon 13	c.1180dupG	p.Glu394GlyfsX6	This work
Exon 14	c.1236delC	p.Glu413AsnfsX9	This work
Exon 15	[c.1469C>A; c.1544T>C]	[p.Pro490Gln; p.Ile515Thr]	Mirghomizadeh et al. [2002]
Exon 16	c.1601delC	p.Pro534GlnfsX4	This work
Exon 16	c.1651delG	p.Glu551SerfsX5	Varga et al. [2003]
Exon 17	c.1886dupA	p.Pro630AlafsX5	Varga et al. [2006]
Intron 18	c.2093+1G>T		Varga et al. [2006]
Exon 19	c.2122C>T	p.Arg708X	Rodríguez-Ballesteros et al. [2003]; this work
Mutations affecting both the long and the short isoforms			
Exon 20	c.2239G>T	p.Glu747X	This work
Exon 21	c.2348delG	p.Gly783AlafsX17	Varga et al. [2006]; this work
Exon 22	c.2485C>T	p.Gln829X	Migliosi et al. [2002]; Rodríguez-Ballesteros et al. [2003]; Rouillon et al. [2006]; Varga et al. [2006]; this work
Exon 23	c.2649C>A	p.Cys883X	This work
Exon 24	c.2684.2685delGG	p.Gly895GlufsX106	This work
Exon 24	c.2732.2735dupAGCT	p.Tyr913AlafsX90	This work
Intron 24	c.2866+1G>A		Adato et al. [2000]
Exon 25	c.2887C>T	p.Arg963X	Hutchin et al. [2005]
Exon 25	c.2891C>A	p.Ala964Glu	This work
Exon 25	c.2905.2923delinsCTCCGAGCGCA	p.Ala969LeufsX30	This work
Exon 26	c.3032T>C	p.Leu1011Pro	Tekin et al. [2005]
Exon 28	c.3400C>T	p.Arg1134X	This work
Intron 28	c.3409-2A>G		Varga et al. [2006]
Exon 29	c.3413T>C	p.Leu1138Pro	This work
Intron 35	c.4227+1G>T		This work
Exon 36	c.4275G>A	p.Trp1425X	Rodríguez-Ballesteros et al. [2003]
Exon 36	c.4351G>T	p.Gly1451X	This work
Intron 36	c.4362+2T>G		Rodríguez-Ballesteros et al. [2003]
Exon 37	c.4483C>T	p.Arg1495X	This work
Exon 37	c.4491T>A	p.Tyr1497X	Yasunaga et al. [1999]; this work
Exon 38	c.4559G>A	p.Arg1520Gln	Rouillon et al. [2006]
Intron 39	c.4799+1G>C		Varga et al. [2003]
Exon 41	c.5011dupT	p.Trp1671LeufsX73	This work
Exon 44	c.5384T>G	p.Phe1795Cys	This work
Exon 44	c.5473C>G	p.Pro1825Ala	Migliosi et al. [2002]
Intron 44	c.5533+1G>A		Rouillon et al. [2006]
Exon 46	c.5800dupC	p.Leu1934ProfsX251[a]	
		p.Leu1934ProfsX185[b]	This work
Exon 48	c.5816G>A	p.Arg1939Gln[b]	Varga et al. [2003]
Exon 48	c.5860.5862delATC	p.Ile1954del[b]	Rodríguez-Ballesteros et al. [2003]
Exon 48	c.5960C>G	p.Pro1987Arg[b]	Varga et al. [2003]

*Nomenclature of mutations is based on cDNA sequence (GenBank accession number AF183185.1), the A of the translation initiation codon being considered as +1.
[a]Isoforms with exon 47.
[b]Isoforms without exon 47.

