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# Analysis of the presence of the GJB6 mutations in patients heterozygous for GJB2 mutation in Brazil

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**Abstract** Mutations in the GJB2 gene, mainly 35delG, are responsible for most autosomal recessive inherited genetic hearing loss. The audiometric standard of these hearing losses remains inconsistent and other genes, such as GJB6, have been involved in association with GJB2. The objective of the study was to identify the deletions del(GJB6-D13S1830) and del(GJB6-D13S1854) in patients heterozygous for 35delG/GJB2 and analyze the phenotype they present. 101 patients with mild to profound degree of sensorineural hypoacusis were evaluated. The allele-specific PCR technique was used to identify 35delG. The del(GJB6-D13S1830) and del(GJB6-D13S1854) were identified through the PCR multiplex technique. 90 % of the subjects presented a normal genotype for the analyzed mutations; 6.93 % were shown to be heterozygous for 35delG/GJB2 and 1 % presented compound heterozygosis GJB2/GJB6). The data found reinforced the hypothesis of an interaction of more than one gene as the cause of autosomal recessive genetic hearing loss and emphasized the importance of an early diagnosis for appropriate intervention.

**Keywords** Deafness · Connexin 26 · Connexin 30 · GJB6 · GJB2

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## Introduction

Hereditary hearing loss affects 1 out of every 1,000 live births and is responsible for more than 50 % of severe deafness in childhood [1, 2], generally being influenced by consanguinity. In Brazil there is an estimated hereditary hearing loss rate of four out of every 1,000 live births [3]. Most cases of genetic deafness are almost exclusively monogenic, that is, due to a single and highly heterogeneous gene [4], which may be dominant, recessive or linked to the X chromosome. The pattern of autosomal recessive inheritance is involved in more than 75 % of the cases; the dominant inheritance makes up 10–20 % of the cases, and 2–3 % in inheritance linked to the X [4, 5].

Genetic hearing impairment has been greatly studied and today it is known that mutation in the connexin 26 (GJB2) gene is one of the principle causes of autosomal recessive hearing loss in non-syndromic patients.

This gene, considered the greatest determinant of non-syndromic recessive hearing loss, presents a prevalence of mutations varying between 0.5 and 5.4 % in different racial groups [6–8]. The specific deletion 35delG includes 70–85 % of the mutations of this gene among northern Europeans [6, 9–12]. This mutation may be present in homozygosis or compound heterozygosis (with other mutations in the GJB2 gene or in other genes). In Brazil, the frequency of those carrying the mutant allele varied from 0.97 to 2.24 % in studies carried out in the state of Sao Paulo [13–15].

Despite several studies in process, a pattern of hearing loss caused by a mutation in the GJB2 gene still has not been established. This inconsistent and incomplete phenotypic pattern causes problems in determining the genetic pattern. In addition, a great part of these patients (10–42 %) present just one mutant allele [16]. It is thought that the presence of biallelic mutations, in other words,

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homozygosis, characterizes a hearing loss that affects all acoustic frequencies, appears in the pre-lingual period, is symmetric, sensorineural, non-progressive and of variable severity. It is assumed that the severity and progress of hearing loss varies according to the genotype [6, 9, 10, 17, 18]. In compensation, heterozygous mutations do not have a defined audiometric pattern.

A mutation involving the GJB6 gene (encoding the protein connexin 30) proved to be the cause of dominant hearing loss (DFNA3). Recent research indicated that deletions in this same gene, adjacent to GJB2 on chromosome 13 (locus DFNB1), could also be causes of recessive hearing loss. It is believed that this mutation may cause hearing loss as a homozygous inheritance or in combination with a mutation in the GJB2 gene. This hypothesis arose from the fact that 10–50 % of individuals with hearing loss secondary to autosomal recessive GJB2 mutations have just one mutant allele. The proteins connexin 26 and connexin 30 have 76 % identical amino acids and their chromosomal location is really close. Therefore, they are considered jointly responsible for certain hearing impairments [19]. Santos et al. [6] also confirmed this association. Among the deletions involving the GJB6 gene, two are considered more prevalent, del (GJB6-D13S1830) and del (GJB6-D13S1854). A multicenter study involving nine countries showed a prevalence ranging from 5.9 to 9.7 % of the del (GJB6-D13S1830) [20]. Other studies have considered the frequency of deletions between 5 and 15 % in subjects with only one mutated GJB2 allele [21]. In European, Jewish, as well as Brazilian populations, the del (GJB6-D13S1830) mutation already appears as the second most frequent one related to the autosomal recessive hearing loss [22]. Thus, studies in several genetic centers are being carried out in patients heterozygous for 35delG GBJ2 in order to identify the del(GJB6-D13S1830) and del(GJB6-D13S1854) deletions in GJB6, trying to explain the variety of phenotypes of heterozygous hearing loss for GJB2 through the interaction between these two genes [6, 23, 24].

The aim of this study was to describe the impact of the GJB6 deletions in association with the GJB2 heterozygosis mutation in the hearing loss. Another objective was also correlate genotypic pattern to the phenotype presented by subjects.

