A novel KCNQ4 mutation and a private IMMP2L-DOCK4 duplication segregating with nonsyndromic hearing loss in a Brazilian family

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Here we describe a novel missense variant in the KCNQ4 gene and a private duplication at 7q31.1 partially involving two genes (IMMP2L and DOCK4). Both mutations segregated with nonsyndromic hearing loss in a family with three affected individuals. Initially, we identified the duplication in a screening of 132 unrelated cases of hearing loss with a multiplex ligation-dependent probe amplification panel of genes that are candidates to have a role in hearing, including IMMP2L. Mapping of the duplication by array-CGH revealed that the duplication also encompassed the 3'-end of DOCK4. Subsequently, whole-exome sequencing identified the breakpoint of the rearrangement, thereby confirming the existence of a fusion IMMP2L-DOCK4 gene. Transcription products of the fusion gene were identified, indicating that they escaped nonsense-mediated messenger RNA decay. A missense substitution (c.701A>T) in KCNQ4 (a gene at the DFNA2A locus) was also identified by whole-exome sequencing. Because the substitution is predicted to be probably damaging and KCNQ4 has been implicated in hearing loss, this mutation might explain the deafness in the affected individuals, although a hypothetical effect of the product of the fusion gene on hearing cannot be completely ruled out.

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INTRODUCTION

Several genes contribute to the physiology of hearing, and, consequently, hearing loss is extremely heterogeneous from a genetic point of view. Over the past two decades, several genes responsible for hereditary hearing loss have been identified. However, a large number of genes remain to be discovered, as evidenced by the many cases of inherited deafness that continue to be unexplained after extensive molecular investigation. Some mutation mechanisms, such as deletions and duplications, have been under-studied, and the contribution of DNA copy-number variations to hearing loss has only recently begun to be investigated.

To evaluate the contribution of microdeletions and microduplications of genes that might have a role in the etiology of syndromic and nonsyndromic deafness, we selected 17 candidate genes among the genes involved in the rare microimbalances detected by Catelani et al. for investigation by multiplex ligation-dependent probe amplification (MLPA). Our investigated cohort comprised 132 probands from pedigrees presenting with syndromic or nonsyndromic deafness. Among these probands, we identified a duplication in a nonsyndromic proband that segregated with hearing loss in her family. A further investigation with whole-exome sequencing identified the breakpoint of the rearrangement, and it also revealed a missense substitution in the KCNQ4 gene, which is one of the most frequently mutated genes in autosomal dominant nonsyndromic hearing loss.

MATERIALS AND METHODS

Subjects

DNA samples were collected from 132 unrelated individuals affected by hearing impairment who were referred to us for genetic counseling by several institutions in the State of São Paulo, Brazil. In all patients, the most frequent mutations associated with hearing loss (m.1555A>G in the mitochondrial MT-RNR1 gene, c.167delT and c.35delG in the nuclear GJB2 gene and Δ(GJB6-D13S1830) and Δ(GJB6-D13S1854) in the GJB6 gene) had been previously excluded as being the cause of the hearing impairment. Among the 132 patients, 43 presented additional clinical signs and were classified as syndromic, but could not be classified into known syndromes. The majority were isolated cases. The remaining 89 patients were probands from pedigrees in which nonsyndromic deafness segregated in an autosomal mode of inheritance (either dominant or recessive). We also investigated 189 Brazilian individuals presenting with normal hearing from several geographical regions and various ethnical backgrounds (African, Asian, European and native American) as controls for the MLPA experiments. Moreover, an admixed sample of 604 Brazilians with whole-exome sequencing was used as a control to filter variants detected after massive parallel sequencing.

This study was approved by the Ethics Committee of the Biosciences Institute, University of São Paulo, São Paulo, Brazil, and written informed consent was obtained from all participants or their legal guardians.

Audiological evaluation

All patients were evaluated using the following hearing measurements: acoustic immittance including tympanometry, acoustic reflex thresholds and tonal and vocal audiometry with conditioning methods.

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Selection of candidate genes and design of synthetic MLPA probes

Seventeen candidate genes mapping within seven microimbalances detected by BAC array among syndromic hearing loss patients were included in the screening. These genes (CTTN, FGF19, FGF3, FOXC1, FOXX2, FOXQ1, IMPPL2, KIFSC, LRRN3, MAP1A, MYLK4, PPP3CA, SHANK2, SLC5A7, STRC, TPCR2 and TUBB2A) were selected because they were functionally related to the physiology of hearing or expressed in the cochlea or nervous system. One synthetic MLPA probe for each candidate gene was designed in accordance with the protocol from MRC-Holland (Amsterdam, The Netherlands); the probes were produced by Invitrogen (Life Technologies, Carlsbad, CA, USA). The sequences of the MLPA probes are available upon request.

