Screening for mutations in the \textit{GJB3} gene in Brazilian patients with nonsyndromic deafness

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\textbf{Abstract.} Deafness is a complex disorder that is affected by a high number of genes and environmental factors. Recently, enormous progress has been made in nonsyndromic deafness research, with the identification of 90 loci and 33 nuclear and 2 mitochondrial genes involved (http://dnalab-www.uia.ac.be/dnalab/hhh/). Mutations in the \textit{GJB3} gene, encoding the gap junction protein connexin 31 (Cx31), have been pathogenically linked to erythrokeratodermia variabilis and nonsyndromic autosomal recessive or dominant hereditary hearing impairment. To determine the contribution of the \textit{GJB3} gene to sporadic deafness, we analysed the \textit{GJB3} gene in 67 families with nonsyndromic hearing impairment. A single coding exon of the \textit{GJB3} gene was amplified from genomic DNA and then sequenced. Here we report on three amino acid changes: Y177D (c.529T \textgreater T), 49delK (c.1227C \textgreater T), and R32W (c.144-146delGAA). The latter substitution has been previously described, but its involvement in hearing impairment remains uncertain. We hypothesize that mutations in the \textit{GJB3} gene are an infrequent cause of nonsyndromic deafness.

\textbf{Key words:} connexin 31, \textit{GJB3}, gap junctions, hearing impairment, mutations.

\textbf{Introduction}

Hearing impairment is the most prevalent sensorial deficit in the general population and its prevalence increases with age (RABIONET et al. 2000). In developed countries, genetic factors are the major cause of hearing loss, accounting for about 60\% of cases (COHEN, GORLIN 1995, MORTON 1991). In Brazil, most cases of hearing loss are due to environmental factors, such as congenital infections.
(mainly rubella), perinatal anoxia, kernicterus, and meningitis (SIMÕES, MACIEL-GUERRA 1992). However, the proportion of genetic causes tends to increase as a result of improvements in health care.

In some cases, different mutations at the same locus have been found to cause syndromic and nonsyndromic forms of deafness. Approximately 90% of cases of genetic deafness are nonsyndromic, and in this group the majority are autosomal recessive forms, in which the patients are born from parents with normal hearing, while the remaining 10% or less are born from deaf parents (ACMG 2002). Recently, some forms of deafness have been described, in which the hearing loss may result from combined effects of genes at two or more loci.

Direct evidence of the critical physiological role of connexins has come through the linkage of several human diseases with pathogenic mutations in genes that encode specific connexins. Connexins are transmembrane proteins involved in formation of intercellular channels. These gap junction channels permit the rapid exchange of ions and are thought to play an important role in maintaining hearing function by circulation of potassium ions between the fluids of the inner ear (GOODENOUGH et al. 1996, BRUZZONE et al. 1996). Blockage of K+ circulation causes hearing impairment.

A number of relevant genes have been cloned. DFNB1 was the first locus implicated in nonsyndromic deafness (KELSELL et al. 1997). The causative gene GJB2, which encodes the protein connexin 26, accounts for up to 50% of recessive nonsyndromic hearing impairments in various populations (ZELANTE et al. 1997, ESTIVILL et al. 1998).

Several connexins are known to be expressed in the cochlea and involved in deafness. The GJB3 gene has recently been found as a deafness gene encoding ion channels regulating the K+ recycling pathway.

Mutations in the GJB3 gene were initially linked to nonsyndromic autosomal dominant hearing loss (XIA et al. 1998) and subsequently, with autosomal recessive deafness (LIU et al. 2000). GJB3 mutations have also been linked to erythrokeratodermia variabilis (RICHARD et al. 1998). In this study, we tested GJB3 mutations in 67 families with nonsyndromic hearing impairment. These patients were previously tested for GJB2 mutations.

Material and methods

Blood specimens and clinical data were collected from human subjects with approval of the appropriate institutional review board and informed consent of those subjects.

For mutation analysis a single coding region of the GJB3 gene was PCR-amplified with the use of five pairs of primers described elsewhere (LIU et al. 2000). PCR was performed in a total volume of 40 µL, containing 200 ng of genomic DNA, 20 pmols of each primer, 200 µM of each dNTP, 1.5 mM
MgCl₂, and 2.5 U of Taq DNA polymerase. The conditions for the reactions were 94°C for 5 min, 30 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. Direct sequencing of PCR products was performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM/PE Biosystems) and the products were resolved on ABI PRISM™ 377 (Perkin Elmer). For mutation analysis, the denatured PCR products were run on a 1 mm thick 12% nondenaturing polyacrylamide gel and SSCP's were detected by silver staining as previously described (LIU et al. 2000).