## Case selection and methods

A previous study carried out from June 2007 to December 2009 involved 264 individuals with apparently non-syndromic autosomal recessive hearing loss that were attended at the Otorhinolaryngology Clinic in the Clinical Hospital at the Medical School in Marilia. Of these patients, 101 were submitted to genetic tests for research of the 35delG mutation in the connexin 26 (GJB2) gene. The inclusion criterion in this study was the absence of evidence of another cause of genetic hearing loss, whether syndromic, non-syndromic, or environmental. To identify the genetic mutation, the DNA of the patients was isolated by means of peripheral blood. The deletion 35delG in the GJB2 gene was identified through the allele-specific PCR method [25].

Of these patients, the individuals that presented heterozygous characteristic for the 35delG mutation in the GJB2 gene were selected to give sequence in the study. Other mutations involving the connexin 30 (GJB6) gene were also studied in these patients. The deletions del(GJB6-D13S1830) and del(GJB6-D13S1854) including part of the GJB6 gene were analyzed essentially as described by Del Castillo et al. [16], through the PCR multiplex technique, using three primers (F: 5′TTT AGG GCA TGA TTG GGG TGA TTT-3′; R1: 5′CAC CAT GCG TAG CCT TAA CCA TTTT-3′; R2: 5′TCA TCG GGG GTG TCA ACA AAC A-3′). The F and R1 primer detected the presence of the deletion and the R2 primer detected the normal allele. When used together, the three primers allow the differentiation between individual normal or mutant homozygotes and heterozygotes.

All of the patients signed a free and clarified consent term according to the 196/96-CNS/MS resolution. In the case of children, the consent of the parents or those responsible was obtained. Furthermore, it is important to note that this project was submitted to analysis by the Committee of Ethics in Research Involving Human Beings from the Medical School in Marilia where it was considered APPROVED in Regular Meeting on October 29, 2007, according to the 196/96 Resolution and its Resolutions from the National Health Council under the protocol study number 625/07.

For the analysis of the results, audiometric asymmetry was defined as a difference of more than 10 dB in at least two frequencies between the ears [26]. The average to define the gravity of hearing loss was calculated using the frequencies of 500, 1,000, 2,000 and 4,000 Hz and hearing loss was classified as the following: 26–40 dB, mild loss; 41–60 dB, moderate loss; 61–80 dB, severe loss; and >81 dB, profound loss [27]. The age of onset for hearing loss was defined as being pre-lingual when it appeared before the acquisition of oral language (usually 2 years), making it impossible to learn, and post-lingual when it appeared after acquiring oral language (after 2 years).

In addition, the progression of these hearing losses was also analyzed.

## Results

Of the 101 patients that underwent molecular tests to detect the 35delG mutation in the GJB2 gene, eight presented

**Table 1** Distribution of the patients according to genetic mutations, gender, type of hearing loss, audiologic symmetry, type of audiometric curve, age of onset, degree of hearing loss, and progression

| Individuals | 35delG GJB2 | Del (D13S1830)/del (D13S1854) GJB6 | Gender | Type of hearing loss | Audiologic symmetry | Type of audiometric curve | Age of onset | Degree of hearing loss | Progression |
|---|---|---|---|---|---|---|---|---|---|
| 01 | Heterozygous | Absent/absent | F | SN | Yes | Flat | PrL | P | No |
| 02 | Heterozygous | Heterozygous/ absent | M | SN | Yes | Flat | PrL | P | No |
| 03 | Heterozygous | Absent/absent | M | SN | Yes | Flat | PoL (adult) | Mi | Yes |
| 04 | Heterozygous | Absent/absent | F | SN | Yes | Flat | PoL (child) | S | Yes |
| 05 | Heterozygous | Absent/absent | F | SN | Yes | Flat | PrL | P | No |
| 06 | Heterozygous | Absent/absent | F | SN | Yes | High | PoL (child) | Mo | No |
| 07 | Heterozygous | Absent/absent | M | SN | Yes | Flat | PoL (child) | Mo | Yes |
| 08 | Heterozygous | Absent/absent | F | SN | Yes | Flat | PrL | S | No |

heterozygous alleles for such mutation, two belonging to the same family (father-03- and son-02- shown by Table 1). All of them presented sensorineural hearing loss (SN) with symmetrical bilateral audiometric curves, seven with flat configuration and one with a drop in high frequencies. There were pre-(PrL) and post-lingual (PoL) hearing loss. The degree of hearing impairment ranged from mild (Mi) to profound (P). Three patients presented a progressive loss (only data reported by anamnesis of patients who did not specify time) and in five of them, the hearing loss remained stable, even in those with non-profound hearing loss who did not show progression until the interview date, when they were adults (subjects 06 and 08). These data are summarized in Table 1.

Of all eight patients that were submitted to the test to detect mutations in the GJB6 gene, one was found to be heterozygous positive for del(GJB6-D13S1830) (subject 02). Figure 1 illustrates this mutation.

