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ORIGINAL ARTICLE

Novel *OTOF* mutations in Brazilian patients with auditory neuropathy

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The *OTOF* gene encoding otoferlin is associated with auditory neuropathy (AN), a type of non-syndromic deafness. We investigated the contribution of *OTOF* mutations to AN and to non-syndromic recessive deafness in Brazil. A test for the Q829X mutation was carried out on a sample of 342 unrelated individuals with non-syndromic hearing loss, but none presented this mutation. We selected 48 cases suggestive of autosomal recessive inheritance, plus four familial and seven isolated cases of AN, for genotyping of five microsatellite markers linked to the *OTOF* gene. The haplotype analysis showed compatibility with linkage in 11 families (including the four families with AN). Samples of the 11 probands from these families and from seven isolated cases of AN were selected for an exon-by-exon screening for mutations in the *OTOF* gene. Ten different pathogenic variants were detected, among which six are novel. Among the 52 pedigrees with autosomal recessive inheritance (including four familial cases of AN), mutations were identified in 4 (7.7%). Among the 11 probands with AN, seven had at least one pathogenic mutation in the *OTOF* gene. Mutations in the *OTOF* gene are frequent causes of AN in Brazil and our results confirm that they are spread worldwide.
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INTRODUCTION

Mutations in the *OTOF* gene encoding otoferlin are an important cause of autosomal recessive non-syndromic hearing loss and are also associated with auditory neuropathy (AN).[1–5]

AN is a type of non-syndromic deafness characterized by an absent or abnormal auditory brainstem response (ABR), with preservation of otoacoustic emissions (OAEs) and/or cochlear microfonics (CMs). The presence of OAE indicates the preservation of the function of outer hair cells. To date, four loci responsible for non-syndromic AN were mapped: DFNB9 (*OTOF* gene) and DFNB59 (*PJVK* gene), responsible for autosomal recessive AN; AUNA1 for autosomal dominant AN; and AUNX1 for X-linked AN.[6–9] Mitochondrial mutations were found to be possibly related to AN.[10]

Structure, splicing patterns and possible roles for the product of the *OTOF* gene, otorferlin, were extensively investigated.[11–14]

To date, 43 different pathogenic mutations in the *OTOF* gene were described from populations of variable origins. A c.2485C>T (p.Q829X) mutation was found at a frequency of ~3.5% among a deaf sample in Spain.[2,15]

Herein, we report on the genetic analysis of 59 Brazilian probands selected for sensorineural non-syndromic autosomal recessive hearing loss or AN, with regard to the *OTOF* gene, and the discovery of six novel pathogenic mutations.

MATERIALS AND METHODS

Participants

From a sample of 342 unrelated Brazilian patients with hearing impairment and without recognizable deafness-related syndromes, we selected 48 probands from families with consanguinity or with two or more affected sibs and unaffected parents (suggestive of autosomal recessive inheritance), four familial cases of AN and seven isolated cases of AN.

All 59 individuals had undergone at least pure-tone audiometry, which showed a severe or profound hearing loss. In a part of the sample, OAE, CM and ABR were also tested. In cases in which OAE and/or CM were present, and in which ABR was absent or abnormal, the patients were diagnosed as having AN.

Mutations c.35delG and c.167delT of the *GJB2* gene, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) deletions of the *GJB6* gene and the A1555G mitochondrial mutation in the 12S rRNA gene were excluded in all individuals.

Individuals were Brazilians of European or African descent, except for one family, which came from Lebanon.

This study was approved by the Ethics Committee of the 'Instituto de Biociências, Universidade de São Paulo.' Written informed consent was obtained from all participants or from their guardians.

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Genotyping and mutation screening

For the detection of the c.2485C>T (p.Q829X) mutation in the *OTOF* gene, PCR, followed by restriction enzyme *Bfa*I digestion, was carried out.[15]

Probands, parents and sibs from the 59 selected families (because of recessive inheritance or AN) were genotyped for five microsatellite markers, linked to the *OTOF* gene (D2S162, D2S168, D2S305, D2S165 and D2S367) and amplified using fluorescent-labeled primers. All PCR products were analyzed in the MegaBACE 1000 DNA Analysis System with the software Genetic Profiler version 1.5 (GE Healthcare, Chalfont St. Giles, UK).

