

Original Article

Spectrum and frequency of *GJB2* mutations in a cohort of 264 Portuguese nonsyndromic sensorineural hearing loss patients

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Abstract

Objective: To assess the spectrum and prevalence of mutations in the *GJB2* gene in Portuguese nonsyndromic sensorineural hearing loss (NSSLH) patients. **Design:** Sequencing of the coding region, basal promoter, exon 1, and donor splice site of the *GJB2* gene; screening for the presence of the two common *GJB6* deletions. **Study sample:** A cohort of 264 Portuguese NSSLH patients. **Results:** At least one out of 21 different *GJB2* variants was identified in 80 (30.2%) of the 264 patients analysed. Two mutant alleles were found in 53 (20%) of these probands, of which 83% (44/53) harboured at least one c.35delG allele. Twenty-seven (10.2%) of the probands harboured only one mutant allele. Subsequent analysis revealed that the *GJB6* deletion del(*GJB6*-D13S1854) was present in at least 7.4% (2/27) of the patients carrying only one mutant *GJB2* allele. Overall, one in five (55/264) of the patients were diagnosed as having DFNB1-related NSSLH, of which the vast majority (53/55) harboured only *GJB2* mutations. **Conclusions:** This study provides clear demonstration that mutations in the *GJB2* gene are an important cause of NSSLH in Portugal, thus representing a valuable indicator as regards therapeutical and rehabilitation options, as well as genetic counseling of these patients and their families.

Key Words: *GJB2*; *GJB6*; *DFNB1*; connexin 26; hearing loss; hereditary; genetic; deafness; mutation

Hearing loss (HL) is the most common congenital sensory impairment, and the most prevalent sensorineural disorder (Hilgert et al, 2009), being a major public health concern.

Regarding nonsyndromic sensorineural hearing loss (NSSLH), more than 140 genetic loci have been mapped, with more than 60 genes identified to date (Alford, 2011; Smith & Van Camp, 2011). Despite this extreme genetic heterogeneity, mutations in the *GJB2* gene, encoding the protein connexin 26 (Cx26), are a major cause of NSSLH in many world populations, which makes *GJB2* the gene most frequently associated with this condition. Two gross deletions, del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854), involving the *GJB6* gene, which codes for connexin 30 (Cx30), have also been associated with nonsyndromic hearing loss (NSHL) in several cases, often in *trans* with a *GJB2* mutation (del Castillo et al, 2005; Lerer et al, 2001; Pallares et al, 2002). Both genes, *GJB2* and *GJB6*,

are localized to chromosome region 13q11, corresponding to the DFNB1 locus.

About two hundred *GJB2* mutations causing nonsyndromic HL have been reported (Stenson et al, 2009), most of them being recessive. A few specific mutations in *GJB2* also have been described in families with autosomal dominant HL (Smith et al, 2012). The carrier rate in the general population for a recessive deafness-causing *GJB2* mutation is approximately one in 33 (Smith et al, 2013).

The spectrum and the prevalence of *GJB2* mutations vary among different populations. The predominant mutations in Europeans, Ashkenazi Jews, East Asians, and Ghanaian population are c.35delG, c.167delT, c.235delC and p.R143W, respectively (Kenneson et al, 2002). A different *GJB2* pathogenic mutation, p.W24X, was found to be the predominant one in NSHL cases in India (RamShankar et al, 2003), and in Gypsies (an ethnic group that traces back to the

Abbreviations

Cx	Connexin
HL	Hearing loss
NSHL	Nonsyndromic hearing loss
NSSHL	Nonsyndromic sensorineural hearing loss
PSDM	PCR-mediated site directed mutagenesis
RFLP	Restriction fragment length polymorphism
SSCP	Single-strand conformational polymorphism

Indian subcontinent) from Eastern Slovakia (Minárik et al, 2003) and Spain (Álvarez et al, 2005). This mutation was also shown to have a high carrier frequency (2.4%) in the Indian population (RamShankar et al, 2003), and in some subpopulations of Gypsies (Minárik et al, 2003; Álvarez et al, 2005).

