Exome Sequencing Identifies a Novel Nonsense Mutation of MYO6 as the Cause of Deafness in a Brazilian Family

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Summary

We investigated 313 unrelated subjects who presented with hearing loss to identify the novel genetic causes of this condition in Brazil. Causative GJB2/GJB6 mutations were found in 12.7% of the patients. Among the familial cases (100/313), four were selected for exome sequencing. In one case, two novel heterozygous variants were found and were predicted to be pathogenic based on bioinformatics tools, that is, p.Ser906* (MYO6) and p.Arg42Cys (GJB3). We confirmed that this nonsense MYO6 mutation segregated with deafness in this family. Only the proband and her unaffected mother exhibited the GJB3 mutation, which is in the same amino acid of a known Erythrokeratodermia variabilis mutation. None of the patients exhibited this skin disease, but the proband exhibited a more severe hearing loss. Hence, the GJB3 mutation was considered to be a variant of uncertain significance. In conclusion, we described a novel nonsense MYO6 mutation that was responsible for the hearing loss in a Brazilian family. This mutation resides in the neck domain of myosin-VI after the motor domain. Thus, our data give further support for genotype-phenotype correlations, which state that when the motor domain of the protein is functioning, the hearing loss is milder and has a later onset. The three remaining families without mutations in the known genes suggest that there are still deafness genes to be revealed.

Keywords: Hearing loss, MYO6 gene, Exome sequencing, GJB3 gene

Introduction

Hearing loss (HL) or deafness is one of the most common sensorineural defects in humans. Nonsyndromic hearing loss (NSHL) is highly heterogeneous in its clinical presentation (i.e., age at onset, progression, and audiological characteristics), pattern of inheritance and underlying genetic causes (Smith et al., 2005). With the progress of prenatal and early postnatal health attention, the majority of occurrences of HL in prelingual children are of genetic etiology (50–60%) (Mehta et al., 2016). With recent advances in molecular genetics, our understanding of the pathogenesis of sensorineural HL has greatly expanded, and to date, mutations in more than 100 genes involved in autosomal dominant or autosomal recessive NSHL have been identified (Van Camp & Smith, 03/2017). The identification of causative genes for hereditary NSHL is important for decisions about the treatment modalities and the counseling of patients.

Except for the relatively common genes GJB2 and GJB6, the most reported mutations are present in only a single or a few families (Vona et al., 2014; Nishio & Usami, 2015; Zazo-Seco et al., 2017), and thus the genetic causes remain unknown for the majority of deaf individuals. The etiologic contributions of the less commonly screened deafness genes in particular have yet to be investigated systematically in many populations. Due to the large number and presumably low mutation frequencies of those genes, it would be highly expensive and time-consuming to address this issue with conventional Sanger sequencing. A growing number of research and clinical diagnostic laboratories are successfully using...
next-generation sequencing technologies for gene/variant identification (Schnekenberg et al., 2013). With the genomic capturing of a wide selection of known deafness genes, it is possible to screen these genes in a high-throughput manner by next-generation sequencing.

In this study, we present the results of the molecular analysis from a family that segregated with autosomal dominant NSHL. This family was one of four familial cases that was selected for exome sequencing from a large multiethnic group consisting of 313 unrelated Brazilian subjects with hearing impairments.

**Patients and Methods**

**Patients data and family ascertainment**

A total of 313 Brazilian unrelated subjects from all over the country who presented with hearing impairment were referred to the Genetic Deafness Counseling Unit of the Otolaryngology Department (Clinics Hospital of University of Sao Paulo School of Medicine). This study was approved by the Ethics Committee for Analysis of Research Projects from the University of Sao Paulo School of Medicine. Written informed consent was obtained from all hearing-impaired individuals or their legal guardians, their relatives, and control individuals. We did not exclude patients with suspected environmental causes or syndromic features from the analysis. Approximately 31% of our cohort (100/313) were familial cases, and four of them in which the segregation pattern was compatible with autosomal dominant inheritance were selected for exome sequencing. All four familial cases had at least three documented affected subjects who agreed to participate in the study.

