Prevalence of *GJB2* (Connexin-26) and *GJB6* (Connexin-30) Mutations in a Cohort of 300 Brazilian Hearing-Impaired Individuals: Implications for Diagnosis and Genetic Counseling

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Objective: Hereditary nonsyndromic deafness is an autosomal recessive condition in about 80% of cases, and point mutations in the *GJB2* gene (connexin 26) and two deletions in the *GJB6* gene (connexin 30), del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854), are reported to account for 50% of recessive deafness. Aiming at establishing the frequencies of *GJB2* mutations and *GJB6* deletions in the Brazilian population, we screened 300 unrelated individuals with hearing impairment, who were not affected by known deafness related syndromes.

Methods: We firstly screened the most frequently reported mutations, c.35delG and c.167delT in the *GJB2* gene, and del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) in the *GJB6* gene, through specific techniques. The detected c.35delG and c.167delT mutations were validated by sequencing. Other mutations in the *GJB2* gene were screened by single-strand conformation polymorphism and the coding region was sequenced when abnormal patterns were found.

Results: Pathogenic mutations in *GJB2* and *GJB6* genes were detected in 41 individuals (13.7%), and 80.5% (33/41) presented these mutations in homozygosis or compound heterozygosis, thus explaining their hearing defect. The c.35delG in the *GJB2* gene was the most frequent mutation (37/300; 12.4%), detected in 23% familial and 6.2% the sporadic cases. The second most frequent mutation (1%; 3/300) was the del(*GJB6*-D13S1830), always found associated with the c.35delG mutation. Nineteen different sequence variations were found in the *GJB2* gene. In addition to the c.35delG mutation, nine known pathogenic alterations were detected c.167delT, p.Trp24X, p.Val37Ile, c.176_191del16, c.235delC, p.Leu90Pro, p.Arg127His, c.509insA, and p.Arg184Pro. Five substitutions had been previously considered benign polymorphisms: c.-15C>T, p.Val27Ile, p.Met34Thr, p.Ala40Ala, and p.Gly160Ser. Two previously reported mutations of unknown pathogenicity were found (p.Lys168Arg, and c.684C>A), and two novel substitutions, p.Leu81Val (c.G241C) and p.Met195Val (c.A583G), both in heterozygosis without an accompanying mutation in the other allele. None of these latter four variants of undefined status was present in a sample of 100 hearing controls.

Conclusions: The present study demonstrates that mutations in the *GJB2* gene and del(*GJB6* D13S1830) are important causes of hearing impairment in Brazil, thus justifying their screening in a routine basis. The diversity of variants in our sample reflects the ethnic heterogeneity of the Brazilian population.

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INTRODUCTION

In developed countries, 60% of congenital deafness has genetic causes, and ~70% are nonsyndromic cases, presenting hearing impairment as the only symptom (Petit, et al., 2001). Autosomal recessive inheritance accounts for about 80% of the genetic cases (Morton, 1991).

Since 1994, 52 loci associated with recessive hearing loss (HL) have been mapped, and 23 different genes have been identified (The Hereditary Hearing Loss Homepage: http: webhost.ua.ac.be/hhh; Khan, et al., 2006; Petersen & Willems, 2006; Petit, 2006). In spite of this extensive genetic heterogeneity, a single locus, DFNB1 (13q11-q12; OMIM 220290), accounts for up to 50% of nonsyndromic autosomal recessive cases. This locus contains two genes associated with hearing impairment, the *GJB2* encoding connexin-26 (Cx26) (OMIM 12011) and *GJB6* encoding connexin-30 (OMIM 604418). Connexins are transmembrane proteins that oligomerize with five other connexin molecules to form a homomeric or a heteromeric connexon. Connexons in adjoining cells fuse through disulfide bonding to form gap junctions, which allow molecules to pass from cell to cell. Connexins 26 and 30 are highly expressed in epithelial supporting cells of the mammalian cochlea and are believed to play a key role in the cycling of potassium from the hair cells back to the endolymph (Petit, et al., 2001). About 90 different *GJB2* mutations have been described (The Connexin-Deafness Homepage: http://davinci.crg.es/deafness/). The types and frequencies of mutations are strongly influenced by the ethnic composition of the population. In the Caucasian populations (European descendants), a single mutation, c.35delG, accounts for the majority of HL caused by *GJB2* mutations, with a carrier frequency varying from 1 to 5% (Gasparini, et al., 2000). Among the Ashkenazi Jewish, in East Asians and in Africans, the predominant mutations are c.167delT, c.235delC mutation, and p.Arg143Trp, respectively (Brobby, et al., 1998; Kudo, et al., 2000; Morell, et al., 1998).

