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GJB2 and *GJB6* Genes: Molecular Study and Identification of Novel *GJB2* Mutations in the Hearing-Impaired Argentinean Population

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Viviana Dalamón[a] Vanesa Lotersztein[b] Agustina Béhèran[c] Marcela Lipovsek[a] Fernando Diamante[c] Norma Pallares[c] Liliana Francipane[b] Gustavo Frechtel[b] Bibiana Paoli[d] Enrique Mansilla[d] Vicente Diamante[c] Ana Belén Elgoyhen[a, e]

[a]Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad de Buenos Aires, [b]Servicio de Genética, Hospital de Clínicas 'José de San Martín', [c]Centro de Implantes Cocleares 'Prof. Dr. Vicente Diamante', [d]Servicio de Otorrinolaringología Infantil, Hospital de Clínicas 'José de San Martín', y [e]Departamento de Farmacología, Facultad de Medicina, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina

Key Words

GJB2 · Connexin 26 · *GJB6* · Connexin 30 · Deafness · Mutation, novel

Abstract

Mutations in the *GJB2* gene are responsible for more than half of all cases of recessive non-syndromic deafness. This article presents a mutation analysis of the *GJB2*, *GJB6*, *OTOF* and *MTRNR1* genes in 252 patients with sensorineural non-syndromic hearing loss. Thirty-one different mutations were identified in *GJB2* and *GJB6* in 86 of the 252 (34%) patients. We describe for the first time two new mutations in *GJB2*: the missense mutation c.29 T>C (p.Leu10Pro) in the N terminal domain and c.326 G>T (p.Gly109Val) in the intracytoplasmic domain of connexin 26. This work shows the high prevalence of *GJB2* mutations in the Argentinean population, with frequencies that are comparable to those of the Mediterranean area. Most important, it adds two novel *GJB2* mutations to be taken into consideration in the genetic diagnosis of non-syndromic sensorineural hearing loss.

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Introduction

Millions of people are affected by hearing impairment (HI), one of the most common sensory disabilities that may drastically limit the quality of life, with an incidence of 1:1000 newborns. Hearing loss is caused by several environmental and genetic factors and the proportion attributed to inherited causes is assumed in at least 50%. Approximately 70% are non-syndromic [Cremers et al., 1991]. The pattern of inheritance of non-syndromic cases is autosomal recessive (DFNB) in about 80%. DFNB non-syndromic hearing loss (ARNSHL) results in the most prevalent form of deafness [Morton, 1991; Van Camp et al., 1997]. To date more than 50 loci have been characterized for ARNSHL (http://webh01.ua.ac.be/hhh/). Mutations in the DFNB1 locus, containing the gap junction β2 gene (*GJB2*, NM_004004.5) that encodes connexin 26 (Cx 26), and the gap junction β6 gene (*GJB6*, NM_001110219.2) that encodes connexin 30 (Cx 30), are the most predominant causes of ARNSHL (50%). More than 100 mutations have been described in the *GJB2* gene (http://www.crg.es/deafness), but the c.35delG accounts for the majority of mutations in deaf Caucasians [Kelley

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Fax +41 61 306 12 34
E-Mail karger@karger.ch
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Viviana Dalamón
Vuelta de Obligado 2490
1428 Ciudad Autónoma de Buenos Aires (Argentina)
Tel. +54 11 4783 2871, Fax +54 11 4786 8578
E-Mail dalamon@dna.uba.ar

et al., 1998; Scott et al., 1998; Zelante et al., 1997]. The combined frequency of all *GJB2* mutations is sufficiently high to make the mutation analysis of this gene a clinically useful and therefore widely available genetic test.

Recent studies have shown that two large deletions of 309 and 232 kb, del(GJB6-D13S1830) and del(GJB6-D13S1854), respectively, involving the 5′-portion of the *GJB6* gene may be common second mutations causing ARNSHL [del Castillo I et al., 2002, 2003; del Castillo FJ et al., 2005; Esmaeili et al., 2007]. Furthermore, the mutation p.Gln829X, usually named Q829X, in the gene encoding otoferlin (*OTOF*, locus DFNB9 on 2p22–p23, OMIM 603681) has been reported as the third most frequent mutation causing prelingual deafness in the Spanish population [Migliosi et al., 2002]. Finally, a high proportion of patients having a familial history of hearing loss related or not to aminoglycoside exposure harbor the c.1555A>G mutation in the gene coding for the mitochondrial 12S ribosomal RNA *(MTRNR1)*. Different clinical phenotypes have been associated with this mutation [del Castillo I et al., 2003; Estivill et al., 1998; Prezant et al., 1993].