Results and discussion

The entire GJB3 gene was sequenced in families with nonsyndromic hearing loss after initial screening for mutation in GJB2 alleles. Two new mutations in the GJB3 gene were found.

One mutation is a substitution leading to a missense mutation Y177D (c.529T > G) situated in the second extracellular loop of the connexin 31 protein (Figure 1). This mutation was found in two unrelated patients with profound hearing loss and no skin lesions in heterozygous form. The first patient has a profound hearing loss and probably inherited the mutation from his mother, whose hearing status is unknown. In the other patient, the heterozygosity for this mutation was found also in his hearing father. Therefore, the pathogenicity of this mutation could not be confirmed. To exclude the possibility that the Y177D (c.529T > G) mutation is a benign polymorphism, we studied samples from 100 unrelated con-

![Figure 1. Sequence analysis of the GJB3 gene illustrating the Y177D (c.529T > G) alteration. A single peak (T) is observed at nucleotide 529 in the GJB3 sequence from a healthy person (a), and a doublet (T/G) is observed at nucleotide 529 (N) in a patient (b and c).](image-url)
trol subjects, but no mutation was found. The role of this mutation remains to be clarified. In fact, there are many other mutations of genes coding for connexins that could act as polymorphisms in different backgrounds, such as R32W (c.1227C > T), which we will discuss later.

The other novel mutation found is a deletion of three nucleotides (c.144-146delGAA) in the GJB3 gene, which results in the lack of a lysine residue in position 49 (49delK) (Figure 2). This patient presents moderate sensorineural deafness with late onset and no other clinical abnormalities.

The R32W (c.1227C > T) mutation was first described and associated with two sequence variants in the GJB2 gene (M34T and D66W) in two affected members of a family with hearing loss and palmoplantar keratoderma (KELSELL et al. 2000). The amino acid substitution R32W (c.1227C > T) is located in the highly conserved sequence of the first transmembrane domain. The C → T transversion at nt 1227 changes the amino acid arginine residue to a noncharged tryptophan residue. Other studies extended the analysis of this mutation to patients with peripheral neuropathy (2/110), deafness (7/153), and control subjects (8/46) (LÓPEZ-BRIGAS et al. 2000). R32W (c.1227C > T) was reported as a common polymorphism with an allelic frequency of 7.5% in the Spanish population, and does not segregate with hearing impairment in several families (LÓPEZ-BRIGAS et al. 2001).

Recently, the biologic relevance of the R32W (c.1227C > T) mutation was studied in terms of gap junction activity, suggesting that this mutation is a functionally inconsequential polymorphism of the GJB3 gene (MHATRE et al. 2003).
In our study, we found heterozygotes of the R32W (c.1227C > T) mutation in a large family with many individuals with various degrees of hearing impairment. The proband and his father were heterozygous for R32W and present moderate sensorineural deafness with late onset. On the other hand, in his sister, who presents profound hearing loss and has a cochlear implant, the R32W (c.1227C > T) mutation was not detected. Therefore, deafness is not segregating with this mutation.

The presence of the R32W (c.1227C > T) mutation can be detected by restriction analysis (digestion with \textit{Hpa} II), which in the absence of R32W (c.1227C > T) digests the PCR product (210 bp) into two fragments: 117 bp and 93 bp, respectively.

We also identified two heterozygotes for single nucleotide substitutions, which do not affect the connexin 31 amino acid sequences P159P and G193G (c.477G > A; c.579C > T).

**Conclusions**

Clinical data of patients with \textit{GJB3} mutations indicate that there is a wide variability in the age of onset of hearing loss, with some mutations being also present in subjects with apparently normal hearing. This variability probably depends on the type and location of the \textit{GJB3} mutation.

In this study, mutations of the \textit{GJB3} gene were not shown to be a major cause of nonsyndromic hearing impairment. In agreement with other authors, we suggest that the \textit{GJB3} gene mutations are not a common cause of deafness. Further studies are necessary to confirm the relevance of mutations in this gene in order to detect whether some of them may act as modifying alleles.

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**REFERENCES**