MLPA analysis

The MLPA reaction was performed following the manufacturer’s instructions (MRC-Holland). Capillary electrophoresis and fragment analysis were performed using the 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). Data analyses were performed using the GeneMarker software (Softgenetics, State College, PA, USA).

Array-CGH analysis

Oligoarray-based comparative genomic hybridization was performed with the sample of the patient with the IMPPL2 duplication detected by MLPA using a whole-genome platform containing ~180,000 oligoarrays (Oxford Gene Technology, Oxfordshire, UK), resulting in an average spacing between oligonucleotides of 17 kb. Hybridization and washing were performed according to the manufacturer’s protocol, and the data were analyzed using the Genomic Workbench software (Agilent Technologies, Santa Clara, CA, USA).

Fluorescence in situ hybridization analysis

The duplication in chromosome 7 was verified by fluorescence in situ hybridization performed according to the standard protocols using the RPS-905M6 PAC clone (111,079,598–111,185,843—GRCh37/hg19). This probe was part of the 1-Mb clone set that has been assembled by the Mapping Core, Map Finishing and Microarray Facility groups of the Welcome Trust Sanger Institute, Hinxton, UK, and has been kindly provided by Dr Nigel Carter.

Reverse transcription PCR

Total RNA was isolated from peripheral blood with the RNeasy Protect Cell Mini kit (Qiagen, Venlo, The Netherlands) and treated with DNase I (Invitrogen). Complementary DNA synthesis was performed using the SuperScript First-strand Synthesis System (Invitrogen) following the manufacturer’s protocol. The following primer pair was used to assess the formation of a hybrid transcript composed of IMPPL2 and DOCK4:

5′-ACAGTCCACAAGGGTGTTGGA-3′ (IMMP2L exon 2) and 5′-AGGCGTCCAGCCTCTCTGAG-3′ (DOCK4 exon 39). The reverse transcription PCR (RT-PCR) products were separated by agarose gel electrophoresis, followed by excision from the gel, purification and sequencing using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) and the 3730xl DNA Analyzer (Applied Biosystems).

Massive parallel sequencing analysis

The DNA sample from the proband with the duplication was submitted to Mendelics (São Paulo, SP, Brazil) for whole-exome sequencing. The library was prepared using the Illumina TruSeq (Illumina, San Diego, CA, USA) library preparation kit. Sequence capture was performed with the Nextera Rapid Capture Exome Kit (Illumina), and sequencing was performed using an Illumina HiSeq 2500 platform. Approximately 92 million reads were generated such that at least 82% of the coding sequence was covered by a minimum of 10 reads. Fastq files were aligned to the human reference sequence (GRCh37/hg19) with BWA (http://bio-bwa.sourceforge.net/) for the generation of the BAM files. Realignment of indel regions, variant detection and recalibration of base qualities were performed with GATK (http://www.broadinstitute.org/gatk/) for the production of variant call format files. Annotation was performed with Annovar (http://annovar.openbioinformatics.org/en/latest/).

The bioinformatics pipeline consisted of a one-step filtering over the set of genotyped variants. The following rules were adopted: (1) minor allele frequency = 0 considering 1,000 Genomes (http://www.1000genomes.org/) frequencies, the Exome Variant Server (ESP6500) (http://evs.gs.washington.edu/EVS/) and a database of 604 Brazilian healthy controls; (2) only non-synonymous, frameshifts/in-frame and stop gain/loss variants; and (3) only genes with OMIM/HGMD/HPO phenotypes related to the keyword ‘deafness’ (these phenotypes were organized as a local private database).

RESULTS

Among the 132 cases of syndromic and nonsyndromic hearing loss screened by MLPA, we identified six heterozygous deletions and one duplication in STRC (5.3%) and one duplication in IMPPL2 (0.7%). The screening of imbalances in these genes in 189 hearing individuals revealed eight heterozygous deletions and three duplications in STRC (5.8%) and three heterozygous deletions involving IMPPL2 (1.6%), but no duplications. Although the STRC gene has been previously related to recessive deafness, the Database of Genomic Variants (http://projects.tcag.ca/variation/) shows that deletions and duplications are present in the normal population at a relatively high frequency. The fact that we found heterozygous losses in similar frequencies in the patients and controls suggest that these imbalances most likely represent polymorphisms with no phenotypic effect rather than an association with deafness.