Although biallelic mutations in the *GJB2* gene account for ~50% of autosomal recessive nonsyndromic hearing impairment, a large number of cases with a single mutation are left unexplained by screening *GJB2* alone. The *GJB6* gene maps adjacent to *GJB2* at the DFNB1 locus, and two large deletions [one of 309 kb, del(*GJB6*-D13S1830) and another of 232 Kb, del(*GJB6*-D13S1854)] upstream the *GJB2* gene are frequently found among individuals who are deaf in Spain (Del Castillo, et al., 2002, 2003, 2005). *GJB6* gene encodes Cx30, and these deletions in trans with mutations in the *GJB2* gene provide an explanation for HL. It is not known whether this deletion

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affects the expression of Cx26 by deleting an upstream regulatory element, thus inactivating the second allele (monogenic inheritance) or whether the mutant Cx26 and the mutant Cx30 act together in determining deafness (digenic inheritance) (Common, et al., 2005; Del Castillo, et al., 2003, 2005; Pallares-Ruiz, et al., 2002). The frequencies of these deletions ranged from 5 to 15% in individuals with only one detected mutation in *GJB2* gene (Del Castillo, et al., 2003; Marlin, et al., 2005). The possibility that other deletions in *GJB6* gene act in conjunction with *GJB2* mutations remains open (Wilch, et al., 2006a,b).

A splice site mutation IVS 1 + 1G/A in the *GJB2* gene was detected in 4% of Czech individuals carrying only one pathogenic mutation in the coding region of the *GJB2* gene, representing the third most common *GJB2* mutation in that population (Seeman, & Sakmaryova, 2006). This mutation has been found in other populations and is predicted to disrupt splicing, yielding no detectable mRNA (Santos, et al., 2005; Shahin, et al., 2002; Snoeckx, et al., 2005).

The Brazilian population was formed by a complex process, which first led to gene mixture deriving from original South American Indians, Portuguese settlers, and African slaves. During the last 100 yr, São Paulo and other regions of Southern Brazil received many foreign migrants, particularly from Europe (e.g., Italians, Spaniards, Germans, Asian Japanese, Chinese) and Middle East (Arabs). The population of São Paulo must then be regarded as representative of this ethnic heterogeneity.

With the aim of evaluating the impact of mutation screening of *GJB2* and *GJB6* genes for determining the etiology of hearing impairment and for genetic counseling in Brazil, we determined the prevalence of *GJB2* mutations and of deletions in *GJB6* gene in a sample of 300 unrelated hearing-impaired Brazilian individuals without known deafness-related syndromes.

PATIENTS AND METHODS

Patients

A total of 300 unrelated Brazilian individuals presenting hearing impairment, without recognizable deafness-related syndromes, were tested for the presence of mutations in the DFNB1 locus, at the Genetic Counseling Unit, Centro de Estudos do Genoma Humano, Institute of Biosciences, University of São Paulo, Brazil. They had been referred by the following institutions: DERDIC (Divisão de Educação e Reabilitação de Distúrbios Auditivos da Comunicação Pontifícia Universidade Católica de São Paulo), CEPRO (Centro de Ensino Profissionalizante Rotary) Hospital das Clínicas da Faculdade de Medicina da USP, all in the State of São Paulo, and Universidade Estadual de Maringá, State of Paraná. Clinical and genealogical data were collected for each affected individual. Only individuals with known deafness-related syndromes were excluded from the analysis. Individuals with other clinical signs and dysmorphic features, but whose clinical presentation did not allow specific syndromic diagnosis, were included. Those with possible environmental causes for hearing impairment were not excluded.

