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ORIGINAL ARTICLE

Screening for the *GJB2* c.-3170 G>A (IVS 1+1 G>A) Mutation in Brazilian Deaf Individuals Using Multiplex Ligation–Dependent Probe Amplification

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Mutations in *GJB2* gene are the most common cause of nonsyndromic sensorineural recessive hearing loss. One specific mutation, c.35delG, is the most frequent in the majority of Caucasian populations and may account for up to 70% of all *GJB2* mutations. However, 10–40% of the patients carry only one pathogenic mutation in the *GJB2* gene. Deletions del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854), truncating the *GJB6* gene, have been detected in *GJB2* heterozygous patients in different populations. The IVS 1+1 G>A splice site mutation in the noncoding region of the *GJB2* gene has been found in heterozygous state in addition to c.35delG mutation. This mutation has not been reported in Brazilian deaf patients. In the present study we investigated the presence of the IVS 1+1 G>A mutation by multiplex ligation–dependent probe amplification in 185 unrelated Brazilian patients with autosomal recessive nonsyndromic sensorineural hearing loss (43 heterozygous patients and 142 without any pathogenic mutation in the *GJB2*-coding region). We have found two patients (4.6%) carrying the IVS 1+1 G>A mutation in compound heterozygous with c.35delG mutation.

Introduction

Despite the enormous heterogeneity of genetic hearing loss, mutations in the *GJB2* gene encoding the protein connexin 26 (Cx26) account for up to 50% of cases of nonsyndromic sensorineural recessive hearing loss (NSRHL). In addition, mutations in *GJB2* gene are responsible for 10–20% off all the prelingual hearing impairment (Wilcox *et al*., 2000).

Many mutations in *GJB2* gene have been identified (Connexin-Deafness HomePage, http://davinci.crg.es/deafness/index.php). The frequency of these mutations is substantially different among populations (Kenneson *et al*., 2002). The c.35delG mutation is the most common, accounting for up to 70% of all the *GJB2* mutations in Caucasian individuals (Seeman *et al*., 2004). In Brazil, mutations in the *GJB2* gene have been found in 22% of the patients with nonsyndromic deafness (Oliveira *et al*., 2002). A previous study to verify the c.35delG carrier frequency in different Brazilian regions revealed an overall frequency of 1:74 among random samples of newborns (Oliveira *et al*., 2007).

However, in different populations, 10–40% of the patients with NSRHL carry only one pathogenic mutation in the *GJB2* gene, which causes a problem in molecular diagnostic and genetic counseling. Searching for other mutations near *GJB2* gene, two large deletions in the *GJB6* gene named del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) have been found (Del Castillo *et al*., 2003). Del(*GJB6*-D13S1830) is more frequent than del(*GJB6*-D13S1854) and is the second causal mutation in monoallelic heterozygous patients in Spain and France (Del Castillo *et al*., 2005). This deletion has been found in about 25% of the c.35delG heterozygous patients in Brazil (Piatto *et al*., 2004).

A significant percentage of monoallelic patients for *GJB2* mutations is negative for deletions in *GJB6* gene or other mutations in coding region of the *GJB2* gene. Therefore, they may have mutations in another region of the gene or an additional unidentified mutation.

The IVS 1+1 G>A mutation in the splice donor site of intron 1 in the *GJB2* gene has been found in heterozygous state in addition to c.35delG mutation in several populations (Seeman and Sakmaryova, 2006; Bajaj *et al*., 2008). This mutation, corresponding to c.-3170 G>A relative to the AUG translation-initiating codon (genomic sequence of this region appears in GenBank U43932), was originally reported by

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Denoyelle *et al*. (1999) and was considered to be rare. Functional studies of IVS 1+1 G>A mutation have revealed a disruptive splicing, yielding no detectable mRNA (Shahin *et al*., 2002).

In the present study we have investigated the presence of the IVS 1+1 G>A mutation in 185 patients with NSRHL. The aim of this screening was to analyze the presence, severity, and clinical discoveries in this affected population carrying this mutation.