## Discussion

Among the 101 patients studied, eight presented to be heterozygous for 35delG/GJB2 and of these eight, one presented to be heterozygous for del(GJB6-D13S1830). This incidence is in agreement with previous studies as shown in Table 2.

In this study, one may observe varied degrees of hearing loss (mild to profound) within the same type of genotype. Literature shows that the severity of hearing loss may vary according to homozygosis, heterozygosis or even to biallelic mutations [6, 11, 29]. The patients that presented profound hearing loss were so since birth; the mild and moderate hearing losses appeared in the post-lingual period; while those with severe hearing loss varied between congenital and post-lingual. Previous studies correlate moderate losses with genotypes heterozygous for GJB2 [6].

All presented bilateral and symmetrical hearing losses, confirming other studies in which the interaural differences in patients with 35delG/GLB2 were rare [9].

Although most of the audiometric tonal thresholds presented flat curves, a descending curve was also observed, in agreement with previous studies that verified that there was no audiometric standard for GJB2 and that flat curves were common [9, 13, 18, 30].

Progressive and stable hearing losses were found, similar to that observed by Janecke et al. [11], even though previous studies defined mutations in the GJB2 as non-progressive [9, 10, 18, 29]. Large cohorts are necessary to evaluate progression.

Literature defends the hypothesis that most hearing loss due to GJB2 mutations are pre-lingual when homozygosis [9]. This study showed that heterozygosis may cause hearing loss in the pre- or post-lingual period.

## Conclusion

In conclusion, this study showed more evidence that the GJB2 mutation in heterozygosis causes a variable degree of sensorineural hypoacusis, progressive or not, generally symmetrical, varying in its audiometric curve and may manifest in the pre- or post-lingual period. The compound heterozygosis phenotype (GJB2/GJB6) appeared as a sensorineural, bilateral, flat, pre-lingual with audiometric flat configuration, and profound degree of hearing loss. Thus, it can be concluded that these autosomal recessive hearing losses may be caused by other mutations in the GJB2,

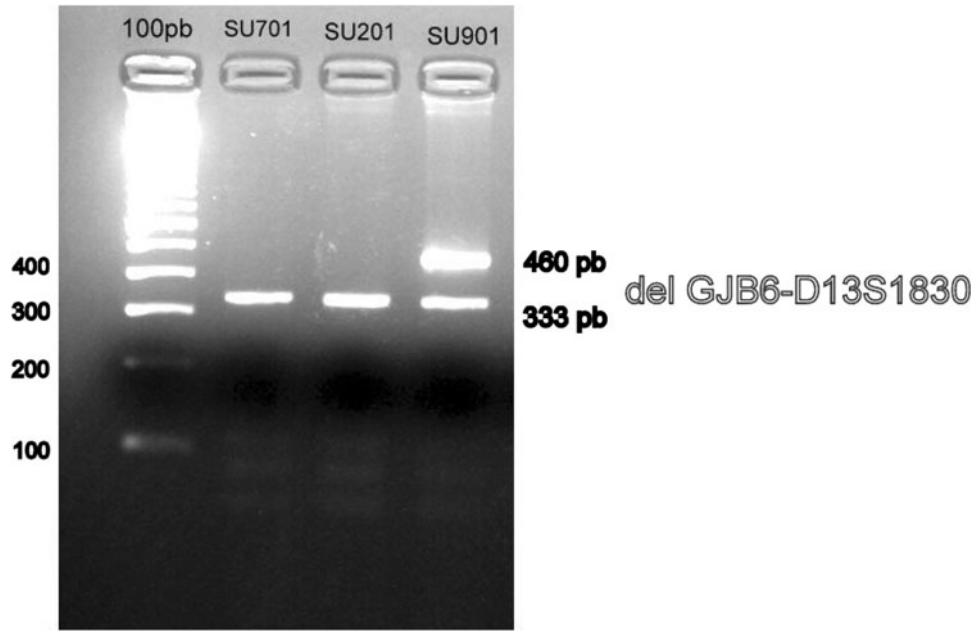


**Fig. 1** Del (GJB6-D13S1830) found in the gene GJB6's patient 02 (SU 901)

**Table 2** Incidence of genotypes found in compared literature

| Genotypes | Present study (%) | Cordeiro-Silva et al. [13] (%) | Batissoco et al. [21] (%) | Piatto et al. [28] (%) |
|---|---|---|---|---|
| 35delG/N | 07/101 (6, 93) | 05/77 (7, 8) | 12/300 (4) | 3/33 (9) |
| 35delG/del (GJB6-D13S1830) | 01/08 (12, 5) | 01/06 (16, 7) | 03/15 (20) | 01/04 (25) |

*N* absence of 35delG/GJB2 mutation and absence of del(GJB6-D13S1830) mutation

GJB6 or even another gene, in addition to speculation on worsening influence of environmental factor upon hearing.

These results reinforce the importance of a molecular diagnosis, not only to establish the cause of hearing loss, but also to provide genetic counseling to the family, and hopefully, to have a better capacity in the future to repair such abnormalities.

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