Among the 300 probands, 59.7% (179/300) were sporadic cases of HL and 37.6% (113/300) had at least one affected relative. Familial data were not available for 2.7% of cases (8/300) because of adoption. Among the 289 individuals with known age of onset of hearing impairment, it was prelingual in 75.4% and postlingual in 24.6%.

Classification of the hearing impairment in the less-affected ear was performed according to Davis and Silverman (1970). According to this classification, the degree of hearing impairment is evaluated through average thresholds in the frequencies of 500, 1000, and 2000 Hz. Among the 274 individuals with complete clinical assessment, the degree of hearing impairment was profound in 59.5% (163), severe in 20.1% (55), moderate in 17.2% (47), and mild in 3.3% (9).

Most individuals (61.6%) were classified as "white" or having European origin, 36.7% were classified as African-Brazilians (negroes and mulattos), and 1.7% were of Asiatic descent (Japanese or Chinese).

The study was approved by the Ethics Committee, Institute of Biosciences, University of São Paulo. Written informed consent was obtained from all hearing-impaired individuals or their legal guardians, their relatives, and control individuals.

Molecular Analysis

Genomic DNA was extracted by standard protocols from peripheral blood leukocytes. The 300 probands were firstly screened for c.35delG and c.167delT mutations in the *GJB2* gene, and del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) deletions in the *GJB6* gene.

c.35delG and c.167delT screening • The c.35delG mutation was detected by allele-specific polymerase chain reaction as described by Scott et al. (1998). For the detection of c.167delT mutation, the coding exon was amplified using primers 1F and 3R, as described below. Amplification was followed by digestion with restriction enzyme *Pst*I.

***GJB6* deletions •** The del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) were tested by amplifying the breakpoint-containing fragments (Del Castillo, et al. 2003, 2005).

Single-strand conformation polymorphism • In hearing-impaired individuals with normal results in the screening of the above described mutations (a and b), and in 50 European-Brazilian and 50 African-Brazilian controls, the coding region of *GJB2* gene was analyzed by single-strand conformation polymorphism (SSCP). A first fragment (425pb) was amplified by PCR with primers 1F-5′ GTG TTG TGT GCA TTC GTC TTT TC-3′ and 3R-5′ ACC TTC TGG GTT TTG ATC TCC TC 3′, and a second fragment (422pb) was amplified with primers 4F-5′ GGA AGT TCA TCA AGG GGG AGA TA 3′ and 2R-5′ CCT CAT CCC TCT CAT GCT GTC TA 3′. PCR products were analyzed after vertical electrophoresis in 27.5% MDETM gel (Bio Whittaker Molecular Applications), at 7 W, for 20 hr.

Sequencing of *GJB2* coding region • The detected c.35delG and c.167delT mutations, in heterozygosis or homozygosis, were confirmed by sequencing the coding region of the *GJB2* gene, and parents and sibship of the probands were investigated. This same protocol was followed when an altered migration pattern was observed after SSCP screening for other alterations. In an attempt to identify the second mutation in heterozygous for the c.35delG mutation or other possibly pathogenic mutation, sequencing of the *GJB2* gene was also performed in the heterozygous hearing parents. When samples from relatives were not available, cloning before sequencing was performed to confirm whether the mutations were in trans.

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Sequencing was performed with the same set of primers described above in Single-Strand Conformation Polymorphism section. The coding region of the *GJB2* gene was sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) and analyzed in the MegaBACE 1000 DNA Analysis System (Amersham Biosciences).

