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Identities and frequencies of mutations of the otoferlin gene (*OTOF*) causing DFNB9 deafness in Pakistan

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Abstract

Mutations in *OTOF*, encoding otoferlin, cause non-syndromic recessive hearing loss. The goal of our study was to define the identities and frequencies of *OTOF* mutations in a model population. We screened a cohort of 557 large consanguineous Pakistani families segregating recessive, severe-to-profound, prelingual-onset deafness for linkage to *DFNB9*. There were 13 families segregating deafness consistent with linkage to markers for *DFNB9*. We analyzed the genomic nucleotide sequence of *OTOF* and detected probable pathogenic sequence variants among all 13 families. These include the previously reported nonsense mutation p.R708X and 10 novel variants: 3 nonsense mutations (p.R425X, p.W536X, and p.Y1603X), 1 frameshift (c.1103_1104delinsC), 1 single amino acid deletion (p.E766del) and 5 missense substitutions of conserved residues (p.L573R, p.A1090E, p.E1733K, p.R1856Q and p.R1939W). *OTOF* mutations thus account for deafness in 13 (2.3%) of 557 Pakistani families. This overall prevalence is similar, but the mutation spectrum is different from those for Western populations. In addition, we demonstrate the existence of an alternative splice isoform of *OTOF* expressed in the human cochlea. This isoform must be required for human hearing because it encodes a unique alternative C-terminus affected by some *DFNB9* mutations.

Keywords

DFNB9; hearing; *OTOF*; otoferlin

Mutations in *OTOF*, encoding otoferlin, cause non-syndromic recessive hearing loss at the *DFNB9* locus (1). *OTOF*-related hearing loss (OMIM 60381) is frequently associated with intact otoacoustic emissions as part of a distinctive phenotype referred to as auditory neuropathy/dys-synchrony (AN/AD) (2–6). There are different hypotheses for the pathogenesis of the DFNB9 phenotype (7–10): some, but not all, authors believe that it reflects a role for otoferlin solely in exocytosis at the cochlear inner hair cell synapse.

There are ‘long’ and ‘short’ isoforms of *OTOF* that result from utilization of alternative transcription start sites either at exon 1 for the long isoform of *OTOF* or in two locations in

the sequence of exon 20, respectively (11). The long isoform encodes a protein containing six predicted C2 domains and a C-terminal transmembrane domain (11). The long isoform is thought to be required for normal hearing because it is affected by all *DFNB9* mutations, whereas only some mutations also affect the short isoform (11).

Alternative splicing also contributes to *OTOF* transcript diversity. There are two classes of transcripts in which either exon 47 or exon 48 encodes alternative C-termini and translation stop codons (11). The only reported human full-length long isoform is a brain-derived transcript that utilizes exon 47 (GenBank AF183185.1), whereas other human (brain, kidney and heart) transcripts utilizing exon 48 have been detected only in short isoforms (11). The existence of transcripts utilizing exon 48 in human cochlea was initially suggested by the presence of such a transcript in mouse cochlea (11) and further supported by the detection of probable *DFNB9* mutations in the open reading frame encoded by exon 48 (2, 3), although such a transcript has not been reported in human cochlea.

Rodríguez-Ballesteros et al. demonstrated that the p.Q829X mutation of *OTOF* accounts for about 5.1% of recessive prelingual deafness in the Spanish population (3). More recently, biallelic *OTOF* mutations were identified in approximately 3.1% of Spanish and Caucasian families segregating recessive non-syndromic hearing loss (5, 6). However, these studies may have overestimated the prevalence of *DFNB9* hearing loss due to an ascertainment bias for subjects with a clinical diagnosis of AN/AD.