transmembrane domain of otoferlin. The list of novel pathogenic mutations also includes one splice site mutation (c.4227+1G>T, in the donor site of intron 35, most likely resulting in skipping of exon 35), five nonsense mutations (p.Glu747X, p.Cys883X, p.Arg1134X, p.Gly1451X, and p.Arg1495X), and four missense mutations (p.Gln255His, p.Ala964Glu, p.Leu1138Pro, and p.Phe1795Cys). These missense mutations represent nonconservative changes that affect conserved residues (Supplementary Fig. S2). A total of three of them (p.Gln255His, p.Ala964Glu, and p.Phe1795Cys) lie in C2 domains (C2B, C2D, and C2F, respectively). In addition, p.Gln255His, p.Ala964Glu, and p.Leu1138Pro (twice) were found in subjects carrying in *trans* a clearly pathogenic allele (Table 2). None of these 18 novel pathogenic mutations was found in at least 50 control Spanish subjects with normal hearing (100 chromosomes). To rule out that the novel missense mutations might be polymorphisms frequent in populations other than the Spanish one, we screened additional population-matched control subjects with normal hearing: 56 Italian controls (112 chromosomes) for mutations p.Gln255His, p.Ala964Glu, and p.Phe1795Cys; and 56 Austrian controls (112 chromosomes) for p.Leu1138Pro. These mutations were not found either in these additional sets of controls.

A total of 14 of the 18 novel pathogenic mutations described here were found each in only one pedigree in this study. Only four were found repeatedly: c.1601delC (two pedigrees), c.2905_2923delinsCTCCGAGCGCA (four pedigrees), c.4227+1G>T (three pedigrees), and p.Leu1138Pro (two pedigrees). To investigate their evolutionary origins, we genotyped the families carrying these mutations for four microsatellite markers intragenic (D2S2350) or very close (D2S158, D2S2223, and D2S174) to the OTOF gene [Dib et al., 1996], and haplotype analysis was performed (Fig. 1). A single haplotype (276-194-94-219) is associated with c.2905_2923delinsCTCCGAGCGCA in all four unrelated Argentinean pedigrees carrying this mutation. Similarly, a single haplotype (276-198-94-219) is associated with c.1601delC in the two pedigrees (one from Austria, one from Argentina) segregating this mutation. As for c.4227+1G>T, it is associated with a single haplotype (276-196-92-219) in two Argentinean pedigrees. In the Spanish pedigree carrying c.4227+1G>T, and in the German pedigree carrying p.Leu1138Pro, the associated haplotypes could not be determined unambiguously.

In a previous study we reported a common founder for p.Gln829X in the Spanish population [Migliosi et al., 2002]. In this work, we have found this mutation in two Argentinean and two Colombian families. The haplotype associated with p.Gln829X in these families (276-196-98-205) is the same as that reported in the Spanish population, supporting the hypothesis of a common origin (Fig. 1).

A total of 21 different nonpathogenic sequence variants were also found in this screening, including 13 silent and eight missense polymorphisms (Supplementary Table S1). Of them, four were novel, two being silent (p.Ser1395Ser, p.Thr1444Thr) and two others being missense (p.Arg1236Gln, p.Thr1688Lys). The frequency of the nonpathogenic missense alleles found in this study was determined in control subjects with normal hearing (Supplementary Table S1).

DISCUSSION

Our results expand the spectrum of sequence variants in the OTOF gene. It now includes 42 pathogenic mutations (Table 3; Fig. 2) and 36 neutral variants (Supplementary Table S1). Although there is not a hotspot for pathogenic mutations in the gene, they are clustered mainly between exons 13 and 29 (23 mutations) and between exons 35 and 48 (15 mutations). Nonpathogenic sequence variants follow a similar distribution, with a majority of changes between exons 16 and 27 (14 variants) and between exons 35 and 45 (13 variants). This information should be helpful when planning effective diagnostic strategies.

Most of the pathogenic mutations (34/42) have been described each in only one family. Four other mutations, c.1601delC, p.Arg708X, c.2348delG, and p.Leu1138Pro, have been found each