IVS 1 + 1G/A noncoding mutation in *GJB2* gene • A noncoding IVS 1 + 1G/A splice site mutation located in exon 1 of the *GJB2* gene was tested in eight heterozygous individuals, with only one detected pathogenic mutation in the coding region of the *GJB2* gene. PCR amplification was followed by digestion with restriction enzyme *Hph* I. Primers were those described by Denoyelle et al. (1997).

Multiplex ligation-dependent probe amplification • Multiplex Ligation-Dependent Probe Amplification (MLPA) using SALSA P0163 GJB-WFS1 kit (MRC-Holland, Amsterdam, The Netherlands) was performed on DNA samples from eight heterozygous individuals, with only one detected pathogenic mutation in the coding region of the *GJB2* gene. This MLPA kit was designed to detect deletions/duplications of one or more exons of the *GJB2*, GJB3, *GJB6*, and WFS1 genes, and also includes several probes for the screening of the screening of chromosome region 13q11. The fragments were analyzed in the MegaBACE 1000 DNA Analysis System with the software Fragment Profiler version 2.2 (Amersham Biosciences).

RESULTS

Table 1 shows the frequencies of *GJB2*-*GJB6* genotypes identified after specific mutation screening, SSCP analysis, and sequencing. Nineteen different sequence variations were detected. Fifteen of these had been previously reported, 10 pathogenic (c.35delG, c.167delT, p.Trp24X, p.Val37Ile, c.176_191del16, c.235delC, p.Leu90Pro, p.Arg127His, c.509insA, and p.Arg184Pro) and five benign variants (c.-15C>T, p.Val27Ile, p.Met34Thr, p.Ala40Ala, and p.Gly160Ser).

We found four substitutions of unknown significance, p.Leu81Val (c.G241C), p.Lys168Arg, p.Met195Va (c.G241C) l, and c.684C>A that were present in heterozygosis, and *GJB2* sequencing did not reveal a mutation in the other allele. We did not find these substitutions in the control sample of 50 European-Brazilian and 50 African-Brazilian hearing individuals. The analysis using the world wide web PolyPhen-Polymorphism Phenotyping (Ramensky, et al., 2002; http://genetics.bwh.harvard.edu/pph/, 2007) indicated that only substitution p.Met195Val as probably pathogenic and the other three being probably benign variants. The substitutions p.Leu81Val (c.G241C) and p.Met195Val (c.G241C) have not been reported before.

In eight individuals, only one pathogenic mutation was found, c.35delG/+ (5), p.Arg127His/+ (2) and c.167delT/+ (1), thus not explaining their HL, considering that these mutations are known for causing autosomal recessive deafness. The IVS 1 + 1G/A mutation in the *GJB2* gene was ruled out in these individuals, as well as deletions and/or duplications that were detectable by the MLPA kit used. Among these, five were sporadic cases (one born from consanguineous parents), and three were familial, and transmission of HL was consistent with autosomal recessive inheritance in two cases and with autosomal dominant inheritance in one case.

TABLE 1. Total results obtained after specific mutation screening, SSCP analysis, and sequencing of *GJB2* gene