Our previously reported Pakistani study population is a powerful resource for recessive hearing loss studies because their large, consanguineous family structures support statistically significant linkage scores (12). The families in our study are ascertained without otoacoustic emissions testing and are thus unselected with regard to AN/AD. We have already estimated the contributions of several other *DFNB* genes to recessive, severe-to-profound, congenital or prelingual-onset deafness in this population (13–18). Mutations of *GJB2*, *SLC26A4*, *MYO15A*, *TMC1*, *TRIOBP*, *MYO6* and *RDX* each account for 0.3–6.1% of recessive deafness in this population. These results reflect the extensive genetic heterogeneity and large genetic load of deafness that is still unaccounted for in this and other populations. The goal of our current study was to characterize the identities and frequencies of *OTOF* mutations in 557 Pakistani families segregating recessive deafness.

Materials and methods

Pakistani families segregating recessive deafness

This study was approved by Institutional Review Boards (IRBs) at the National Centre of Excellence in Molecular Biology (CEMB, Lahore, Pakistan) and the National Institutes of Health (Combined Neuroscience IRB, Bethesda, MD, USA). A total of 557 Pakistani families segregating recessive, congenital or prelingual-onset, severe-to-profound sensorineural deafness were ascertained by the CEMB and evaluated for linkage to known *DFNB* loci as previously reported (15). Phenotype evaluations included medical and developmental history interviews, physical examinations and pure tone audiometry. *DFNB9*-linked short tandem repeat (STR) markers were *D2S2144*, *D2S2223*, and *D2S174*.

Mutation analysis

We performed bidirectional nucleotide sequence analysis of the 48 reported exons of *OTOF* (GenBank AF183185.1) and additional exonic sequence that encodes the 5′-untranslated region (UTR) of the short isoform (GenBank AF183187.1) in families segregating deafness linked to *DFNB9*. We polymerase chain reaction (PCR) amplified and sequenced *OTOF* exons as described (1, 11) with a few exceptions. A new primer was designed for PCR amplification of exon 38, and nested primers were used for sequencing exons 18, 20 and 21

and the 5'-UTR of the short isoform. The primer sequences were 38F, 5'-CTTCACTGTCATAGGACCCTCAG; 38R, 5'-GATTGGCTAGGGTGGGAGGT; 18F_{nested}, 5'-CTGGTGGGA-ATGCACTCTA; 18R_{nested}, 5'-CGGGAGGTGAGGTCTTGG; 20F_{nested}, 5'-ATCAACAGGGAGGAGGCATT; 20R_{nested}, 5'-TTTGTCCAGTTCCGCCTCAT; 21F_{nested}, 5'-TTTGTCCAGTTCCGCCTCAT; 21R_{nested}, 5'-TGTGACACCTTCTCACAACCA; 5'-UTR(F)_{nested}, 5'-GTCCTAGGGTTTCCCTCTGTCT; and 5'-UTR (R)_{nested}, 5'-GTGAATCAGGAGTGTGGGTGAT.

All participating affected and unaffected family members were included for sequence analyses of variant exons. Numbering of mutations in exons 1–46 was based upon the human brain long isoform (GenBank AF183185.1). Mutations in exon 48 were numbered according to exons 1–46 of the human brain long isoform followed by exon 48 of the human short isoform (GenBank AF183187.1). Names of all variants were checked using Mutalyzer (<http://www.LOVD.nl/mutalyzer/>). Splice site variants, intronic variants and synonymous changes were considered non-pathogenic when they were detected in normal Pakistani population, or the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html) and ESEFINDER (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>) bioinformatic tools did not predict a change that creates or eliminates an acceptor or donor splice site.

RT-PCR analysis of exon 47 of *OTOF* in human tissues

Human cochlea complementary DNA (cDNA) was derived from a human fetal cochlea cDNA library (19), and human brain cDNA was purchased from Clontech (human multiple tissue cDNA panel 1, Palo Alto, CA, USA). We PCR amplified *OTOF* cDNA with forward and reverse primers corresponding to exons 46 (5'-GAGGCAGAGAAGAACCCAGTG) and 48 (5'-AAGCCACTGAAAGGAAATGC), respectively, using HotStarTaq DNA polymerase (Qiagen Sciences, MD) with an initial 15-min activation step of 95°C, followed by 35-step cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 min. The cDNA amplification products were separated by agarose gel electrophoresis and purified, and the nucleotide sequence was confirmed by direct sequencing.