**Clinical evaluation**

Pure tone audiometry was performed to test for air conduction (250–8000 Hz) and bone conduction (250–4000 Hz). Syndromic features were assessed after physical examinations and complete anamneses. No symptoms of vestibular dysfunction were reported. Malformations of the inner ear were examined by high-resolution computed tomography of the temporal bones in the proband.

**Molecular analysis**

Genomic DNA was extracted with commercial kits from the peripheral blood leukocytes. All subjects were screened for the following: mutations in the coding region of the \( \text{GJB2} \) gene by conventional Sanger sequencing, the \( \Delta(\text{GJB6-D13S1830}) \) and \( \Delta(\text{GJB6-D13S1854}) \) deletions by multiplex PCR (del Castillo et al., 2005), and the m.1555A>G mitochondrial mutation (MT-RNR1) by restriction enzyme digestion analysis (Estivill et al., 1998).

**Exome sequencing**

The DNA qualities and quantities were verified with a NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE, USA) and a Qubit fluorometer (Life Technologies, Grand Island, NY, USA). Amplicon libraries were prepared according to the manufacturer’s instructions using the Ion PI Library kit plus an Ion PI Ampliseq Exome RDY 1 × 8 (Applied Biosystems, ThermoFisher Scientific Inc., Waltham, MA, USA), and an Ion PI Hi-Q OT2 200 kit was used for template preparation. Sequencing was conducted with an Ion PI Hi-Q Sequencing 200 kit and an Ion PI Chip v3 in a Proton Semiconductor Sequencer–Ion Torrent (Life Technologies, Thermo Fisher Scientific Inc.).

**Base call and data analysis**

Variant filtering was performed as follows. First, the VCF files were parsed into a database management system (MySQL, http://www.mysql.com/) in which we integrated the cohort- and batch-wise variant annotations. We then excluded the variants that were expected to have a low pathogenicity (intergenic, intronic located away from the splice sites, and synonymous variants that did not interfere with the splice sites). The next step was to remove variants with minor allele frequencies (MAFs) in the Exome Aggregate Consortium (ExAC) database greater than 0.5% as well as the variants with a MAF greater than 0.5% in a local variant database to account for the local population-specific variants or systematic errors in sequencing or mapping methods. Finally, we filtered the variants found in the genes implicated in hereditary hearing loss as listed in http://hereditaryhearingloss.org/ (Van Camp & Smith, 2017) and ranked them according to the presumed inheritance patterns in each family.

**Sanger sequencing**

Sanger sequencing was performed to screen for \( \text{GJB2} \) mutations, to validate the exome results and to perform segregation analysis of the candidate variants. The primers were designed with Primer3 (bioinfo.ut.ee/primer3-0.4.0/) using the following reference sequences: the \( \text{MYO6} \) gene (NM_004999), the \( \text{GJB3} \) gene (NM_024009), and the \( \text{OTOA} \) gene (NM_144672). After purification of the PCR products with the enzymes Exonuclease I and FastAP Thermosensitive Alkaline Phosphatase (Fermentas, ThermoFisher Scientific Inc., Waltham, MA), they were sequenced with an

Conservation and pathogenicity

Five bioinformatics tools were used for pathogenicity prediction (PolyPhen2, SIFT, Mutation Taster, VEP, and Provean). The evolutionary conservation of each variant position was measured with PhyloP (Siepel et al., 2005) and PhastCons found in the Mutation Taster analysis. The missense variant was also evaluated for potential pathogenicity using protein prediction tools, such as SNPs3D (http://www.snp3d.org), PhD-SNP (http://snps.biofold.org/phd-snp/phd-snp.html), and SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/cgi-bin/adv_sosui.cgi). We used the standards and guidelines from Richards et al. (2015) for the interpretation of the sequence variants regarding the evidence for pathogenic or benign classification status.