Genotypes	No. probands/ total sample	No. probands/ probands with alterations in *GJB2* and *GJB6* genes
Pathogenic		
c.35delG/c.35delG	22/300 (7.5%)	22/63 (35.1%)
c.35delG/del*GJB6* (D13S1830)	3/300 (1.0%)	3/63 (4.7%)
c.35delG/p.Trp24X	1/300 (0.3%)	1/63 (1.6%)
c.35delG/p.Val37Ile	1/300 (0.3%)	1/63 (1.6%)
c.35delG/c.167delT	1/300 (0.3%)	1/63 (1.6%)
c.35delG/c.176-191del16	1/300 (0.3%)	1/63 (1.6%)
c.35delG/c.235delC	1/300 (0.3%)	1/63 (1.6%)
c.35delG/ p.Leu90Pro	1/300 (0.3%)	1/63 (1.6%)
c.35delG/c.509insA + p.Met34Thr	1/300 (0.3%)	1/63 (1.6%)
p. Arg184Pro/ p.Arg184Pro	1/300 (0.3%)	1/63 (1.6%)
c.35delG/+	5/300 (1.7%)	5/63 (7.9%)
p.Arg127His/+	2/300 (0.8%)	2/63 (3.1%)
c.167delT/+	1/300 (0.3%)	1/63 (1.6%)
Total	41/300 (13.7%)	41/63 (65.2%)
Nonpathogenic		
p.Met34Thr/p.Met34Thr	1/300 (0.3%)	1/63 (1.6%%)
p.Met34Thr/p.Val27Ile	1/300 (0.3%)	1/63 (1.6%%)
p.Met34Thr/+	5/300 (1.7%)	5/63 (7.9%)
p.Val27Ile/+	5/300 (1.7%)	5/63 (7.9%)
c.-15C>T (5′UTR)/+	3/300 (1.0%)	3/63 (4.7%)
Ala40Ala/+	1/300 (0.3%)	1/63 (1.6%)
p.Gly160Ser/+	1/300 (0.3%)	1/63 (1.6%)
Total	17/300 (5.6%)	17/63 (26.9%)
Undefined pathogenicity		
p.Lys168Arg/+	2/300 (0.8%)	2/63 (3.1%)
p.Leu81Val (c.G241C)/ +*	1/300 (0.3%)	1/63 (1.6%)
p.Met195Val (c.A583G)/+*	1/300 (0.3%)	1/63 (1.6%)
c.684C>A (3′UTR)/+	1/300 (0.3%)	1/63 (1.6%)
Total	5/300 (1.7%)	5/63 (7.9%)
Total	63/300 (21%)	63/63 (100%)

*The signal + indicates that sequencing revealed a normal allele. The signal * indicates novel variants.*

The severity of hearing impairment in probands carrying pathogenic mutations or the two novel mutations of unknown pathogenicity first reported in this study and in probands without mutations detected in *GJB2* and *GJB6* genes is presented in Table 2, for comparison.

DISCUSSION

The prevalence of mutations in *GJB2* and *GJB6* genes in individuals with HL has been determined in different populations (Liu, et al., 2002; Morell, et al., 1998; Ohtsuka, et al., 2003; Pandya, et al., 2003; Park, et al., 2000). The frequency of the c.35delG mutation varied from 28 to 63% among individuals with nonsyndromic autosomal recessive HL, and from 10 to 30%, among sporadic cases (Feldmann, et al., 2004; Gasparini, et al., 2000; Gualandi, et al., 2002; Samanich, et al., 2007).

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TABLE 2. Severity of hearing impairment in probands with pathogenic mutations in the *GJB2* and *GJB6* genes (or with the novel mutations) and in probands without detected mutations of these genes

Severity of HL	*GJB2* and *GJB6*	*GJB2*	*GJB2* homozygotes or compound heterozygotes (*GJB2* and/or *GJB6*)
Mild	Negative	Heterozygotes	Heterozygotes (*GJB2* and/or *GJB6*)
	7	0	1 (c.35delG/p.Val37Ile)
			1 (c.35delG/p.Leu90Pro)
Moderate	40	1 (c.35delG/+)	3 (c.35delG/c.35delG)
		1 (c.35delG/+)	1 (p.Arg184Pro/p.Arg184Pro)
			1 (c.35delG/c.167delT)
Severe	46	1 (c.167delT/+)	5 (c.35delG/c.35delG)
		1 (p.Arg127His/+)	1 (c.35delG/p.Trp24X)
		1 (p.Leu81Val/+*)	
Profound	139	2 (c.35delG/+)	14 (c.35delG/c.35delG)
		1 (p.Arg127His/+)	3 [c.35delG/del(*GJB6*-D13S1830]
		1 p.Met195Val/+*)	1 (c.35delG/c.176-z191del16)
			1 (c.35delG/c.235delC)
			1 (c.35delG/c.509insA + p.Met34Thr)
Nonevaluated	25	1 (c.35delG/+)	0
Total	257	10	33

*The signal + indicates that sequencing revealed a normal allele. The signal * indicates the two novel variants of unknown pathogenicity.*

In the present study, we identified the c.35delG mutation in 12.4% of 300 unrelated cases of hearing impairment, either sporadic or familial, thus corresponding to 72% (57/79) of the pathogenic alleles detected (Table 1).