In samples from the probands of the seven familial cases in which microsatellite segregation was consistent with linkage to *OTOF*, from the probands of the four familial cases of AN (also compatible with linkage) and in samples from the seven isolated cases of AN, the 48 exons of the *OTOF* gene were sequenced using previously published PCR primers.[15] PCR products were sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare) and analyzed in the MegaBACE 1000 DNA Analysis System (GE Healthcare). In the same 18 selected samples, all coding exons of the gene, *PJVK* (pejvakin), were also sequenced using previously published PCR primers.[7] To assess the possible damaging effect of amino-acid substitution, Polyphen was used (www.polyphen.com, Heidelberg, Germany).

Non-synonymous novel substitutions were screened in unrelated normal-hearing control individuals by restriction enzyme assays or by single-nucleotide polymorphic analysis, using the MegaBACE SNuPe genotyping Kit (GE Healthcare). The hearing control population consisted of 50 unrelated Brazilians of African descent and 50 unrelated Brazilians of European descent.

RESULTS AND DISCUSSION

The variant, c. 2485C>T (p.Q829X), is the third most frequent mutation causing non-syndromic prelingual hearing impairment in the Spanish population,[2,15] being also identified in two French, one Mexican, two Argentinean and one English patient.[16–18] In our sample, it was absent in the 342 deaf individuals. De Oliveira *et al.*[19] also failed to identify this mutation in 645 unrelated deaf Brazilians. Thus, this mutation is not a frequent cause of deafness in the Brazilian population. It could be expected to be detected in Brazil, as it was found in a neighboring country, Argentina.[16] If we postulate that p. Q829X results from a founder effect, being spread first in Spain, we could explain its absence in Brazil by the fact that the European fraction of Brazilian ancestors is mainly represented by the Portuguese. Spanish colonization was not as prevalent as it was in Argentina. Besides, it is well known that among Brazilians who are classified as 'white' on the basis of their skin color, a high percentage of genetic admixture with Africans and Native Americans is detected through ethnic-specific molecular markers;[20,21] however, our own data[22] on Connexin 26 mutations indicated that c. 35delG is the most frequent causative mutation in hearing impairment, because it may also be frequent in Portugal as well as in many other countries that resulted from European colonization.[23]

Among the 59 selected families, microsatellite segregation analysis excluded linkage to DFNB9 in 34, it was inconclusive in 14 and it was compatible with linkage in the remaining 11 families, including one case with consanguinity and diagnosis of AN, and three familial cases of AN.

Sequencing of all exons in the 18 selected cases, in 7 isolated cases with diagnosis of AN and in 11 cases with putative linkage to the *OTOF* (including four families with a diagnosis of AN) revealed 38 different variants. Ten of them, found in eight families, are probably pathogenic (Table 1).

We identified four frameshift and two nonsense mutations, which are obviously pathogenic (Table 1).

The nonsense mutation, c.3400C>T (p.R1134X), identified by Rodriguez-Ballesteros *et al.*,[2] was detected in homozygosis in patient 3 with AN, born from a consanguineous marriage. The mutations

Table 1 Ten pathogenic mutations in the *OTOF* gene identified in eight Brazilian families

Subjects	Associated genotype based on nucleotide change	Associated genotype based on amino-acid change	Phenotype
1	[c.2905-2923del19ins11+c.?]	[p.A969fs+c.?]	NSHL
2	[c.1552-1567del16[a]+c.?]	[p.R518fs[a]+c.?]	NSAN
3	[c.3400C>T+c.3400C>T]	[p.R1134X+p.R1134X]	NSAN
4	[c.4960G>A[a]+c.?]	[p.G1654S[a]+p.?]	NSAN
5	[c.2348delG+c.5800-5801insC]	[p.G783fs+p.L1934fs]	NSAN
6	[c.1841G>A[a]+c.3239G>C[a]]	[p.G614E[a]+p.R1080P[a]]	NSAN
7	[c.5431A>T[a]+c.?]	[p.K1811X[a]+p.?]	NSAN
8	[c.5785A>C[a]+c.?]	[p.N1929H[a]+p.?]	NSAN

Abbreviations: NSAN, non-syndromic auditory neuropathy; NSHL, non-syndromic hearing loss.
[a]Novel mutations.

led to a premature stop codon in exon 28 and was confirmed in heterozygosis in both parents. The proband was diagnosed as having AN, confirmed through the presence of OAE and CM, and abnormal ABR.

The mutations, c.2348delG (p.783fs) and c.5800-5801insC (p.L1934fs), were inherited by patient 5 paternally and maternally, respectively This was an isolated case of AN. Patient 5 is an isolated case of hearing loss, diagnosed with AN. The combination of the paternal mutation, described by Varga *et al.*,[1] and the maternal mutation in exon 46, identified by Rodriguez-Ballesteros *et al.*,[2] probably caused the AN.