Variant databases

All variants were searched in the Deafness Variation Database (deafnessvariationdatabase.com), which compiles pathogenic and non-pathogenic variants of known deafness genes, the ExAC browser (exac.broadinstitute.org/), the 1000 Genome Project Database (http://www.1000genomes.org/node/home), and the ABrOM (Brazilian genomic variants, http://abraom.ib.usp.br, 03/2017). The ABrOM comprises the exome variants from 609 elderly healthy individuals from the Brazilian population.

Results

We molecularly investigated 313 unrelated Brazilian subjects who presented with hearing impairment. Among these subjects, 8% had syndromic features, and the remaining subjects were nonsyndromic. From the group of 313 subjects, 100 were familial cases (31%). From among the familial cases, four were selected for exome sequencing because they had at least three documented affected individuals who agreed to participate in the study. All participants exhibited postlingual onset and autosomal dominant inheritance as the most likely inheritance mechanism. After exome sequencing, variants in known deafness genes were found in only one of the four families. This family had 13 affected individuals in four generations who supported an autosomal dominant mode of inheritance (Fig. 1).

Clinical description

Computed tomography scan analyses of the proband ruled out the presence of inner ear malformations. Audiograms were available from 12 affected individuals, including one individual with a distinct clinical phenotype (II:11) and two unaffected family members (Fig. 2). We divided the audiograms into two figures based on the age at assessment, that is, 19–37 years (Fig. 2A) and 55–87 years (Fig. 2A). The hearing loss was sensorineural and bilateral, except for subject II:1. At the onset, the hearing impairment mainly affected the high frequencies with a downsloping audiometric configuration in all family members. The referred age of onset varied between 12 years (IV:1) and 60 years (II:7). Progression was well documented in the proband (audiograms III:1-19y and III:32y, Fig. 2A), who exhibited asymmetrical hearing loss that was profound for high frequencies in the right ear by the age of 32 years. The remaining audiograms from three family members aged in their 30s revealed mild hearing loss, whereas the other seven audiograms from three family members aged between 55 and 65 years old revealed moderate hearing loss that also indicated progressive hearing loss. Thus, the proband’s phenotype was more severe compared with her cousins’ of the same age. The most severe hearing impairment was demonstrated by the oldest affected subject at 87 years of age who presented with severe impairment in the low–mid frequencies and profound impairment in the high frequencies. A medical history revealed that the proband had rheumatic fever at 18 years of age, or approximately 1 year before noticing the hearing impairment. The other affected individuals did not refer to any disease prior to the hearing loss onset. To date, there have been no reports on the association between rheumatic fever and hearing loss (Schubert et al., 2001).

Patient II:11 suffered a tympanic perforation during childhood because of a tonsil infection. A tonsillectomy was performed at 12 years old, and a tympanoplasty was performed at 16 years old. Because she experienced recurrent ear infections that resulted in unilateral mixed hearing loss (Fig. 2C), her hearing loss was considered a phenocopy.

Genetic findings

Mutations in the GJB2/GJB6 genes explained 12.7% of the 313 cases, including one syndromic subject who presented with palmoplantar keratoderma. Considering the familial cases, regardless of which was the most likely inheritance mechanism, GJB2/GJB6 causative mutations explained 13%, and among the sporadic cases, 12.2%. Among the biallelic GJB2/GJB6 subjects, 65% were c.35delG mutation homozygotes, and 24% were compound heterozygotes for this mutation. The p.Trp24* mutation was the second most common mutation in GJB2, with one homozygote.
Figure 1 (A) Pedigree of the family segregating with autosomal dominant bilateral sensorineural hearing loss. The referred ages at onset are indicated inside the brackets. The +/- indicates which individuals had the MYO6 mutation. The grey shading of symbol II:11 indicates that she presented unilateral mixed hearing loss and her hearing loss was considered a phenocopy. (B) Electropherograms of the GJB3 and MYO6 mutations showing the novel variants identified in the present study. [Colour figure can be viewed at wileyonlinelibrary.com]

(~3% of the sample) and 11% compound heterozygotes [c.35delG/ p.Trp24*, p.Trp24*/p.Arg184Pro, and p.Trp24*/p.Val95Met]. We found three cases of GJB6 deletions in combination with the c.35delG mutation, including two with delGJB6-D13S1830 and one with delGJB6-D13S1854 mutations. Six subjects were monoallelic for c.35delG. The m.1555A>G mitochondrial mutation was present in 1% (3/313).