The duplication involving the IMPPL2 gene at 7q31.1 detected in a proband with nonsyndromic hearing loss was inherited from the mother and also shared with the proband’s older sister; all three were affected by different clinical presentations of hearing loss (Figure 1). The proband’s (II-2) hearing impairment was sensorineural, prelingual, bilateral and severe, whereas the mother (I-2) presented postlingual, bilateral and mild sensorineural deafness (Figure 1b). The sister (II-1) presented a normal audiogram with the exception of elevated thresholds (40 dB) in frequencies higher than 6 kHz (Figure 1b). Ophthalmological assessment did not detect evidence of any visual problems, such as retinitis pigmentosa or neuropathy. The proband’s mother reported other cases of hearing loss in the family related to her father, but she could not provide precise information to build the extended pedigree.

We performed array-CGH to determine the extent of the rearrangement at 7q31.1. The results revealed that DOCK4 was also partially duplicated, in addition to IMPPL2 (Figure 2a,b). Fluorescence in situ hybridization analysis showed that both copies of the duplicated segment mapped to a single location at 7q31.1, suggesting the duplicated segments were in tandem (data not shown). In an attempt to investigate other variants that could explain the deafness in this family, we performed whole-exome sequencing on the proband’s sample. The breakpoint could be precisely mapped by two reads, which revealed that the duplication size was exactly 459,020 bp and encompassed exons 1–3 of IMPPL2 and exons 39–52 of DOCK4 (110,942,457 to 111,401,476 bp; GRCh37/hg19 assembly). Because the whole-exome sequencing indicated that the duplication was in tandem and directly oriented, we predicted that a fusion gene composed of the promoter, the 5′-end of IMPPL2 and the 3′-end of DOCK4 could have arisen from the duplication (Figure 2b). The union between exon 3 of IMPPL2 and exon 39 of DOCK4 was predicted to produce a transcript with a shift in the reading frame leading to a premature stop codon in exon 39 of DOCK4 that presumably encoded a hybrid 101-residue protein. To test the existence of
such transcript, we performed RT-PCR on complementary DNA derived from the RNA of peripheral blood lymphocytes using primers that amplify a hybrid segment of 283 bp. RT-PCR products were detected only in samples of the affected individuals tested (Figure 2c). In addition to the expected product of 283 bp (Figure 2c,d), two longer variants were also obtained. Sanger sequencing of the 364-bp product identified an additional 81-bp fragment corresponding to intron 3 of IMMP2L (data not shown) between exon 3 of IMMP2L and exon 39 of DOCK4. This may be due to a splice error that retained part of intron 3 or an alternative splicing of this hybrid transcript. We were not able to identify the sequence of the third variant due to the presence of multiple peaks.

In addition to the duplication, after filtering, whole-exome sequencing identified a list of 18 candidate variants. Taking into account the biological relevance, inheritance and phenotype database information, the missense variant (c.701A>T or p.His234Leu) in exon 4 of KCNQ4 (one of the two genes at the DFNA2 locus) arose as the most plausible candidate to explain nonsyndromic deafness with dominant transmission. The variant was confirmed by Sanger sequencing (Figure 2e). The proband's mother and sister also presented the variant, which was absent in the father. This substitution occurred at an evolutionarily conserved site (Figure 2f) and had not been described in public databases nor was it present in a local control database composed of whole-exome sequencing samples from 604 Brazilian individuals. Evaluation with web-based tools predicted that this variant was probably damaging (PolyPhen-2, value 0.968), deleterious (SIFT, value 0.01) and disease causing (MutationTaster2, value 0.998786).

**DISCUSSION**

In this study, we detected a novel missense substitution in the KCNQ4 (DFNA2A) gene, which is one of the most frequent causative genes in autosomal dominant nonsyndromic hearing loss, in a family with three affected individuals. In addition, we report a rare 459-kb duplication spanning the promoter, the 5′-end of IMMP2L and exon 39 of DOCK4 that was also found to segregate with hearing loss. The duplication created a fusion gene that comprised parts of IMMP2L and DOCK4 and probably encoded three-hybrid transcriptional variants (Figure 2c). Importantly, although IMMP2L and DOCK4 are located within 7q31, these genes do not overlap the critical region of any of the three deafness loci previously mapped in the area (DFNB4, DFNB14 and DFNB17).