Due to the high frequency of *GJB2* mutations among NSHL cases in several populations worldwide, mutation analysis of this gene is a widely available genetic test, representing the first step in the molecular diagnosis of this pathology.

Since most *GJB2* mutations described so far localize to the coding region (totally included within exon 2), only this region has been systematically analysed. Nonetheless, a few noncoding pathogenic mutations have already been identified, contributing to the elucidation of the genetic etiology of the HL in some patients who harboured only one recessive mutation in the *GJB2* coding region (Denoyelle et al, 1999; Mani et al, 2009; Matos et al, 2007).

We present here a comprehensive molecular analysis of the *GJB2* gene in a cohort of 264 Portuguese NSSHL patients.

Materials and Methods*Patients*

A total of 264 Portuguese unrelated individuals (sporadic and familial cases), presenting with bilateral mild to profound NSSHL were tested for the presence of mutations in the *DFNB1* locus. They had been referred over the last ten years, from various otolaryngology departments and genetic units, and come from different regions of the country. A recessive mode of inheritance was predominantly observed in the familial cases.

Diagnosis of NSSHL was established by accepted clinical criteria. Infection, oto-trauma, or neonatal diseases causing acquired deafness were excluded by collecting detailed clinical history. Individuals referred as not having been born in Portugal were also not considered.

Written informed consent was obtained from all the participants, or from parents in case of those younger than 18 years.

Audiological methods

All patients involved in the study underwent otoscopic and audiometric examinations by using age-appropriate methods. Pure-tone audiometry, tympanometry and auditory brainstem response were obtained in a sound-proof room according to current clinical standards. We were not able to retrieve medical history and audiometric records for all the patients. For some patients family histories were unknown.

Molecular analysis

Blood samples were obtained from most subjects. DNA extraction from whole blood has been performed since the beginning of the

study using either Quiagen or JetQuick DNA extraction kits. For some patients DNA was extracted from buccal swabs using the Chelex method.

All DNA samples were first screened for the c.35delG mutation by PCR-mediated site-directed mutagenesis (PSDM) followed by restriction fragment length polymorphism (RFLP) analysis (Storm et al, 1999).

The c.35delG homozygotes were diagnosed as having *GJB2*-related HL and, consequently, no other studies were performed.

Initially, the c.35delG heterozygotes and the negative cases had been screened by single-strand conformational polymorphism (SSCP) analysis, according to Scott et al (1998), followed by sequencing of the relevant *GJB2* fragment. In more recent years, all new cases falling into the two above mentioned situations have instead been analysed by direct sequencing of the entire coding region and flanking acceptor splice site, as previously described (Matos et al, 2010). Those c.35delG heterozygotes first analysed by SSCP and in which no second variant had been identified, as well as 29 of the 105 c.35delG negative cases which revealed no changes in the subsequent SSCP analysis, were further sequenced for the entire coding region and flanking acceptor splice site.

If the cause of deafness remained non-elucidated, the patients were then screened for the del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) *GJB6* deletions using multiplex PCR, as described in the literature (del Castillo FJ et al, 2005). Twelve cases could not be analysed for any of the *GJB6* deletions (eight cases negative for *GJB2* mutations plus four c.35delG heterozygotes), and two cases (both negative for *GJB2* mutations) could only be screened for del(*GJB6*-D13S1830), since the corresponding DNAs had meanwhile run out, or had degraded, especially those DNAs extracted from buccal swabs.

Ninety patients among those found not to harbour any *GJB6* deletion have been screened by sequencing for mutations in the *GJB2* basal promoter, exon 1 and donor splice site. Results concerning 83 of these 90 patients have been reported (Matos et al, 2007, 2008, 2010, 2011).