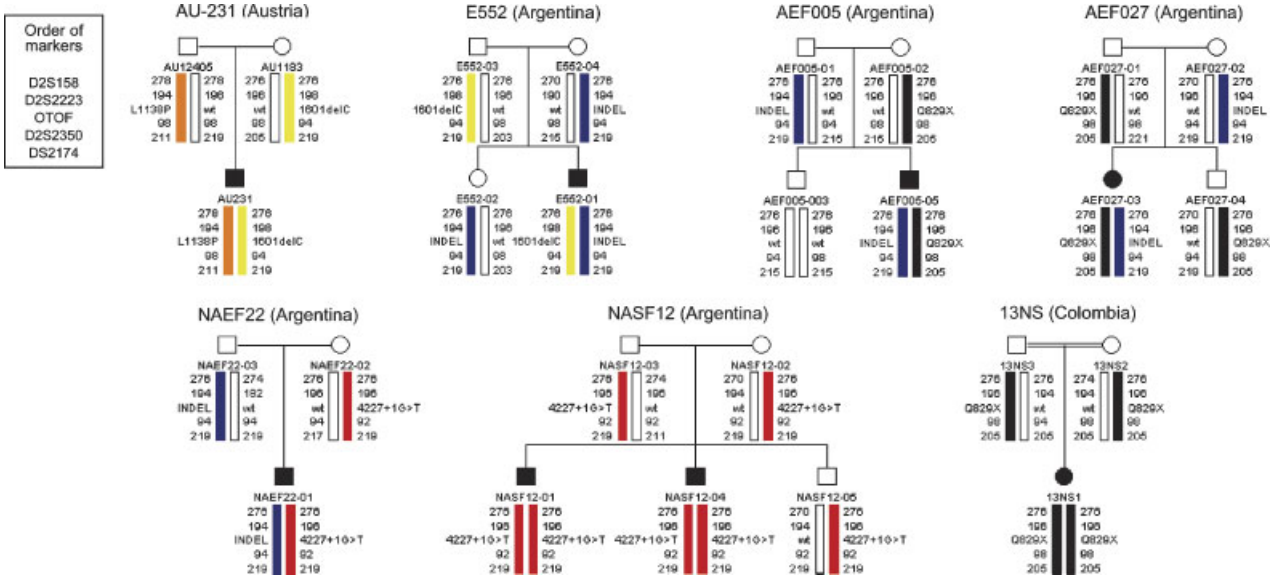

FIGURE 1. **Haplotype analysis of families segregating the mutations that were found repeatedly in this work. The code and geographical origin of each family are shown above the pedigree. Allele numbers indicate allele size, using as reference CEPH subject 134702 [Dib et al., 1996]. Haplotypes are indicated by vertical bars. The haplotype associated with each mutation is indicated with a distinctive color: orange, p.Leu1138Pro; yellow, c.1601delC; blue, c.2905_2923delinsCTCCGAGCGCA (abbreviated as INDEL); black, p.Gln829X; red, c.4227+1G>T.**