**IMMP2L** encodes a protein homologous to the yeast Imp2p, which is one of the catalytic components of the mitochondrial inner membrane protease, and is required for the maturation of mitochondrial proteins that are delivered to the intermembrane space. IMMP2L has been proposed as a candidate gene for Tourette syndrome and autism spectrum disorder. With the exception of the deletion described by Catelani et al., IMMP2L has not been associated with deafness. However, because
mitochondrial mutations are estimated to be responsible for at least 1% of all hereditary deafness cases, any gene with mitochondrial function (i.e., IMMP2L) can be a candidate to explain deafness. Furthermore, mouse mutants with impaired Immp2l were shown to exhibit an early onset of aging-associated phenotypes (i.e., loss of subcutaneous fat, kyphosis and ataxia) as a consequence of an excess of the generation of mitochondrial reactive oxygen species, although hearing impairment was not mentioned. The second gene found to be involved in the duplication (DOCK4) is a member of the CED-5, DOCK180 and MBC (CDM) family of unconventional guanine nucleotide exchange factors that activate the small GTPase Rac. Previous studies have suggested a role for DOCK4 in tumorigenesis and, likewise IMMP2L, susceptibility to autism. In a relevant study of hearing physiology, Yan et al. identified an isoform of DOCK4 that localized to the stereocilia in the inner ear and interacted with harmonin. Mutations in harmonin are responsible for Usher syndrome type 1C (USH1C) and autosomal recessive nonsyndromic deafness (DFNB18).

If translated, the hybrid proteins encoded by the IMMP2L-DOCK4 duplication share the first 80 residues with the wild-type 175-residue IMMP2L protein, including the transmembrane domain. The remaining out-of-frame residues translated from DOCK4 do not correspond to the sequence of any known protein. Since the duplication includes the IMMP2L promoter, the fusion gene would presumably be transcribed in the tissues or organs in which this gene is expressed. In fact, IMMP2L had been detected among expressed sequence tags derived from a human fetal cochlear library (Morton Human Fetal Cochlea complementary DNA). As demonstrated by the RT-PCR results (Figure 2c), the mutant transcripts escaped nonsense-mediated messenger RNA decay (NMD) surveillance, but the reason is unclear. Previous studies have shown that escaping NMD may lead the translated protein to exert dominant-negative or gain-of-function effects. Therefore, one could hypothesize that a truncated protein may convey a dominant-negative effect over the wild-type IMMP2L or even DOCK4 in the cochlea, although this would be difficult to demonstrate in a human cochlea.

The reason why NMD was not triggered even though a premature stop codon was hypothetically introduced after the shift in the reading frame is intriguing. A recent study that evaluated the effects of predicted protein-truncating variants (PTVs) on the human transcriptome brought some new insights to NMD predictions. The authors found that 30.5% of rare PTVs predicted to trigger NMD did not lead to detectable differences in allelic expressivity, suggesting that escaping NMD might not be a rare event. In addition, they observed a tissue specificity in NMD activity, which implied that the same PTV-containing transcripts may have different fates across tissues. Therefore, we could speculate that the mutant transcripts detected in the blood of

Figure 2. (a) 180 K aCGH profile of part of chromosome 7 in the proband showing the duplicated segment at 7q31.1. (b) Schematic representation of the duplication. IMMP2L and DOCK4 are represented by red and blue bars, respectively, and are shown relative to the reference genome (above) and the duplication (below). The dashed-line boxes depict the duplicated region that led to the formation of a fusion gene composed of the 5′ of IMMP2L and 3′ of DOCK4. (c) Reverse transcription PCR (RT-PCR) products corresponding to hybrid IMMP2L-DOCK4 transcripts were detected in samples from the sister and the mother and were absent in the negative control. (d) Top: partial electropherogram of the expected 283-bp RT-PCR product (lower band in c) in the sister’s sample, confirming the formation of a fusion transcript with parts of IMMP2L and DOCK4. Bottom: reference sequences of IMMP2L and DOCK4 at the breakpoint junction with a shift in the reading frame after duplication. (e) Electropherograms of KCNQ4 exon 4 in which a missense mutation (c.701A>T or p.His234Leu) was detected in all affected individuals of the family in heterozygosis (sister’s electropherogram not shown). (f) Conservation analysis of residues 208–237 of the KCNQ4 protein. The 234 position is highlighted by an open-red rectangle.
family members might actually be subjected to NMD in other tissues, as it would be expected.