The novel mutation, c.5431A>T (p.K1811X), was inherited paternally in family 7. The proband and his sister were both affected with AN, as indicated by alterations of the ABR waves and the presence of OAE and CM, indicating typical AN. They both inherited the probably pathogenic variant, which substitutes a lysine with a stop codon at exon 44, leading to a putative shorter otoferlin, causing the loss of a part of the last C2 domain, which is important for the normal function of the protein. However, a second mutation was not found in the other allele.

A novel deletion of 16pb (c.1552-1567del16) was found in heterozygosis in patient 2, who also had a diagnosis of AN. This deletion in exon 15 leads to a premature stop codon at exon 16 and causes the loss of the last three C2 domains of the active otoferlin. A second mutation was not found. The clinical findings of patient 2, who presents AN, were detailed in Favero *et al.*[24]

An indel mutation, c.2905-2923del19ins11, described by Rodriguez-Ballesteros *et al.*,[25] was detected in heterozygosis in family 1. This family had a Lebanese origin and both affected children had absent ABR and OAEs. Their audiological evaluations were carried out when they were approximately 3–4 years of age. Knowing that OAE might decline with age,[2] this mutation might also have been responsible for AN, which was not detected in the hearing-impaired children, as OAEs were not investigated before. Besides, recent ABR revealed cochlear microfonism in the affected girl, which reinforces the idea that she might have presented AN.

Sixteen substitutions were silent coding variants and probably benign (alleles in bold are those that have been newly identified in this work): **c.51C>T** (**p.G17G**), c.372A>G (p.T124T), c.2022C>T (p.D674D), **c.2512C>T** (**p.L838L**), c.2580C>G (p.V860V), c.2613C>T (p.L871L), c.2703G>A (p.S901S), c.2736G>C (p.L912L), c.4332C>T (p.T1444T), **c.4341G>A** (**p.E1447E**), **c.4537A>G** (**p.K1512K**), c.4677G>A (p.V1559V), c.4767C>T

Table 2 Results of the screening of the control samples for the novel missense variants, reporting Polyphen and conservation analysis

Exon	*Nucleotide alteration*	*Amino-acid alteration*	*No. of substitutions/ no. of chromosomes*	*No. of homozygotes/total no. of subjects*	*Polyphen*	*Conservation between vertebrates*
2	c.98G>A	p.R33Q	1/200	0/100	Benign	Yes
17	c.1841G>A	p.G614E	0/200	0/100	Possibly damaging	Yes
21	c.2401G>T+c.2402A>T	p.E801L	18/200	2/100	Possibly damaging	Yes
27	c.3239G>C	p.R1080P	0/200	0/100	Possibly damaging	Yes
31	c.3752T>G	p.C1251G	5/200	2/100	Probably damaging	No
40	c.4960G>A	p.G1654S	0/200	0/100	Benign	Yes
41	c.4981G>A	p.E1661K	2/200	0/100	Benign	Yes
41	c.5063C>T	p.T1688M	2/200	0/100	Benign	Yes
46	c.5785A>C	p.N1929H	0/200	0/100	Benign	Yes

(p.R1589R), c.5391C>T (p.F1797F), **c.5553G>C** (**p.L1851L**) and c.5655C>T (p.R1885R).

We identified five missense alterations described in the Spanish and North-American populations as neutral polymorphic changes: c.158C>T (p.A53V), c.244C>T (p.R82C), c.2371C>T (p.R773C), c.2464C>T (p.R822W) and c.4936C>T (p.P1646S).[17,15]

In addition, we also found 11 novel missense coding changes: c.98G>A (p.R33Q), c.1841G>A (p.G614E), c.2401G>T+c.2402A>T (p.E801L), c.3239G>C (p.R1080P), c.3751T>G (p.C1251G), c.4582G>A (p.D1528N), c.4960G>A (p.G1654S), c.4981G>A (p.E1661K), c.5063C>T (p.T1688M), c.5785A>C (p.N1929H) and c.5938G>T (p.A1980S).

The c.4582G>A (p.D1528N) alteration was found in patient 3, in whom we identified another nonsense pathogenic mutation (c.3400C>T) in homozygosis, and it was postulated to be benign according to Polyphen. Thus, it was considered a neutral variant. The c.5938G>T (p.A1980S) substitution was identified in exon 47 and it is not present in the cochlear isoform of the *OTOF* transcript.[11] Thus, it is probably non-pathogenic.