Results

At least one out of 21 different *GJB2* variants (18 coding and three noncoding) was found in 80 (30.2%) of the 264 patients representing a total of 133/528 (25%) mutated alleles (see Table 1). All the variants here identified were previously described, with the exception of the novel variant c.24G>A, which results in a silent mutation at protein level (p.T8T). Two of the reported mutations (c.-259C>T and p.M163L), listed in Table 1, were first identified in Portuguese patients (Matos et al, 2008, 2007) included in the sample under analysis. As expected, c.35delG was the mutation most frequently found amongst the NSSHL patients, being present in 84 of the 528 chromosomes analysed, which corresponds to a prevalence of 15.9% (see Table 1) and a frequency of 63% of all mutated alleles.

Table 2 shows the frequencies of *GJB2* genotypes identified in a total of 264 individuals presenting with bilateral NSSHL. Fifty-three probands (20%) harboured two *GJB2* mutations, of which 83% (44/53) harboured at least one c.35delG allele. The most prevalent mutated genotype was [c.35delG]+[c.35delG], accounting for 52.8% (28/53) of the individuals with two *GJB2* mutations. Next to this genotype, [c.35delG]+[p.E47X], [c.35delG]+[p.W172X], and [c.35delG]+[p.V37I] genotypes were the most represented among patients with two *GJB2* mutations (respectively 5.7%, 5.7%, and 3.8%).

Table 1. Frequency of GJB2 variant alleles (n = 528 chromosomes).

Variant		Location	Effect	Alleles No. (Frequency)
Nucleotide change	Amino acid change			
c.-259C>T ^{a,b}	–	Basal promoter	Pathogenic (T ^c)	1 (0.2)
c.-23 + 1G>A	–	Donor splice site	Pathogenic (T ^c)	2 (0.4)
c.-22-12C>T	–	Intron	Polymorphism (NT ^c)	1 (0.2)
c.24G>A	p.T8T	N-terminus	Novel (NT)	1 (0.2)
c.35delG	p.G12Vfs*2	N-terminus (frameshift)	Pathogenic (T)	84 (15.9)
c.71G>A ^d	p.W24X	M1 (nonsense)	Pathogenic (T)	5 (0.9)
c.101T>C	p.M34T	M1	Controversial (NT)	7 (1.3)
c.109G>A	p.V37I	M1	Pathogenic (NT)	5 (0.9)
c.139G>T	p.E47X	E1 (nonsense)	Pathogenic (T)	3 (0.6)
c.167delT	p.L56Rfs*26	E1 (frameshift)	Pathogenic (T)	1 (0.2)
c.250G>A	p.V84M	M2	Pathogenic (NT)	1 (0.2)
c.269T>C	p.L90P	M2	Pathogenic (NT)	1 (0.2)
c.283G>A	p.V95M	M2	Pathogenic NT	2 (0.4)
c.358_360delGAG	p.E120del	IC	Pathogenic (NT ^c)	3 (0.6)
c.380G>A	p.R127H	IC	Controversial (NT)	6 (1.1)
c.457G>A	p.V153I	M3	Controversial (NT)	1 (0.2)
c.468G>A	p.G160S	E2	Unclear (NT)	1 (0.2)
c.487A>C ^a	p.M163L	E2	Pathogenic (T)	1 (0.2)
c.516G>A	p.W172X	E2 (nonsense)	Pathogenic (T)	3 (0.6)
c.550 C>G	p.R184W	E2	Pathogenic (NT)	1 (0.2)
c.617A>G	p.N206S	M4	Pathogenic (NT)	3 (0.6)

E1-2 = extracellular domain 1 or 2; M1-4 = transmembrane domain 1, 2, 3, or 4; IC = intracellular domain (between M2 and M3); T = truncating mutation; NT = nontruncating mutation. ^aMutations identified for the first time in the Portuguese population; ^bMutation up to now private to one Portuguese family; ^cnoncoding variant; ^dFound in this study only in patients of Gypsy or Indian ethnicity/ancestry; ^edeletion of one nonterminal codon, thus considered to be a nontruncating mutation.