Biallelic *GJB2*/*GJB6* mutations were present in 42.8% (33/77) of cases that probably had autosomal recessive inheritance. Among the 179 sporadic cases, biallelic mutations were detected in nine individuals (5.0%; Fig. 1) and the c.35delG mutation in 11 (6.2%). These sporadic cases would not be scored as genetic cases of deafness in the absence of molecular screening. The importance of molecular tests for genetic counseling is reinforced by these cases, although environmental causes of HL may be highly frequent in this group of patients in Brazil.

Reports on *GJB2*/*GJB6* mutations in Brazil focused mostly on selected samples, because individuals with environmental causes were excluded; besides, family history was not always reported. (de Oliveira, et al., 2007; Oliveira, et al., 2002; Piatto, et al., 2004). Our study comprises a sample that included sporadic and familial cases, and individuals presenting only

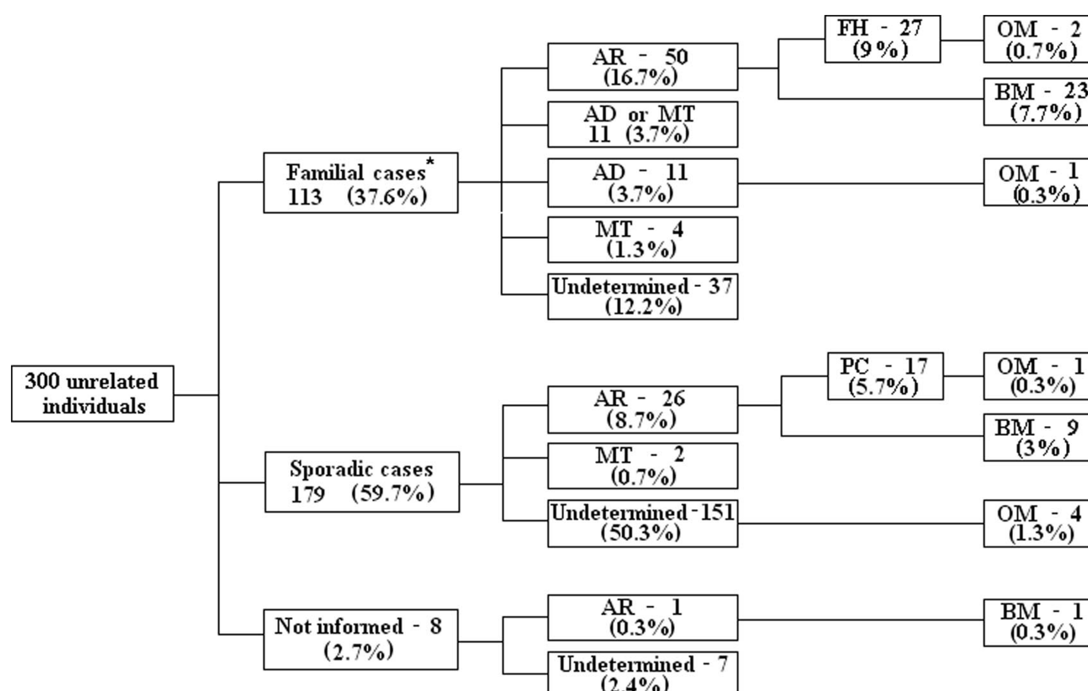

Fig. 1. Characterization of the sample according to probable patterns of transmission of hearing impairment.*With at least one more affected relative. AR, autosomal recessive; AD or MT, autosomal dominant or mitochondrial; MT, mitochondrial, with A1555G mutation; FH, Family history; PC, parental consanguinity; BM, biallelic mutations in *GJB2*/*GJB6* gene; OM, only one pathogenic mutation in *GJB2* gene.