Results

DFNB9 and *OTOF* mutation prevalence

We identified 13 (2.3%) of 557 families (Fig. 1a) cosegregating deafness with homozygosity for *DFNB9*-linked STR marker genotypes. We detected probable pathogenic sequence variants of *OTOF* in affected subjects from all 13 Pakistani *DFNB9* families (Table 1). Homozygosity for these variants cosegregated with deafness in each of the families.

Pathogenic variants

We found the previously reported p.R708X mutation (3, 6) in two families. The other 10 probable pathogenic variants were novel: these comprised 1 frameshift (p.G368AfsX2), 3 nonsense mutations (p.R425X, p.W536X, and p.Y1603X), 1 single amino acid deletion (p.E766del) and 5 missense substitutions (p.L573R, p.A1090E, p.E1733K, p.R1856Q and p.R1939W). Nine of 10 novel variants were identified in only one family and were not present in 196 to 334 Pakistani control chromosomes (Table 1). One of 10 novel variants (p.E1733K) was found in two families and was detected in 1 of 304 control chromosomes (Table 1), but this low carrier frequency is consistent with a role as a pathogenic recessive allele. p.E1733K and p.R1856Q affect the C2F domain and are the only non-truncated mutations that affect a predicted functional (C2) or transmembrane domain of otoferlin in this study. The missense substitutions and the single amino acid deletion affect amino acids

that are conserved in known *OTOF* orthologs (Fig. 1b). These data suggest that the missense variants and p.E766del are pathogenic. However, p.E766del does not affect a C2 domain, and the sequence from rat otoferlin shows the absence of this particular residue (Fig. 1b). It is possible that the human protein may still be functional without E766. However, we did not find another potentially pathogenic variant in the 48 exons or donor or acceptor splice sites. Moreover, deafness segregating in family PKDF527 was linked to markers for *OTOF* and achieved a LOD score of 3.92. Together, these data suggest that in humans, the p.E766del allele of *OTOF* may be pathogenic. Among the 11 pathogenic variants, 5 affect only the long isoform and 6 affect both long and short isoforms.

Alternative splicing of exon 47 in human cochlear *OTOF* transcripts

p.R1939W affects the same amino acid as the previously reported p.R1939Q mutation encoded by exon 48 (2). Exon 48 is predicted to encode the C-terminal 60 amino acids of an alternative *OTOF* isoform lacking exon 47. We investigated alternative splicing of exon 47 in human cochlear *OTOF* transcripts by reverse transcription-polymerase chain reaction analysis (Fig. 1c). Human cochlear transcripts exclusively utilize exon 48 to encode the C-terminus as previously described for mouse cochlear *OTOF* transcripts (11). Thus, p.R1939W affects the protein-coding region of *OTOF* transcripts expressed in human cochlea.

Non-pathogenic variants

In addition to previously published non-pathogenic variants, we also detected six unreported nonpathogenic sequence variants among different families (Table 1). A novel splice site variant (c.766-3C>T) in *cis* configuration with p.E766del was found in PKDF527. c.766-3C>T was predicted to have a slight effect upon a canonical splice acceptor site by the ESEFINDER program, but this variant was present in 7 of 182 normal Pakistani control chromosomes, indicating it is likely to be a benign variant. Conversely, c.2316-29C>T was detected only once in normal Pakistani control chromosomes, but this variant did not show an effect upon the splice acceptor activity *in silico*.

Discussion

Including the 10 novel mutations in this study, 50 pathogenic variants of *OTOF* have now been reported. Although p.Q829X is a recurrent mutation in Spanish study populations (3, 6, 20), the other known mutant alleles of *OTOF* are either private or very rare. Because these mutations are widely dispersed among many different exons, it may be difficult to implement efficient hierarchical screening strategies in other populations.