Twenty-seven out of 264 (10.2%) probands harboured only one *GJB2* variant. Subsequent analysis revealed that the *GJB6* deletion del(*GJB6*-D13S1854) was present in two of those patients (one heterozygous for c.35delG plus one heterozygous for p.M34T), both with prelingual, profound HL. One other patient solely harboured the dominant p.M163L mutation which has been previously described (Matos et al, 2008).

Discussion

A wide spectrum of *GJB2* variants, mostly pathogenic, was observed in the cohort of 264 NSSHL Portuguese individuals included in this study. Some variants of unclear effect (insufficient published data to infer pathogenicity) or of controversial pathogenicity (contradictory published data regarding pathogenicity) were also identified.

The DFNB1 genotype explained the HL, or was a likely cause of the pathology, in 20.8% (55/264) of the cases. All but two of these cases (53/55), corresponding to 20.8% of all the patients analysed, were presumed to be due to the *GJB2* genotype and thus considered as *GJB2*-related HL. For the remaining two patients (2/55) the HL could be explained by the presence of a coding *GJB2* mutation (c.35delG or c.Met34Thr) and the *GJB6* del(*GJB6*-D13S1854), both in heterozygosity. The *GJB2*-related cases also include the patient harbouring the above referred dominant p.M163L mutation (Matos et al, 2008). Noteworthy, the HL in three patients with one coding *GJB2* mutation was explained by the presence of a second *GJB2* mutation in the basal promoter (c.-259C>T) or in the donor splice site (c.-23 + 1G>A). To our knowledge, the c.-259C>T mutation is so far restricted to the Portuguese family here presented. This

mutation was not found in 91 normal-hearing Portuguese individuals from the general population (Matos et al, 2011) and reporter gene assays have shown that the mutation greatly impairs the promoter function (Matos et al, 2007). The finding of *GJB2* pathogenic mutations in the basal promoter and donor splice site highlights the relevance of screening those noncoding regions in addition to the coding region.

The deletion c.35delG was the predominant *GJB2* mutation among *GJB2*-related HL cases, in accordance with other European, or of mainly European descent populations (Dalámon et al, 2010; Pandya et al, 2003; Roux et al, 2004). Individuals homozygous for the c.35delG mutation accounted for about 53% of the *GJB2*-related HL cases, and represented 10.6% of the total Portuguese NSSHL patients. This predominance had also been observed in a different study conducted on a small number (n = 53) of NSSHL Portuguese patients (Nogueira et al, 2011), and in a sample of 100 Portuguese cochlear implanted children, in which *GJB2* genotype was shown to correlate to better oral performance (Chora et al, 2011).

Interestingly, the dominant *GJB2* mutation p.M163L, causing mild/moderate high-frequency HL (Matos et al, 2008), has meanwhile also been found in one Argentinean patient presenting with prelingual, profound NSSHL (Dalámon et al, 2010), suggesting that the severity of the HL due to the p.M163L mutation might be variable and/or progressive.

As regards the controversial but potentially pathogenic p.M34T (Feldmann et al, 2004; Snoeckx et al, 2005; Bicego et al, 2006; Pollak et al, 2007) and p.R127H (RamShankar et al, 2003; Tóth et al, 2004; Bicego et al, 2006; Matos et al, 2010) variants, they were identified in patients harbouring an accompanying *GJB2*

Table 2. GJB2 genotypes of the 80 patients carrying GJB2 variants.