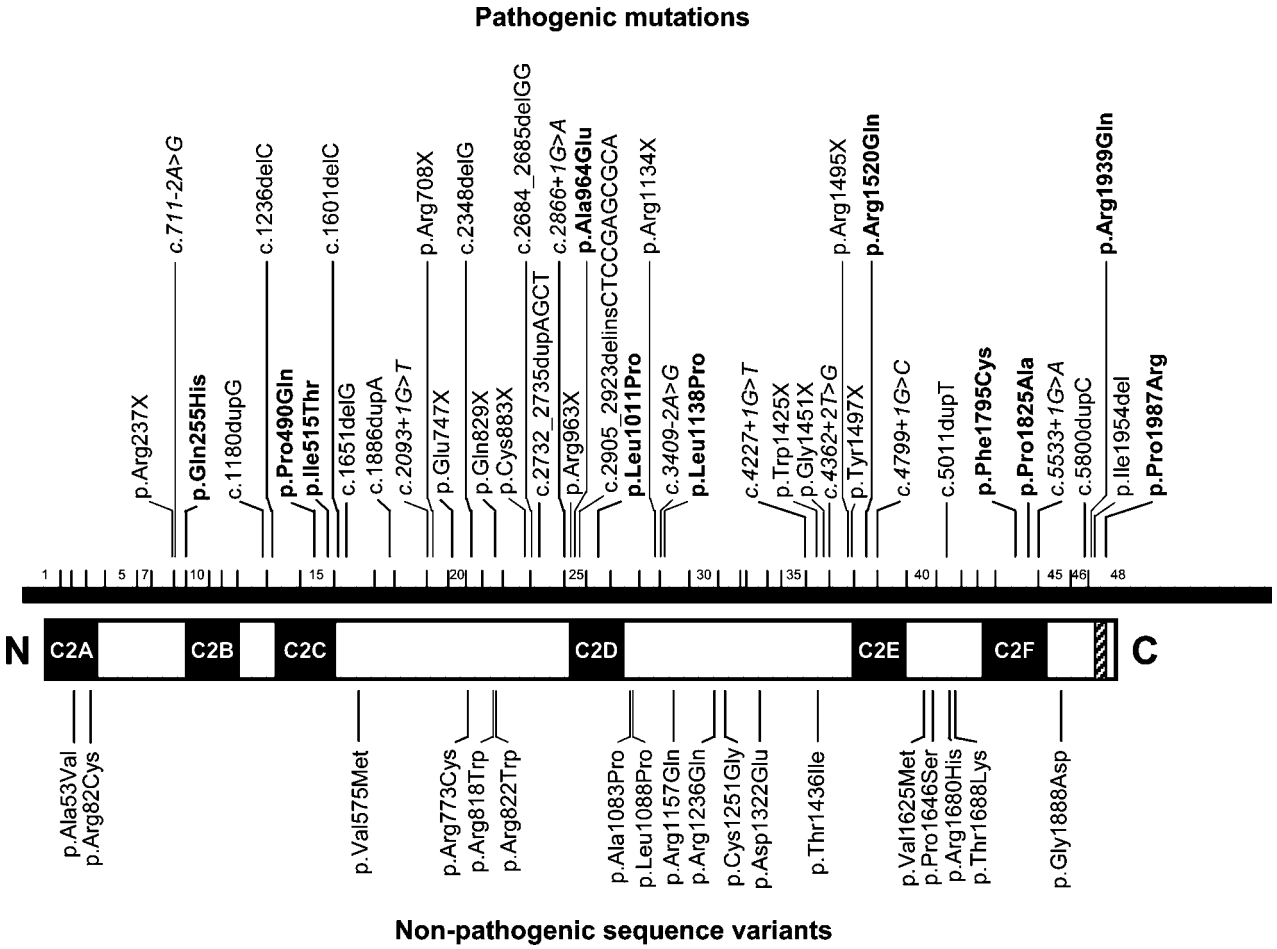

FIGURE 2. **Location of sequence variants in the coding region of the OTOF gene. Vertical lines indicate the position of each mutation on the reference cDNA (accession number AF183185.1) and on the correlated position in the protein. A horizontal bar depicts the cDNA, small vertical bars delimiting the exons, which are numbered. The reference cDNA does not contain exon 6. Exon 47 has been removed from the figure to show the effects of the mutations in exon 48 on the cochlear isoforms of otoferlin. The protein is represented by a rectangle, boxes indicating the predicted domains of the protein. Black boxes, C2 domains; cross-hatched box, transmembrane domain. All pathogenic mutations known to date are shown, but only polymorphisms resulting in amino acid substitutions are depicted. Missense pathogenic mutations are shown in bold, splice site mutations are in italics. The position of splice site mutations is indicated on the border between the exons flanking the affected intron.**

in two families, and c.4227+1G>T has been found in three. On the other hand, mutations p.Tyr1497X (with a common founder in the Lebanese population) [Yasunaga et al., 1999] and c.2905_2923delinsCTCCGAGCGCA appear repeatedly in several families, whereas p.Gln829X is the mutation which has been most frequently reported. It has been found in unrelated families from Spain (47 cases), Argentina (two cases), Colombia (two cases), Cuba (one case), France (two cases), Germany (one case), the United Kingdom (one case), and the United States (one case) [Migliosi et al., 2002; Rodríguez-Ballesteros et al., 2003; Rouillon et al., 2006; Varga et al., 2006; and this work]. The analysis of four polymorphic microsatellite markers close to the OTOF gene has allowed us to identify a single haplotype associated with p.Gln829X in the Spanish, Argentinean, Colombian, and Cuban families, revealing a common founder of Spanish ancestry [Migliosi et al., 2002] (and this work). This could be also the case for the family carrying the mutation in the United States, of Mexican origin [Varga et al., 2006]. The four cases from France, Germany, and the United Kingdom could be due to the radiation of the mutation from Spain or they could represent independent mutational events.