Our identification of probable pathogenic *OTOF* variants in 13 of 13 Pakistani families segregating DFNB9-linked deafness strongly suggests that this is a genetically homogenous locus. This is in contrast with previous studies that detected mutations of *OTOF* only in 17–30% of smaller families with possible linkage to *DFNB9* (5, 20). Based upon our cohort of 557 study families, we estimate that *OTOF* mutations account for 2.3% (13/557) (95% CI: 1.4–4.0%) of the genetic load of recessive, congenital or prelingual-onset, severe-to-profound deafness in this population. This estimate falls within the mid-range among estimates (0.3–6.1%) for other DFNB genes that we have studied in this population: *GJB2*, *SLC26A4*, *TMCI*, *MYO15A*, *TRIOBP* and *RDX* (Table 2).

Our estimated DFNB9 prevalence of 2.3% is not significantly different from those of other studies in other populations. Although Varga et al. identified *OTOF* mutations in 6 (9.2%) of 65 families, only 2 families (3.1%) showed biallelic *OTOF* mutations (5). Moreover, their study preferentially recruited families with a clinical phenotype of AN/AD. Rodriguez-

Ballesteros identified biallelic *OTOF* mutations in 23 (3.2%) of 708 Spanish subjects and 2 (2.4%) of 83 Colombian subjects with non-syndromic hearing loss (6). Their studies also might have preferentially recruited subjects with a clinical phenotype of AN/AD without evidence of linkage. In contrast, we ascertained subjects based upon hearing loss severity and onset without regard to otoacoustic emissions status or a possible diagnosis of AN/AD. We may have therefore underestimated the prevalence of DFNB9 because our ascertainment missed milder or delayed-onset cases of hearing loss. High rates of consanguinity might also have influenced our estimate in either direction. Nevertheless, these similar estimates derived from different ascertainment strategies for different populations indicate that *OTOF* mutations are an important contributor to the genetic load of deafness in diverse populations.

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Fig. 1.

(a) Pedigrees of Pakistani families segregating recessive sensorineural hearing loss (filled symbols) linked to *DFNB9*. (b) ClustalW alignment of otoferlin orthologs in six non-contiguous regions (separated by dots) containing leucine-573 (p.L573R), glutamic acid-766 (p.E766del), alanine-1090 (p.A1090E), glutamic acid-1733 (p.E1733K), arginine-1856 (p.R1856Q), and arginine-1939 (p.R1939W) (indicated by arrows). Regions of amino acid sequence identity are shaded. (c) Reverse transcription-polymerase chain reaction analysis and genomic structure of 3' end of *OTOF* including exon 47. Amplification products derived from human cochlea or brain were separated by 1.3% agarose gel electrophoresis. Human cochlear transcripts exclusively utilize exon 48 to encode the C-terminus (white arrowhead: 301 bp), whereas human brain also has transcripts that utilize exon 47 (white arrow: 499 bp). Black box, protein-coding region; open box, 3'-UTR.