GJB2 genotypes	Probands	Frequency % (n = 264)	HL better ear	History
<i>Two variants</i>				
[c.35delG]+[c.35delG]	28	10.6	6 Moderate 5 Severe 12 Profound	9 FC; 9 SC; 10 Un
[c.35delG]+[p.E47X]	3	1.1	5 Unreported 1 Moderate 1 Profound 1 Unreported	1 FC; 2 Un
[c.35delG]+[p.W172X]	3	1.1	1 Moderate 2 Profound	Un SC
[c.35delG]+[p.V37I]	2	0.8	1 Mild	Un
[c.35delG]+[c.-23 + 1G> A]	1	0.4	1 Moderate	FC
[c.35delG]+[p.W24X]	1	0.4	1 Profound	SC
[c.35delG]+[p.M34T]	1	0.4	1 Moderate	SC
[c.35delG]+[c.167delT]	1	0.4	1 Unreported	SC
[c.35delG]+[c.167delT]	1	0.4	1 Severe	FC
[c.35delG]+[p.L90P]	1	0.4	1 Moderate	SC
[c.35delG]+[p.V95M]	1	0.4	1 Profound	SC
[c.35delG]+[p.E120del]	1	0.4	1 Moderate	SC
[c.35delG]+[p.N206S]	1	0.4	1 Moderate	FC
[p.W24X]+[p.W24X]	1	0.4	1 Moderate	FC
[p.W24X]+[p.R127H]	1	0.4	1 Moderate	FC
[p.M34T]+[p.V95M]	1	0.4	1 Moderate	FC
[p.V37I]+[p.R184W]	1	0.4	1 Severe	SC
[p.V37I]+[p.N206S]	1	0.4	1 Severe	SC
[p.V84M]+[c.-259C> T]	1	0.4	1 Profound	FC
[p.E120del]+[p.E120del]	1	0.4	1 Moderate	SC
[p.R127H]+[c.-23 + 1G> A]	1	0.4	1 Profound	SC
[p.R127H]+[p.R127H]	1	0.4	1 Unreported	FC
<i>Total</i>	53	20.1		
<i>One variant</i>				
[=]+[c.35delG]	12	4.5	1 Mild 3 Moderate 3 Severe 3 Profound 2 Unreported	4 FC; 5 SC; 3 Un
[=]+[p.M34T]	5	1.9	1 Moderate 1 Severe 2 Profound 1 Unreported	4 FC; 1 Un
[=]+[p.R127H]	2	0.8	2 Moderate	1 FC; 1 SC
[=]+[c.-22-12C> T]	1	0.4	1 Severe	Un
[=]+[p.T8T]	1	0.4	1 Unreported	
[=]+[p.W24X]	1	0.4	1 Severe	
[=]+[p.V37I]	1	0.4	1 Profound	
[=]+[p.V153I]	1	0.4	1 Mild	
[=]+[G160S]	1	0.4	1 Moderate	
[p.M163L]+[=]	1	0.4	1 Mild	
[=]+[p.N206S]	1	0.4	1 Moderate	
<i>Total</i>	27	10.2		

FC = Familial case; SC = Sporadic case; Un = Unreported; Symbols: [=] wild type allele.

variant or the del(*GJB6*-D13S1854) deletion. Those genotypes were here considered to be the likely cause of the HL, with some reservations regarding the only p.R127H homozygous patient (negative for the *GJB6* deletions). Since other hearing-impaired relatives of this patient were not available for study, we cannot know whether the p.R127H homozygous genotype is segregating with HL in the family.

The unclear p.V153I (RamShankar et al, 2003; Meşe et al, 2004; Snoeckx et al, 2005; Guerri et al, 2007) and p.G160S (Janecke et al, 2002; Snoeckx et al, 2005) were identified in heterozygosity in two of our patients, respectively. No inference regarding the relation of their *GJB2* genotype with the HL could be made.

In which concerns genotype-phenotype correlation, truncating (T) mutations are usually associated with a greater degree of HL than

nontruncating (NT) mutations (Cryns et al, 2004; Putcha et al, 2007; Snoeckx et al, 2005). However, variability in the degree of the hearing loss in T/T genotypes has been noticed, and is well exemplified by the significant intra- and inter-familial variation, ranging from mild to profound impairment, observed in c.35delG homozygotes (Cryns et al, 2004; Murgia et al, 1999). In the present study, the majority (n = 17/23) of homozygotes for c.35delG with a reported phenotype had severe or profound HL, as expected.