As for c.2905_2923delinsCTCCGAGCGCA, it has been found in four unrelated Argentinean cases, all of them sharing the same haplotype associated to the mutation, suggesting also a common founder. Although the presence of this mutation has not been specifically investigated in large cohorts of Spanish subjects with nonsyndromic HI, it has not been detected to date in the compound heterozygous state with the highly prevalent p.Gln829X mutation, suggesting that the common founder is not of Spanish origin. It has also not been found in our three Italian cases carrying two mutant alleles of OTOF, but this sample size is too small to exclude an Italian origin. In fact, this study and numerous other reports support the existence of many differences among the spectra of deafness-causing mutations, as well as in the ranking of the most prevalent ones in different populations, even from neighboring geographical regions. Founder effects are major contributors to these differences, as shown by this work and other studies [Friedman et al., 2003]. Accordingly, the prevalence of each different clinical subtype of HI, such as auditory neuropathy, is predictable to be highly variable among populations.

Most of the pathogenic mutations in OTOF (30/42 mutant alleles, including nonsense point mutations, deletions, indels, duplications, and splice-site mutations) are predicted to result in the synthesis of truncated polypeptides, in the synthesis of polypeptides carrying longer and abnormal C-terminal tails, or in no protein synthesis at all because of the action of nonsense-mediated mRNA decay mechanisms. Therefore, these sequence variants must be considered inactivating mutations. Only 12 nontruncating pathogenic mutations have been described in OTOF, including 11 missense mutations and one in-frame deletion removing the Ile-1954 amino acid residue. Interestingly, all but one of these mutations affects the predicted functional domains of otoferlin. A total of eight mutations affect conserved residues in the calcium-binding domains: C2B (p.Gln255His), C2C ([p.Pro490Gln; p.Ile515Thr] and p.Ile515Thr), C2D (p.Ala964-Glu and p.Leu1011Pro), C2E (p.Arg1520Gln), and C2F (p.Phe1795Cys and p.Pro1825Ala). The three other mutations (p.Arg1939Gln, p.Ile1954del, and p.Pro1987Arg) affect the transmembrane domain of otoferlin. On the contrary, 16 out of the 18 nonpathogenic missense sequence variants do not affect any of the predicted functional domains of otoferlin. The exceptions are variants p.Ala53Val and p.Arg82Cys, which are located in C2A, an incomplete domain that lacks the β1 strand of the canonical C2 domain [Yasunaga et al., 2000]. This distribution of nontruncating pathogenic mutations and missense neutral polymorphisms confirms the importance of the C2 domains B to F and the transmembrane anchor for the function of otoferlin. Consistently, a missense mutation in the homologous murine gene (Otof), affecting the C2F domain of otoferlin, is the cause of deafness in the *pachanga* mouse [Schwander et al., 2007].

In a previous study, we reported that mutations in the OTOF gene are responsible for a very homogeneous phenotype of prelingual, profound NSHI, without associated inner ear malformations. In addition, many affected subjects presented with auditory neuropathy [Rodríguez-Ballesteros et al., 2003]. In this work, we have further substantiated this correlation. Among our Spanish, Colombian, and Argentinean subjects with autosomal recessive NSHI, prelingual profound HI accounts for only 39%, 66%, and 77% of the cases, respectively (Table 1). Significantly, all subjects carrying two mutant alleles of OTOF (23, 2, and 2, respectively) belong to this group (Table 2). It should be noted that the two Spanish cases carrying pGln829X in the heterozygous state and lacking a second mutated OTOF allele have postlingual moderate NSHI and prelingual severe NSHI, respectively, which do not coincide with the expected DFNB9 phenotype, and so most likely they are just coincidental carriers of this prevalent mutation. Of note, according to our specific screening for p.Gln829X, OTOF mutations could be responsible for at least 8% (23/276) of the cases of autosomal recessive prelingual, profound NSHI in the Spanish population.