Table 1

*OTO*F sequence variants in Pakistani families with DFNB9 hearing loss^a

Location	Nucleotide variation ^b	Predicted effect	Allele frequency ^c	Family	Reference
Pathogenic variants					
Frameshift					
Exon 13	c.1103_1104delinsC	p.G368AfsX2	0/196	PKDF236	
Nonsense					
Exon 14	c.1273C>T	p.R425X	0/252	PKDF014	
Exon 16	c.1607G>A	p.W536X	0/272	PKDF083	
Exon 19	c.2122C>T	p.R708X		PKDF328 PKSR43A	(3)
Exon 40	c.4809C>A	p.Y1603X	0/258	PKDF099	
Deletion					
Exon 20	c.2295_2297del	p.E766del	0/208	PKDF527	
Missense substitution					
Exon 16	c.1718T>G	p.L573R	0/272	PKDF604	
Exon 27	c.3269C>A	p.A1090E	0/320	PKDF334	
Exon 43	c.5197G>A	p.E1733K	1/304	PKDF602 PKSN8	
Exon 45	c.5567G>A	p.R1856Q	0/254	PKDF525	
Exon 48	c.5815C>T	p.R1939W	0/334	PKDF339	
Non-pathogenic variants ^d					
Exon 4	c.244C>T	p.R82C		6 PK families	(20)
Exon 5	c.372A>G	p.T124T		5 PK families	(20)
IVS9 ^g	c.766-3C>T	IVS9-3C>T	7/182	PKDF527	
Exon 11	c.945G>A	p.K315K		PKDF604	(20)
5'-UTR ^e	c.-137_-136del^f	None		8 PK families	(21)
5'-UTR ^e	c.-224G>C^f	None	54/174	8 PK families	
IVS20 ^g	c.2315+24G>A	IVS20+24G>A	4/138	PKDF083	
IVS20 ^g	c.2316-29C>T	IVS20-29C>T	1/184	PKDF527	
IVS21 ^g	c.2406+36G>A	IVS21+36G>A	17/172	PKSN8	

Location	Nucleotide variation ^b	Predicted effect	Allele frequency ^c	Family	Reference
Exon 22	c.2512C>T	p.L838L	3/188	PKDF236	
Exon 23	c.2580C>G	p.V860V		9PK families	(20)
Exon 24	c.2736G>C	p.L912L		6PK families	(22)
Exon 40	c.4936C>T	p.P1646S		PKDF099	(5)
Exon 44	c.5391C>T	p.F1797F		PKDF083	(20)
Exon 45	c.5655C>T	p.R1885R		PKDF525	(20)

UTR, untranslated region.

^aAll the variants were found in the homozygous state, and novel variants are shown in bold.

^bNumbering from exon 1 through 46 according to GenBank AF183185.1 and the sequence thereafter was based upon the human heart isoform transcript skipping exon 47 (GenBank AF183187.1).

^cAllele frequency in Pakistani control population.

^dc.227+54T>C (rs1879760), c.509+39A>T (rs7575020), c.510–39C>T (rs884390), c.711–49A>C (rs6547079), and c.2215–83C>T (rs4665855) were also found in this study.

^e5'-UTR refers to an additional exon upstream of exon 20, which contributes to the 5'-UTR of the transcript encoding the human otoferlin short isoform 1 (GenBank NM AF183187.1). The first 27 nucleotides of exon 20 also contribute to the 5'-UTR of the short isoform transcripts.

^f5'-UTR variants were numbered according to GenBank NM AF183187.1. Complementary DNA number +1 corresponds to the A of the ATG translation initiation codon.

^gIVS, intervening sequence, intron.

Table 2Percentage of Pakistani families segregating mutant alleles of *DFNB* genes

Gene	Locus	Families (%)	95% CI	References
<i>GJB2</i>	<i>DFNB1</i>	6.1 (12/196)	3.5–10.3	(23)
<i>SLC26A4</i>	<i>DFNB4</i>	4.7 (10/212)	2.6–8.4	(13)
<i>TMC1</i>	<i>DFNB7/11</i>	3.4 (19/557)	2.2–5.3	(15)
<i>MYO15A</i>	<i>DFNB3</i>	3.3 (20/600)	2.2–5.1	(14)
<i>OTOF</i>	<i>DFNB9</i>	2.3 (13/557)	1.4–4.0	This study
<i>TRIOBP</i>	<i>DFNB28</i>	1.6 (9/557)	0.8–3.0	(16)
<i>MYO6</i>	<i>DFNB37</i>	1.2 (3/250)	0.4–3.4	(17)
<i>RDX</i>	<i>DFNB24</i>	0.3 (2/557)	0.1–1.3	(18)