As opposed to T/T *GJB2* genotypes, NT/NT *GJB2* genotypes appear to be often associated with mild or moderate HL (Putcha et al, 2007; Snoeckx et al, 2005). When considering NT/NT genotypes including at least one p.M34T or p.V37I allele, it was observed that they are usually, but not always, associated with less severe HL (Snoeckx et al, 2005). One patient of this study, with genotype [p.M34T] + [p.V95M], presented indeed with moderate HL. Two sporadic cases, compound heterozygous for p.V37I plus one of two other NT mutations, p.R184W and p.N206S, however presented with severe HL, thus supporting previous observations (Snoeckx et al, 2005).

Regarding T/NT *GJB2* genotypes, the variability on HL degree seems to depend on the NT mutation, at least when the T mutation is c.35delG (an early frameshift mutation). The two patients who were compound heterozygous for the p.V37I and c.35delG displayed mild and moderate HL, respectively, which is in accordance with the phenotypes previously found associated with this compound genotype by Cryns and co-workers (Cryns et al, 2004). The association of the [c.35delG]+[p.V37I] genotype with less severe HL phenotypes was corroborated by other studies (Snoeckx et al, 2005; Pollak et al, 2007). One other T/NT *GJB2* genotype, [c.35delG]+[p.L90P], is most commonly associated with mild or moderate HL (Cryns et al, 2004; Snoeckx et al, 2005; Pollak et al, 2007). Our patient presenting this genotype had a moderate hearing impairment. As regards the genotype [c.35delG]+[p.N206S], it apparently follows the same trend as the [c.35delG]+[p.L90P] genotype (Snoeckx et al, 2005; Marlin et al, 2005). The [c.35delG]+[p.N206S] patient reported here presented with moderate HL. Finally, the [c.35delG]+[p.V95M] genotype, found here in two patients with severe HL, effectively appears to be more often associated with severe to profound HL (Cryns et al, 2004; Snoeckx et al, 2005) than with less severe HL (Cheng et al, 2005).

In general, our data meet the view that the severity of *GJB2*-related HL can be correlated with specific mutation combinations (Cryns et al, 2004; Snoeckx et al, 2005). Variation in the phenotype for a given genotype would likely be dependent on more than one major modifier gene, as suggested by the study of Hilgert and co-workers (Hilgert et al, 2009), as well as on environmental factors.

In conclusion, *GJB2* mutations are a major cause of NSSHL in the Portuguese population, c.35delG being the predominant mutation, and each of the 19 other pathogenic/controversial variants occurring at much lower frequencies. Screening for the *GJB2* donor splice site and basal promoter, and for the two common *GJB6* deletions, allowed the elucidation of the HL etiology in some of the cases with only one coding mutation identified. Therefore, such screening, in patients harbouring only one coding mutation, is advisable and should be performed.

Overall, more than one in five of the Portuguese patients have been diagnosed as having DFNB1-related NSSHL. Analysis of *GJB2* and *GJB6* may thus represent a valuable indicator for clinicians, as regards therapeutical and rehabilitation options, as well as for genetic counselors of Portuguese patients and their families.

In fact, the current clinical assessment of hearing-impaired children/patients includes regular and sometimes invasive clinical investigations due to the possibility of the occurrence of dysfunction in other organs (syndromic HL). A conclusive genetic diagnosis can avoid these follow-ups, leading to a significant reduction of clinical investigations and inherent costs. Furthermore, the higher oral performance scores above referred, obtained in Portuguese cochlear implanted patients with DFNB1-associated deafness, show that individuals with this genetic etiology are good candidates for cochlear implantation, which represents an important prognostic tool.

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