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with known deafness-related syndromes were excluded. In spite of the difference in sample selection criteria, the frequency of individuals presenting c.35delG mutation in the *GJB2* gene (12.4%) in our study was similar to that found (13.9%) in the report of de Oliveira et al. (2007).

Our study agrees with these previous reports (de Oliveira, et al., 2007; Oliveira, et al., 2002; Piatto, et al., 2004), showing that the c.35delG mutation is the most frequent among causative mutations of hearing impairment in the Brazilian population. This is a common mutation causing HL in Caucasian populations, mainly from Mediterranean Europe and the United States, and seems to be rare in the Asian and African populations. Our cohort derives from the ethnically admixed population of São Paulo, and the majority of our patients were considered as "white," thus explaining the high prevalence of c.35delG. The c.167delT mutation that is frequent in Ashkenazi Jews, and the c.235delC mutation, frequent among Asiatic were also found in individuals who reported Jewish and Japanese origins, respectively. Surprisingly, the p.Arg143Trp mutation reported to be frequent among Africans was not found, although 36% of our sample was considered as African descent. The frequency of c.35delG alleles among this group of negroes or mulattos (0.055) was smaller than in the "white" group (0.134), as expected (data not shown), confirming that c.35delG is less frequent in African-derived groups (Samanich, et al., 2007).

The 309 kb del(*GJB6*-D13S1830) mutation was identified in three individuals (3/300), among those heterozygous for the c.35delG mutations, being the second most frequent mutation in our sample (Table 1). The del(*GJB6*-D13S1854) mutation was not detected in our cohort. Recently, de Oliveira et al. (2007) identified the del(*GJB6*-D13S1830) and del(*GJB6*D13S1854) mutations, each one in 5 of 645 individuals, a frequency that does not significantly differ from ours.

A single pathogenic *GJB2* mutation had been previously reported in 10 to 50% of individuals with nonsyndromic autosomal recessive HL (Del Castillo, et al., 2003, 2005). In our study, we identified 37 individuals with the c.35delG mutation, 22 homozygotes, and 15 heterozygotes. In 5 of 15 heterozygotes, a second mutation in the *GJB2* gene was not identified. The frequency of these individuals, heterozygous for c.35delG heterozygotes, 5/300, is higher than expected by chance alone, given that the frequency of c.35delG heterozygotes in Brazilian hearing population was estimated to be 1.35% (Oliveira, et al., 2007). In addition, two p.Arg127His and one c.167delT were the only *GJB2* mutations detected in three individuals (Table 1). The IVS 1 + 1G/A splicing site mutation was excluded. These eight individuals carrying mutations in one allele could be explained by limitations in the detection techniques: large deletions are not detected by SSCP or sequencing, because polymerase chain reaction amplifies only the normal alleles. However, the MLPA technique used allowed exclusion of large deletions and duplications in *GJB2*/*GJB6* genes. We believe it is unlikely that these patients carry deletions. Mutations in other regions of the gene *GJB2* that were not investigated (5′ UTR, noncoding exon 1, 3′ UTR and introns) could be present in those individuals, thus contributing to their hearing impairment.

Nineteen different variants in the *GJB2* gene were found in this study. Four were considered of unknown pathogenicity: p.Leu81Val, p.Lys168Arg (two cases), p.Met195Val, and c.684C>A, because they were not reported in The Connexin-Deafness Homepage, two of them being reported for the first time (p.Leu81Val and p.Met195Val). In these five individuals, a second mutation was not detected. Only one of these variants (p.Met195Val) is potentially pathogenic, according to Polyphen. The p.Lys168Arg and c.684C>A variants were recently reported by Samanich et al. (2007), but, as in our study, their significance remained undetermined. The individuals carrying the p.Lys168Arg (2), p.Met195Val, and c.684C>A variants were sporadic cases of deafness and the meaning of the substitutions remained to be clarified. The proband with p.Leu81Val was born from a pedigree in which family history was compatible with both autosomal dominant and mitochondrial inheritance This variant was not detected in her equally affected sister, excluding the possibility that their hearing impairment was related to this variant.