In family 1, after exome sequencing, we detected four variants in three different genes that had already been associated with deafness: the nonsense heterozygous variant c.2717C>A (p.Ser906*, NM_004999) in exon 26 of the MYO6 gene (DFNA22/DFNB37), the missense heterozygous variant c.124C>T (p.Arg42Cys, NM_024009) in exon 2 of the GJB3 gene (DFNA2B), and two variants in the OTOA gene (DFNB22) including one nonsense [c.2401G>T, p.Glu801*, rs200988634, NM_144672] and one missense [c.2395A>C, p.Thr799Pro, rs464696, NM_144672] gene both in exon 21.

Regarding the OTOA gene, the p.Thr799Pro variant was considered a polymorphism/neutral/tolerated by bioinformatic tools (MutationTaster, Sift, Provean) and benign by the Deafness Variation Database, but the p.Glu801* variant was considered to be disease-causing by MutationTaster, because it was a nonsense mutation, but was listed as benign by the Deafness Variation Database. Additionally, both OTOA variants were detected in the ABraOM database from the Brazilian population with a frequency of approximately 2–3% as well as in ExAC [p.Thr799Pro – 0.17% and p.Glu801* – 0.12%]. Moreover, segregation analysis ruled out OTOA gene as the causative gene because mutations in this gene cause autosomal recessive deafness, and all affected and unaffected family members, including the proband’s mother and one control individual with normal hearing, have both variants, suggesting that they might be in the same allele (cis).

Both the GJB3 and MYO6 variants described herein have not been reported so far in the Deafness Variation Database, ExAC, 1000 Genomes, or ABraOM. The MYO6 mutation, p.Ser906*, was predicted to be pathogenic by all bioinformatic programs (MutationTaster, VEP) as well as the GJB3 mutation, p.Arg42Cys (MutationTaster, Sift, PolyPhen2, Provean).
Figure 2  Audiograms of family members: (A) The individuals' ages when tested were between 19 years and 37 years. (B) The individuals' ages when tested were between 55 years to 87 years, and the audiograms that were within the normal range are represented with dotted lines. (C) Individual II:12 who exhibited a unilateral mixed hearing loss due to a tympanic perforation. [Colour figure can be viewed at wileyonlinelibrary.com]
The MYO6 variant changed a highly conserved nucleotide across vertebrate species (PhyloP = 5.718 and PhastCons = 1), whereas the GJB3 variant changed a nucleotide that was also conserved but with weaker scores (PhyloP = 0.692 and PhastCons = 0.977). The p.Arg42Cys variant was also predicted to be deleterious by protein prediction tools: with a SNP3D score of −0.2; a disease 8 by PhD-SNP; and the generation of an increased average hydrophobicity of the protein from 0.047778 to 0.073704, as calculated by SOSUI. We confirmed that the MYO6 mutation (p.Ser906*) segregated with deafness within the pedigree, as illustrated in Figure 1, whereas the GJB3 variant was confirmed to be heterozygous in the proband (Figure 1) as well as in her unaffected mother but was not detected in any other affected family member.

Discussion

Pathogenic mutations in GJB2/GJB6 exhibited a similar frequency (~12%) in our sample when compared to a study that was previously conducted in Brazilian patients who were also from the city of São Paulo (Batissoco et al., 2009). In the city of Campinas, the frequency of GJB2/GJB6 mutations were found to be higher (22%) by Oliveira et al. (2002). In contrast to the present study and the study from Batissoco et al. (2009), these authors excluded from the analysis syndromic cases and cases with possible environmental causes. If both cases were excluded from our sample, GJB2/GJB6 mutations would be responsible for 16% of the cases. Oliveira et al. (2002) encountered 50% of her familial cases with GJB2 mutations, but only 11.5% were sporadic cases. We found similar frequencies in both the familial and sporadic groups of approximately 12%. We hypothesize that the small sample size and a possible ascertainment bias toward prelingual cases and/or familial cases with autosomal recessive inheritance in Oliveira et al.’s study might explain the different GJB2 mutation prevalence between their study and the present study. The m.1555A>G mitochondrial mutation was less frequent in our sample (1%) than in the Brazilian studies from Abreu-Silva et al. (2006) (i.e., 2%) and from Alves et al. (2016).