Materials and Methods

We evaluated 185 unrelated patients with NSRHL, all with Brazilian origin, except for one, whose origin is United States. The majority of patients studied were Caucasian; however, the composition of the Brazilian population is difficult to be established due to its high ethnic diversity composed of individuals of Caucasian, African, and Amerindian origin (Parra *et al*., 2003).

The protocol was approved by the Ethics Committee, and written informed consent was obtained from the tested subjects or their parents. Syndromic hearing impairment and other nonhereditary causes were excluded by medical history, physical examination, and audiologic testing.

All samples were previously tested for entire coding region of the *GJB2* gene and were analyzed by direct sequencing. Patients were divided into two groups: 43 patients comprised group I and 142 formed group II. In all the 43 patients of group I, the c.35delG mutation in *GJB2* gene was detected in only one allele. In patients of group II, no pathogenic mutation in the *GJB2*-coding region was found. All the 185 samples were also tested for the presence of the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) mutations by polymerase chain reaction (PCR) following the procedure described by Del Castillo *et al*. (2005). Homozygous or compound heterozygous patients were excluded from this study since the genetic cause was identified.

We performed screening of the IVS 1+1 G>A mutation by multiplex ligation–dependent probe amplification (MLPA). We used MLPA P163-B1 GJB Kit (MRC-Holland, Amsterdam, The Netherlands) with specific probes to IVS 1+1 G>A and c.35delG point mutations. Possible large deletions or duplications of Cx26, 30, and 31 genes were also assessed with this MLPA Kit. DNA samples from five healthy individuals were used as normal controls and one patient as positive control (c.35delG and del(*GJB6*-D13S1830), both in heterozygosis). Fragment analysis was performed on an ABI 310 Genetic Analyser and results were examined using the Genescan and Genotyper softwares (Applied Biosystems, Foster City, CA). Peak areas of the amplicons representing the respective probe, analyzed by Genotyper softwares, were exported to a Microsoft Excel spreadsheet and calculations were performed according to the method described by Taylor *et al*. (2003).

To confirm the presence of IVS 1+1 G>A mutation, the exon 1 of *GJB2* gene was amplified with primers flanking donor splicing site previously described by Denoyelle *et al*. (1999). The PCR products were sequenced using the same primers and ABI BigDye Terminator and analyzed on an ABI PRISM® 3700 DNA sequencer (Applied Biosystems). The PCR products were also analyzed by restriction analysis using *Hph*I enzyme.

Results

MLPA screening for IVS 1+1 G>A mutation revealed the presence of IVS 1+1 G>A mutation in 2 patients out of the 43 carrying c.35delG mutation in the other allele (group I). Direct sequencing analysis of noncoding region of *GJB2* gene and restriction analysis were consistent with MLPA results. In both compound heterozygous patients, the separate segregation of each allele could be confirmed in the parents. Testing for the IVS 1+1 G>A mutation explained previously unclear hearing loss in 4.6% of the patients with one pathogenic mutation in only one allele of the coding region of the *GJB2* gene.

We did not detect the IVS 1+1 G>A mutation in any of the 142 patients from group II without mutations in the *GJB2*-coding region. Both groups have showed negative results for the presence of the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) mutations by MLPA and PCR tests.

MLPA method detected the c.35delG mutation in all the 43 heterozygous patients and the known heterozygous presence of the del(*GJB6*-D13S1830) deletion in positive control (Fig. 1). Average value of peak areas of amplicons, computed in comparison to controls, corresponding to Cx30 (*GJB6*) showed a 50% reduction. These alterations were correctly identified with no false positive. Thus, a sensitivity and specificity of 100% could be postulated for this screening method.

Discussion

Cx26 mutations are responsible for a substantial proportion of hereditary hearing impairment in all studied populations. Mutation screening in the *GJB2* gene, especially the frequent c.35delG mutation, has become the standard for the genetic diagnosis of patients with nonsyndromic hearing loss. Most mutations in the *GJB2* gene occur in the coding region.