Auditory neuropathy is a distinctive clinical sign in subjects with DFNB9 HI. Among the 23 Spanish subjects with two mutant alleles of OTOF, TEOAEs were present in 13 cases (56.5%), were absent in four cases (17.5%), and could not be tested in six cases (26%) (Table 3). A similar result was obtained in our previous study, showing that normal TEOAE records could be obtained from 11 out of 21 (52.4%) subjects with two mutant alleles of OTOF [Rodríguez-Ballesteros et al., 2003]. In an affected child ascertained during that study, we could determine that TEOAEs were present in both ears at age 19 months, but they had been lost by age 26 months [Rodríguez-Ballesteros et al., 2003]. The variability in the preservation of TEOAEs may result from the action of modifier factors, environmental or genetic, which remain to be identified. Of note, it seems that there is not a specific type of mutation in the OTOF gene which is responsible for auditory neuropathy. As shown in this work, it has been observed in subjects carrying any combination of mutations, i.e., truncating/truncating (the most prevalent subgroup), truncating/nontruncating, and nontruncating/nontruncating, as well as in subjects carrying two mutations affecting all otoferlin isoforms, or one mutation affecting all isoforms and the other affecting only the long isoforms. It should be noted that auditory neuropathy was not reported in the only three unrelated cases in the literature which carry two mutations affecting only the long isoforms [Houseman et al., 2001; Yasunaga et al., 2000; Mirghomizadeh et al., 2002], but they were not specifically tested for this condition. Although auditory neuropathy is also observed in other genetic conditions (see below), the finding of this clinical sign is a helpful clue to direct the genetic study of the subject to the screening for OTOF mutations. Consistently with data obtained in humans [Rodríguez-Ballesteros et al., 2003; Varga et al., 2003], mutant mice defective for otoferlin have auditory neuropathy [Roux et al., 2006; Schwander et al., 2007].

Auditory neuropathy can be caused by environmental or genetic factors. It can be part of the clinical signs of a systemic neurodegenerative disease (Charcot-Marie-Tooth peripheral neuropathy, Friedreich ataxia, mitochondrial disorders, etc.), or constitute an isolated clinical entity. To date, two genes and one genetic locus have been involved in isolated auditory neuropathy: OTOF and DFNB59 (the gene encoding pejvakin on 2q31) [Delmaghani et al., 2006] in autosomal recessive subtypes, and the AUNA1 locus on 13q14–q21, in an autosomal dominant form [Kim et al., 2004]. Our results suggest that mutations in OTOF are a major cause of isolated auditory neuropathy. In the Spanish cohort of subjects with auditory neuropathy, 13 out of 15 subjects (87%) carried two mutant alleles of OTOF. In the cohort from other countries, this result was obtained in 11 out of 20 subjects (55%). Differences between these cohorts are due mainly to the high frequency of p.Gln829X in Spain. As discussed above, founder effects are responsible for the different prevalences of the various subtypes of NSHI between populations.

In auditory neuropathy, preservation of TEOAEs indicates a normal function of the outer hair cells in the organ of Corti [Kemp, 2002]. Therefore, the primary lesion can be located in the inner hair cells, in the auditory nerve, or in the synapse in between [Cone-Wesson and Rance, 2000; Starr et al., 2000; Rapin and Gravel, 2003]. In the organ of Corti of adult mice, otoferlin is expressed almost exclusively in the inner hair cells [Yasunaga et al., 1999; Roux et al., 2006]. Based on these data and on the good outcome of cochlear implantation in subjects carrying two mutant alleles of OTOF [Rodríguez-Ballesteros et al., 2003; Rouillon et al., 2006], we hypothesized that the primary lesion in this subtype of auditory neuropathy would be cochlear, specifically located in the inner hair cells [Rodríguez-Ballesteros et al., 2003]. Consistently, recent data suggest that otoferlin is required for the exocytosis of the synaptic vesicles generated in the inner hair cells, at the auditory ribbon synapse [Roux et al., 2006]. Functional analysis of the missense mutations in OTOF that are found when screening subjects with NSHI and auditory neuropathy will contribute to advances in understanding of the precise role of otoferlin in these physiological processes.

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