Exome sequencing of four familial cases of autosomal dominant hearing loss identified two potentially pathogenic variants in two different deafness genes (i.e., MYO6 and GJB3) in one case. The MYO6 mutation segregated with deafness within the family, but the GJB3 was only harbored by the proband and her unaffected mother. Both variants resided in conserved positions based on the PhyloP and PhastCons, although the MYO6 variant had much higher values. Mutations in the MYO6 and GJB3 genes have previously been reported in families that segregate autosomal dominant and autosomal recessive hearing loss (Tables 1 and 2). Mutations in MYO6 have also been associated with an inherited form of hypertrophic cardiomyopathy (Mohiddin et al., 2004; Hegan et al., 2015), whereas mutations in GJB3 are associated with a skin disease known as Erythrokeratodermia variabilis (Richard et al., 1998).

The MYO6 gene has 35 exons (NM_004999) with 40 pathogenic mutations that have been described to date. This gene codifies the myosin VI protein with 1285 amino acids, which is essential for the development, structural integrity and appropriate functioning of the inner ear hair cells, and for moving and transporting cargo toward the minus end of actin filaments (Wells et al., 1999; Sweeney & Houdusse, 2007). Myosin VI localizes to the cuticular plate of hair cells and is important for providing protection by restraining mechanical forces (Avraham et al., 1995; Hasson et al., 1997; Self et al., 1999). The protein consists of a motor domain containing an ATP- and an actin-binding site, followed by a neck domain and a globular tail, which interacts with other proteins (Sweeney & Houdusse, 2007). One hypothesis to explain the genotype–phenotype correlation for the dominant versus recessive mutations in the MYO6 gene was proposed by Top-sakal et al. (2009). If the protein motor domain is not affected and functions normally the hearing loss is milder and has a later onset. Indeed, the MYO6 mutation reported here only affects the neck domain and keeps the motor domain intact. Our family resembles the DAN and BEL2 families analyzed by Topsakal et al. (2009) as well as the family described by Yang et al. (2013) in which the mutations leading to premature stop codons after the motor domain caused autosomal dominant late-onset hearing loss. In addition, this mutation is predicted (MutationTaster) to lead to mRNA degradation of some or all of the mRNA from the mutated alleles by NMD and thus result in less protein in affected individuals and hearing loss.

The GJB3 gene encodes connexin 31 (Cx31), which is also known as the gap junction beta-3 protein. Gap junctions are clusters of intercellular channels that allow direct diffusion of ions and small molecules between adjacent cells (Bennett et al., 1991; Harris, 2001). Although the p.Arg42Cys variant has not been reported, a mutation changing the same amino-acid (p.Arg42Pro) has been described to be associated with Erythrokeratodermia variabilis without deafness (Wilgoss et al., 1999; Richard et al., 2000). Both the proband and her normal hearing mother exhibited the p.Arg42Cys variant, but none had this skin disease. The fact that the unaffected mother also carried this variant indicates that this variant was not the main cause of hearing loss that segregated in the family, but it might have contributed to the phenotype as a modifier (Richards et al., 2015). Indeed, the proband presented a more severe hearing loss at a younger age (32 years) when compared to the other affected family members. There are many examples of the action of modifier genes in the disease expressivity for a number of diseases (i.e., pancreatitis, breast cancer, Gaucher disease, retinitis pigmentosa, hemochromatosis, hypertrophic
### Table 1 Mutations in MYO6 reported to date.

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Abbreviations: AD, autosomal dominant; AR, autosomal recessive; NSHL, nonsyndromic hearing loss; DVD, deafness variation database; RP, retinitis pigmentosa; HL, hearing loss.