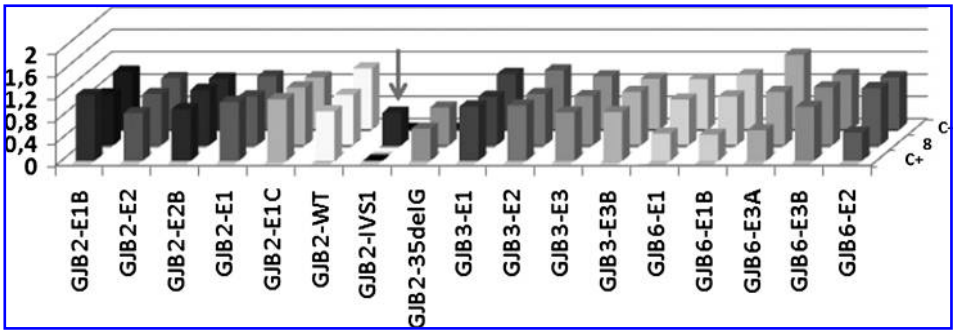

FIG. 1. Graphic showing the MLPA results of the healthy control (C−), of the compound heterozygous c.35delG/IVS 1+1 G>A patient (8) and a 50% reduction of the peak amplicons of Cx30 (C+), corresponding to a compound heterozygous c.35delG/c.del(*GJB6*-D13S1830). Arrow indicates the amplification of the IVS 1+1 G>A probe.

However, the search of mutations in noncoding regions may be important to clarify the molecular cause, to improve genetic counseling and to provide prognostic information.

We have reported the detection of the IVS 1+1 G>A mutation in noncoding region of the *GJB2* gene in two unrelated patients with NSRHL. One of these patients has North American origin and the other has Brazilian origin. The IVS 1+1 G>A mutation was previously reported in American population but was never described in Brazilian population before (Tang *et al*., 2006).

These patients have the c.35delG mutation in the other allele. The IVS 1+1 G>A mutation has been reported in several other published studies, usually in compound heterozygosity with other *GJB2* mutation (Hismi *et al*., 2006; Seeman and Sakmaryova, 2006). We have not detected the IVS 1+1 G>A mutation in group II, confirming this trait. To date, homozygotes for the IVS 1+1 G>A mutation have not been reported.

In this study, both patients carrying the IVS 1+1 G>A mutation have severe-profound prelingual bilateral hearing loss. These clinical data were consistent with functional studies of c.35delG and IVS 1+1 G>A, mutations that have been demonstrated to not yield detectable amounts of the Cx26 protein and mRNA, respectively (D'Andrea *et al*., 2002; Shahin *et al*., 2002). Some genotype–phenotype correlation studies have reported that several mutation combinations, including IVS 1+1 G>A/c.35delG, result in less severe hearing loss compared to c.35delG homozygous (Cryns *et al*., 2004; Snoeckx *et al*., 2005). On the other hand, some other studies have found similar results to our findings (Santos *et al*., 2005). We have hypothesized that modifier genes or even environmental factors cause these variations, but none has been identified to date.

The MLPA technique is a new method that allows a large screening of genes involved in nonsyndromic sensorineural hearing loss in a single reaction. The MLPA technique in this study has proved to be a highly accurate method to detect c.35delG and IVS 1+1 G>A mutation as well as del(*GJB6*-D13S1830). Similar results were recently published (Gürtler *et al*., 2008).

The direct sequencing method is very useful for molecular diagnosis but is limited by slow throughput, which makes extensive sequencing of all known deafness-causing genes very expensive and time consuming. Based on our results, the MLPA technique could be used for a simultaneous analysis in these three connexin genes; however, the results should be confirmed by other methods.

This study has reported for the first time the presence of the IVS 1+1 G>A mutation in one Brazilian patient with hearing impairment using MLPA technique. Despite its low frequency, this finding highlights the importance of searching for *GJB2* mutation in noncoding regions of the gene to explain hearing loss in monoallelic patients for *GJB2* mutation.

Disclosure Statement

No competing financial interests exist.

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