Many studies have demonstrated that the severity of hearing impairment varies among individuals with c.35delG, usually from severe to profound. In the present study, the majority of the homozygotes for the c.35delG mutation had severe to profound deafness, as expected. However, in three of the homozygous probands, hearing impairment was moderate (Table 2). An interesting finding was that the affected sister of one of the probands, who was homozygous for the c.35delG, did not present hearing impairment when examined at the age of 4 yr. This exceptional case was fully documented by Lezirovitz et al. (2006). Bolz et al. (2004) had reported an individual, homozygous for the c.35delG mutation, who did not have any hearing impairment before the age of 12 yr. These findings indicate that screening of DFNB1 mutations exclusively in patients with prelingual or severe hearing impairment would overlook some cases. Cryns et al. (2004) reinforced the extreme variation of severity of the HL in patients with some combinations of *GJB2* mutations, many patients presenting mild hearing impairment, which could easily mislead to the conclusion that the onset was postlingual. This was the case of the individual in our sample carrying the c.35delG/p.Leu90Pro mutations (Table 2), whose parents reported that hearing impairment was recognized after 4 yr of age, when she was first clinically evaluated.

The degree of hearing impairment in individuals with *GJB6* deletions vary from moderate to profound (Bolz, et al., 2004, Del Castillo, et al., 2005; Marlin, et al., 2005). In three of our probands with del(*GJB6*-D13S1830)/c.35delG, hearing impairment was profound (Table 2), being in average more severe than in individuals carrying two *GJB2* mutations (Feldmann, et al., 2004; Roux, et al., 2004).

In summary, pathogenic biallelic mutations in *GJB2* and *GJB6* genes explained with certainty 11% (33) of the cases in a Brazilian series of 300 hearing-impaired individuals. The high frequency of the c.35delG mutation indicated its screening, regardless severity of hearing impairment or familial history. The c.167delT and del(*GJB6*-D13S1830) mutations were also found (2/300 and 3/300, respectively), thus justifying their screening. On the other hand, the screening of mutations in the *GJB2* gene by SSCP analysis followed by sequencing is time consuming, and in our sample enabled the diagnosis in one case (an Arg184Pro/Arg184Pro homozygote). From the point of view of establishing a cheap laboratory routine, aiming at genetic counseling, a simple screening protocol that includes c.35delG and c.167delT mutations in the

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GJB2, a multiplex PCR for the detection of del(*GJB6*-D13S1830) and del(*GJB6*D13S1854) in *GJB6* gene, followed by sequencing when mutations are in heterozygosis, seems effective for the diagnosis of the majority of cases of hearing impairment in Brazil. In addition, previous data from our laboratory showed that the screening for the A1555G mitochondrial mutation was also worth performing, because it was found to be causative of HL in 2% of unrelated individuals referred to our genetic counseling unit (Abreu-Silva, et al., 2006). In an unselected series of Brazilians attending to a special school for the deaf, Braga et al. (1999) estimated that genetic causes explained 16% of the cases. Considering the general data from our laboratory in which pedigree analysis was associated to molecular tests, genetic factors were estimated to contribute to about 35% of cases (biallelic mutations in DFNB1, mitochondrial mutations or recognized monogenic patterns of inheritance, as depicted in Fig. 1). Although bias resulting from overrepresentation of familial cases in genetic counseling services must be considered, the detected increase of the contribution of genetic factors to deafness might be partly due to the introduction of *GJB2*-*GJB6* molecular tests, at least in the fraction of sporadic cases, but might also be explained by recent changes in health assistance, a factor that can significantly reduce the fraction of cases resulting from environmental injuries and increase the importance of genetic cases in developing countries.

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