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A novel missense mutation p.L76P in the *GJB2* gene causing nonsyndromic recessive deafness in a Brazilian family

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Mutations in the *GJB2* gene, encoding connexin 26 (Cx26), are a major cause of nonsyndromic recessive hearing loss in many countries. We report here on a novel point mutation in *GJB2*, p.L76P (c.227C>T), in compound heterozygosity with a c.35delG mutation, in two Brazilian sibs, one presenting mild and the other profound nonsyndromic neurosensorial hearing impairment. Their father, who carried a wild-type allele and a p.L76P mutation, had normal hearing. The mutation leads to the substitution of leucine (L) by proline (P) at residue 76, an evolutionarily conserved position in Cx26 as well as in other connexins. This mutation is predicted to affect the first extracellular domain (EC1) or the second transmembrane domain (TM2). EC1 is important for connexon-connexon interaction and for the control of channel voltage gating. The segregation of the c.227C>T (p.L76P) mutation together with c.35delG in this family indicates a recessive mode of inheritance. The association between the p.L76P mutation and hearing impairment is further supported by its absence in a normal hearing control group of 100 individuals, 50 European-Brazilians and 50 African-Brazilians.

Key words: *GJB2* gene; Connexin 26; Hearing impairment; p.L76P; c.227C>T

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Hearing impairment is the most frequent and genetically heterogeneous neurosensory disorder. Epidemiologic studies have shown that hereditary nonsyndromic neurosensorial hearing impairment is predominantly inherited in an autosomal recessive manner (80%), *GJB2* being the most frequently mutated gene. This gene encodes the gap junction protein connexin 26 (Cx26) (1). With the exception of the *GJB6* (connexin 30, Cx30) gene, connexin genes share the same architecture. A single large exon contains the entire coding region and is separated from its 5' untranslated region by an intron of variable size.

Most *GJB2* mutations are located in the coding region of the gene (exon 2), with the exception of two splice site mutations (IVS 1+1G>A, IVS 1+3G>A) at the end of the noncoding exon 1, and the mutation -3438C>T in the basal promoter region (2-4). To date, about 90 mutations of the *GJB2* gene have been described, causing recessive hearing impairment (5). The c.35delG is particularly common in many populations, especially in the Mediterranean region (6), where it accounts for approximately 60% of *GJB2* mutated alleles. In Brazil, it represents near 50% of the *GJB2* mutations (7).

Connexins are constituents of connexons. Six connexins assemble into a connexon hemichannel, and the docking of two connexons from adjacent cells establishes gap junctions that constitute a major system of intercellular communication for the exchange of electro-

lytes, second messengers and metabolites. Gap junctions seem to be essential for recycling potassium ions that are needed to initiate action potentials in hair cells (8). One postulated key role of these gap junctions in the sensory epithelia of the inner ear is in recycling potassium ions from the hair cells back to the endolymph in the auditory process (9). Immunochemical experiments have shown *GJB2* expression in the stria vascularis, basement membrane, limbus, and the spiral prominence of the cochlea (10). A defective cochlear gap junction system can lead to hearing impairment.

We report here on a novel missense mutation, p.L76P (c.227C>T), in the coding region of the *GJB2* gene associated with autosomal recessive nonsyndromic hearing loss. This mutation, which has not been previously described, was identified in two sibs with hearing impairment who carried the c.35delG mutation *in trans*.

The proband (Figure 1A, II-1) was a boy, born to nonconsanguineous clinically normal parents, and was referred to us at age of 12 years presenting hearing impairment. His 2-year-old sister also had hearing loss. The parents, both born in the city of São Paulo, were not affected by hearing impairment. No other cases of hearing impairment were reported in the family, and clinical evaluation of the children did not reveal symptoms or malformations that could suggest syndromic features. The following hearing measurements were performed in I-1, I-2, II-1 and II-2: acoustic immittance, including tympanometry and acoustic reflex thresholds, tonal and vocal audiometry with conditioning methods according to patients age. Pure tone audiometry was carried out to test

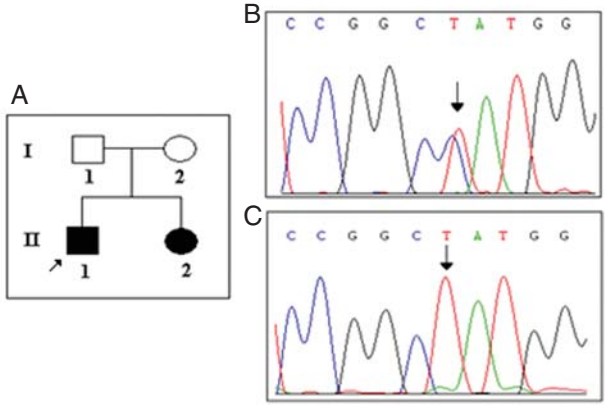

Figure 1. Novel mutation in the *GJB2* gene. *A*, Pedigree of the family carrying the p.L76P (c.227C>T) mutation. The father (I-1) carries p.L76P in heterozygosis and the mother (I-2) carries c.35delG in heterozygosis. The sibs (II-1 and II-2) both carry p.L76P and c.35delG mutations. Sequence showing nucleotides corresponding to positions 222-231 of the *GJB2* gene in the heterozygous father (B) and wild-type sequence (C).

for air (250-8000 Hz) and bone conduction (250-4000 Hz). Transiently evoked oto-acoustic emissions and distortion product were recorded for patients II-1 and II-2. A detailed clinical history was obtained and other causes of hearing impairment were excluded (neonatal complications, bacterial meningitis or other infections, use of ototoxic medication, and head trauma).