Another study that fine-mapped 184 germline duplications from individuals referred for diagnostic cytogenetic testing revealed that most duplications were in tandem and directly oriented. Among the 21 duplications that were presumed to generate fusion genes at the breakpoint junctions, 15 were predicted to be out of frame, likewise the duplication found in our study. Although messenger RNA or protein studies were not performed, the authors suggested that the out-of-frame transcripts might produce proteins by alternative splicing using cryptic splice donor and/or acceptor sites. This speculation might explain why we observed three transcriptional variants in our RT-PCR experiments (Figure 2c). In addition to donor and acceptor sites, changes in more distant sites affecting splicing, such as enhancer or silencer motifs, may also affect the splicing of out-of-frame transcripts.

Another fact that points to a possible effect of the IMMP2L-DOCK4 duplication in hearing loss is that although copy-number variations comprising parts of IMMP2L or DOCK4 in healthy individuals have been reported in the Database of Genomic Variants (particularly microdeletions in the S′ half of IMMP2L), duplications partially involving both genes have not been described to date. This finding might suggest that the copy-number variation found in our family was private or rare. Our investigation of copy-number changes in IMMP2L or DOCK4 in an ethnically admixed Brazilian cohort of 189 hearing individuals detected three individuals with deletions in IMMP2L, but no duplications in either gene. In addition, no IMMP2L-DOCK4 duplication was found by array-CGH among 1,000 Brazilian individuals who were either normal or investigated for reasons other than hearing impairment.

Despite a possible functional implication for the private IMMP2L-DOCK4 duplication, a complementary whole-exome sequencing investigation revealed a missense substitution in KCNQ4 that could alone explain deafness in this family. Pathogenic mutations in this gene are usually associated with high-frequency progressive hearing loss, which is compatible with the hearing loss phenotype presented by the family described here. This is the first report of a KCNQ4 mutation in a Brazilian family.

KCNQ4 is one of the six members of the voltage-gated potassium channel gene family that has important roles in regulating electrical signaling and the ionic composition of biological fluids. The protein encoded by KCNQ4 has six transmembrane domains (S1–S6) and a hydrophobic P-loop region between S5 and S6 that forms the ion-selective channel pore. To date, 21 mutations were identified in subjects with progressive hearing loss; most were concentrated in the pore region. The c.701A>T (p.His234Leu) mutation found in our case is located at the cytoplasmic loop between S4 and S5.

Among the 21 KCNQ4 mutations described to date, only two occurred at the S4–S5 linker region: an 18 nt deletion (c.664_681del) and a missense substitution (c.689T>A or p. V230E). The 18-nt deletion was found in a Korean family with 11 affected individuals of which the majority showed mild or moderate hearing loss for low and mid frequencies and severe hearing loss at high frequencies. Conversely, the Japanese family with the p.V230E mutation detected by Naito et al. showed mid-frequency hearing loss.

Almost half of all KCNQ4 pathogenic mutations are located in the pore region. Therefore, mutations affecting this domain appear to be more crucial to normal hearing due to its key role in the ion selectivity of the channel. Baek et al. performed functional in vitro experiments to evaluate the effects of the S4–S5 linker mutation (c.664_681del) identified in their study, in addition to two previously described mutations (p.W276S and p.G285C) located in the pore region. The authors reported that all mutants inhibited normal channel function by a dominant-negative effect.

However, because the c.664_681del mutation eliminated six residues at the S4–S5 loop, one could speculate that an 18 nt deletion might have a more harmful effect than a missense substitution in the same region.

The hearing loss phenotype of the affected individuals investigated here is remarkably variable, especially when the proband and her sister are compared: although the proband showed prelingual and severe hearing loss, the proband’s sister showed mild hearing loss at higher frequency ranges (Figure 1b). Since the three affected individuals in the pedigree present both the KCNQ4 substitution and the IMMP2L-DOCK4 duplication, the reason for the difference in expressivity of the phenotype is not clear. Nevertheless, intrafamilial variability in the degree of deafness has been well documented in several nonsyndromic hearing loss families. Moreover, one should never discard a possible role of modifying genes or environmental factors in phenotypic variability even within families.

In conclusion, we identified a novel missense mutation in KCNQ4 and a partial duplication of IMMP2L and DOCK4 in a family with variable severity of nonsyndromic hearing loss. The KCNQ4 mutation alone might be the cause of the hearing loss presented by the family, although a role for the IMMP2L-DOCK4 duplication cannot be completely ruled out. Our study illustrates how complementary investigations including whole-exome sequencing can help to address questions related to the elucidation of the pathogenicity of copy-number variations.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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