The remaining nine novel missense variants were screened in 100 unrelated normal-hearing control individuals, analyzed by Polyphen, and the conservation of the amino-acid position was examined in mouse, chicken and zebrafish otoferlin (Table 2).

The variant, c.98G>A (p.R33Q), was found in low frequency in hearing controls and was pointed out as being benign according to Polyphen; c.2401G>T+c.2402A>T (p.E801L) and c.3752T>G (p.C1251G) were pointed out as being possibly or probably damaging according to Polyphen, but they were found in high frequencies in hearing controls, including homozygotes; c.4981G>A (p.E1661K) and c.5063C>T (p.T1688M) were found in 1% of the chromosomes of normal control and were indicated as benign changes, according to Polyphen (Table 2). We consider unlikely that any of these five variants could explain the hearing impairment of affected individuals.

However, the two non-synonymous mutations, c.1841G>A (p.G614E) and c.3239G>C (p.R1080P), are absent in 100 hearing controls and are possibly damaging according to Polyphen (Table 2). Although they are not located in a C2 domain, we considered them as being probably pathogenic. They were found in compound heterozygosis in patient 6 (Table 1). The proband with AN reports that her hearing loss is more severe when she is febrile. When afebrile, her audiometry indicates a mild hearing loss, the OAEs are present but ABR is abnormal, indicating AN. This is the second case of temperature-sensitive AN associated with mutations in *OTOF*.[17]

Two substitutions were found in heterozygosis: c.4960G>A (p.G1564S) in proband 4 and her two sibs and c.5785A>C (p.N1929H) in proband 8 (Table 1). These patients were diagnosed as having AN. Although Polyphen postulated that these variants have probably no effect on otoferlin and they do not affect C2 domains, we failed to identify them in 100 hearing controls. In addition, the reference amino acid was conserved across vertebrate lines. No accompanying mutation was found in these two cases, which may be coincidental carriers or may present more complex mutations, such as mutations in promoter regions, in the regulatory sequences of splicing, and large deletions or other rearrangements, which would be missed by the usual detection methods. We postulate that these two substitutions may be related to hearing loss.

The 18 probands selected for sequencing of the *OTOF* gene had all the coding exons of the *PJVK* gene also sequenced. No possibly damaging mutations were found. It is unlikely that the remaining cases of AN, without mutations in *OTOF*, could be because of mutations in *PJVK*. Besides, this indicates that mutations in *PVJK* are not a frequent cause of AN in Brazil, as mutations in *OTOF* were revealed to be.

Summing up, we succeeded in identifying four frameshift, two nonsense and four missense pathogenic variants in eight families (Table 1). Six of the 10 mutations are novel (p.R518fs, p.G1654S, p.G614E, p.R1080P, p.K1811X and p.N1929H). In the group of 8 patients who presented the 10 identified pathogenic mutations, 7 fulfilled the diagnostic criteria of AN.

Among the 11 cases selected because of AN, 7 had at least one pathogenic mutation in the *OTOF* gene. In the remaining four cases, we cannot rule out the possibility of undetected pathogenic variants in the *OTOF* gene. Owing to technical limitations, whole-exon deletions and complex sequence rearrangements were not investigated, neither were mutations in the untranslated regions or introns. Four familial cases with AN were compatible with linkage to the *OTOF* gene. We found probably pathogenic mutations in three of them (families 3, 4 and 7). As mutations in *PJVK* were not detected, other undetected rearrangements in the *OTOF* gene or in other genes could explain the findings.

Among the seven families without AN, selected after linkage analysis, only one family (1) revealed a pathogenic mutation in the *OTOF* gene. The compatibility with linkage in the remaining six cases probably occurred by chance.

Finally, among the 52 probands from familial cases suggestive of autosomal recessive inheritance (48 familial cases of deafness and 4 familial AN), mutations in the *OTOF* gene were identified in four cases (7.7%). In the group of probands with AN, 63.6% had at least one pathogenic mutation in the *OTOF* gene. If we take into account one familial case of AN with probable recessive inheritance but no

detected mutation in *OTOF*, the fraction of cases of AN that are probably genetic in our sample is 72.7%. Thus, mutations in the *OTOF* gene are frequent causes of AN in the Brazilian population, and the fraction of AN cases that can be attributed to genetic causes is much higher than that suspected earlier. On the other hand, mutations in the *PJVK* gene were not detected to be associated with AN in our population.

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