All variant positions refer to NM_004999. NMD information of Mutation Taster.

*Information provided by Hilgert et al., 2008
Table 2 Mutations in exon 2 of GJB3 reported to date (NM_024009). NMD is not predicted in any of these mutations.

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<th>Ancestry</th>
<th>Onset</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs746219527</td>
<td>chr1:35250529:A&gt;C</td>
<td>c.166A&gt;C</td>
<td>p.Lys56Gln</td>
<td>AD</td>
<td>Hearing loss</td>
<td>German</td>
<td>–</td>
<td>Beck et al., 2015</td>
</tr>
<tr>
<td>rs69979083</td>
<td>chr1:35250680:G&gt;A</td>
<td>c.317G&gt;A</td>
<td>p.Arg106His</td>
<td>AD</td>
<td>Hearing loss</td>
<td>German</td>
<td>–</td>
<td>Beck et al., 2015</td>
</tr>
<tr>
<td>rs74315318</td>
<td>Chr1:35250910:G&gt;A</td>
<td>c.547G&gt;A</td>
<td>p.Glu183Lys</td>
<td>AD</td>
<td>Deafness, non-syndromic</td>
<td>Japanese</td>
<td>early onset</td>
<td>Miyagawa et al., 2013</td>
</tr>
<tr>
<td>rs4135319</td>
<td>Chr1:35250902:G&gt;A</td>
<td>c.539G&gt;A</td>
<td>p.Arg180*</td>
<td>AD</td>
<td>Deafness, non-syndromic</td>
<td>Taiwanese</td>
<td>–</td>
<td>Yang et al., 2007</td>
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<tr>
<td>rs4135318</td>
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<td>c.547G&gt;A</td>
<td>p.Glu183Lys</td>
<td>AD</td>
<td>Progressive Deafness, non-syndromic</td>
<td>Chinese</td>
<td>20-30 years</td>
<td>Xu et al., 1998</td>
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<td>rs117385632</td>
<td>Chr1:35250943:G&gt;A</td>
<td>c.580G&gt;A</td>
<td>p.Ala194Thr</td>
<td>AR (digenic with GJB2-235delC+/+ or 299delAT+/+)</td>
<td>Deafness, non-syndromic</td>
<td>Chinese</td>
<td>Congenital</td>
<td>Lin et al., 2009</td>
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<td>rs373815705</td>
<td>chr1:35251030:C&gt;A</td>
<td>c.667C&gt;A</td>
<td>p.Pro223Thr</td>
<td>AR (second mutation not detected)</td>
<td>Deafness, non-syndromic</td>
<td>Turkish</td>
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<td>Uyguner et al., 2003</td>
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<td>rs74315319</td>
<td>Chr1:35251129:G&gt;A</td>
<td>c.766G&gt;A</td>
<td>p.Gly256Ser</td>
<td>Deafness</td>
<td>Chinese</td>
<td>–</td>
<td>Li et al., 2010</td>
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</table>

All variants were described in exon 2 (NM_024009) and no NMD
and cardiomyopathy among others) as remarkably reviewed by Cooper et al. (2013). The variant p.Arg42Cys was considered as of uncertain significance.

Finally, the lack of mutations in known deafness genes in the remaining three families with autosomal dominant inheritance suggests that the majority of those genes remain to be revealed in the Brazilian population. Zazo-Seco et al. (2017) were unable to identify the causative variants in 66.5% (133/200) of HL patients after exome sequencing, and this rate was not so different from our study given the differences in the sample size.

Conclusion

In conclusion, exome sequencing of four familial cases of autosomal dominant hearing loss revealed a novel nonsense MYO6 mutation in one family. The exome sequencing of deaf families, after the pre-exclusion of known deafness genes, is one strategy that is advancing the determination of the contribution of each known deafness gene in a given population, advancing the establishment of new genotype-phenotype correlations and the discovery of novel deafness genes.

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Conflicts of interest statement

We declare no conflicts of interest.

References


Novel mutation in MYO6 as a cause of deafness


splice site mutation causes autosomal dominant mutations. Curr Opin Cell Biol 64