The study was approved by the Ethics Committee of Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil, and written informed consent was obtained from all individuals or their legal guardians.

Genomic DNA was extracted from peripheral blood leukocytes by standard protocols. The proband and his sister were first screened for the c.35delG mutation in the *GJB2* gene (11), and the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) deletions in the *GJB6* gene (12). The screening of the children revealed c.35delG in heterozygosis in both. Sequencing of the *GJB2* coding region confirmed the c.35delG and indicated a C>T substitution at nucleotide 227 (c.227C>T), leading to the replacement of a leucine by a proline at residue 76 (Figure 1B) in the 2 children. Their mother and father were found to be carriers of the c.35delG and the p.L76P mutation, respectively, and of normal alleles. The detected substitution has not been reported before (5, OMIM 121011).

In order to investigate the frequency of the novel c.227C>T in the general population, we screened a control sample consisting of 100 unrelated normal hearing individuals, 50 European-Brazilian and 50 African-Brazilians. Individuals who declared that the 4 grandparents were of European ancestry were considered to be European-Brazilians, and those who declared at least 1 grandparent of African ancestry were considered to be African-Brazilians. The analysis was performed with the MegaBace SNuPe genotyping kit (GE Healthcare, USA). None of the 100 controls carried the c.227C>T (p.L76P) mutation, thus decreasing the probability of it being polymorphic in the population.

The possible pathological nature of the substitution was pointed out by the analysis using PolyPhen (*Poly*morphism *Phen*otyping) (13,14), which indicated the c.227C>T (p.L76P) mutation to be possibly pathological.

The leucine amino acid at residue 76 of Cx26 is conserved among the different species (Figure 2) and among alpha and beta connexins (15), suggesting that this residue is crucial for the protein to be functional.

Connexins contain four transmembrane domains (TM1-TM4), two extracellular domains (EC1-EC2), one cytoplasmatic loop (CL), and N- and C- cytoplasmic termini (NT-CT). The N-terminal domain is involved in the process of membrane integration and hexamer forma-

tion (16,17) and, together with the first transmembrane domain, determines voltage gating. The extracellular loops regulate the connexon-connexon interactions, including heterotypic channel formation. Each loop contains three cysteine residues, conserved across all connexins and that form essential intramolecular disulfide bonds (18). The intracellular loop and C-terminal domains regulate pH gating (1). The TM domains are important for protein folding. The p.L76P mutation is predicted to affect the second transmembrane domain (TM2), but, according to some data bases, this position may be located at the highly conserved first extracellular loop (EC1) of Cx26, a region suggested to play a role in protein targeting. Other reported mutations that cause dominant (R75W and R75Q) and recessive (W77X, W77R) hearing loss are located in the same region.

The patient described here is of predominantly European ancestry. Since the Brazilian population results from an admixture of three main geographic groups, Africans, Europeans and Native Americans, we cannot rule out a genetic contribution from Native Americans or Africans in this family. It is premature to speculate about the origin of the mutation. It is certainly a rare mutation. We have not detected it in a serial sample of 300 probands with hearing impairment, who had the complete coding region of the *GJB2* gene investigated after SSCP analysis and sequencing (19). The screening of the c.35delG mutation in a total of 600 probands revealed 21 other heterozygotes (our unpublished data), and complete sequencing of the coding region of the *GJB2* gene did not reveal the p.L76P mutation.

p.L76P	K	N	V	C	Y	D	H	Y	F	P	I	S	H	I	R	P	W	A	L
Human	K	N	V	C	Y	D	H	Y	F	P	I	S	H	I	R	L	W	A	L
Rhesus	K	N	V	C	Y	D	H	Y	F	P	I	S	H	I	R	L	W	A	L
Mouse	K	N	V	C	Y	D	H	H	F	P	I	S	H	I	R	L	W	A	L
Dog	K	N	V	C	Y	D	H	Y	F	P	I	S	H	I	R	L	W	A	L
Horse	K	N	V	C	Y	D	H	Y	F	P	I	S	H	I	R	L	W	A	L
Armadillo	K	N	V	C	Y	D	H	Y	F	P	I	S	H	I	R	L	W	A	L
Opossum	K	N	V	C	Y	D	H	F	F	P	I	S	H	I	R	L	W	A	L
Platypus	K	N	V	C	Y	D	H	V	F	P	I	S	H	I	R	L	W	A	L
Lizard	K	N	V	C	Y	D	A	F	F	P	V	S	H	I	R	L	W	A	L
Chicken	R	N	V	C	Y	D	H	F	F	P	I	S	H	I	R	L	W	A	L
X_tropicalis	K	N	V	C	Y	D	H	H	F	P	V	S	H	I	R	L	W	C	L
Stickleback	K	N	V	C	Y	D	H	F	F	P	V	S	H	I	R	L	W	C	L

Figure 2. Alignment analysis of the p.L76P mutation in the connexin 26, *GJB2* gene, in different species. Residue 76 is boxed.

The hearing impairment was mild in the proband (I-1) and profound in his sister (II-2). The segregation of the c.227C>T (p.L76P) mutation together with c.35delG in this family indicates a recessive mode of inheritance, since the c.35delG heterozygous mother and the p.L76P heterozygous father both presented normal hearing.

Summing up, the presented family, population and bioinformatic data provide strong evidence for a causative association of the p.L76P mutation in the *GJB2* gene with hearing impairment. The characterization of novel mutant alleles may contribute to a better understanding of the function of the connexin 26 domains.

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