DFNB1 is characterized by a prelingual onset. The severity of deafness varies generally from mild to profound and may vary among siblings. Hearing loss is generally stable but occasionally progressive or fluctuant. Audiometric curves are either flat or sloping, and hearing loss generally is symmetric and affects all frequencies [Denoyelle et al., 1999; Snoeckx et al., 2005]. Understanding the underlying causes of the variability in DFNB1 is of major importance in terms of genetic counseling. In an earlier study, with only 46 patients, we found that mutations in the *GJB2* gene, alone or associated with the del-GJB6-D13S1830 mutation, are present in the Argentinean population [Dalamon et al., 2005]. In this study, we describe the prevalence of mutations in *GJB2* and *GJB6*, and we analyze the correlation with the phenotype in a large series of non-syndromic sensorineural HI patients. In addition, we describe for the first time two new mutations in *GJB2*, which adds to the complexity of already known mutations in the DFNB1 locus.

Material and Methods

Subjects and Selection Criteria

Clinical data and samples were obtained from a prospective collection compiled between 2004 and 2007. The cohort includes the 46 patients previously described by Dalamon et al. [2005]. Clinical features included age at onset, hearing thresholds, audiometric configuration, pedigree and genetic assessment. All data were reviewed by a clinical geneticist. Patients participating in the study were from Argentina. Two hundred and fifty-two unrelated patients with non-syndromic bilateral sensorineural HI were included (211 congenital-prelingual, 1 perilingual and 40 postlingual). Patients with HI related to environmental causes were excluded. Written informed consent was obtained from all participants or parents in case of minors. The study was approved by the Ethics Committee of the Hospital de Clínicas 'José de San Martín'. Hearing loss was sporadic in 191 cases and familial (2 or more affected members) in 61 families. The degrees of HI varied from mild (n = 5), moderate (n = 39), severe (n = 42) to profound (n = 166).

Audiological Assessment

All patients underwent otoscopic and audiological examination comprehensive of pure tone audiometry, otoacoustic emissions and auditory brainstem evoked potentials (ABR). Audiological features of hearing loss were analyzed according to the GenDeaf Study Group [2003] recommendations. The severity of deafness was considered as: mild (20–40 dB), moderate (41–70 dB), severe (71–95 dB) and profound (≥95 dB).

Molecular Genetic Analysis

DNA from the 252 patients was extracted from peripheral blood according to the hexadecyltrimethylammonium bromide technique [Murray and Christman, 1980]. Mutations in *GJB2* were analyzed by direct sequencing of the coding region (exon 2). A 783-bp PCR product was amplified using primers CxP1F: 5′GAAGTCTCCCTGTTCTGTCCT and CxP4R: 5′TCTAACAACTGGGCAATGC spanning the entire coding region and flanking intronic regions, and purified from the remaining nucleotides and primers using QIAquick PCR purification Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol. Bidirectional DNA sequencing was performed on an automatic sequencer (3730xl DNA Analyzer, Applied Biosystems, Foster City, Calif., USA). The sequence obtained was aligned to the wild-type sequence of the *GJB2* gene (NCBI accession No. M86849) using the NCBI interface (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). The PCR reaction mix contained: 200 μM dNTPs, 1.5 mM $MgCl_2$, 20 mM Tris-ClH (pH 8), 50 mM KCl and 1 U Taq polymerase (Invitrogen, Life Technologies, São Paulo, Brazil) in a final volume of 25 μl. A Bio-Rad PTC200 thermal cycler (Hercules, Calif., USA) was used for 30 cycles with annealing at 60°C for 30 s, extension at 72°C for 30 s, denaturation at 94°C for 40 s, with an initial denaturing step at 95°C for 5 min, and a final extension step of 72°C for 5 min.

In the samples detected as heterozygous for *GJB2* mutations, we also looked for the presence of the c.–23+1G>A splice site mutation in the non-coding region flanking exon 1 of the *GJB2* gene [Green et al., 1999]. Untranslated exon 1, including surrounding splice sites was amplified by PCR using the primers: Cx26E1F 5′CAGTCTCCGAGGGAAGAGG and Cx26E1R 5′AAGGACGTGTGTTGGTCCAG with the same PCR conditions, purification protocol and sequence reaction as reported above.

The PCR-based tests for the two *GJB6*/connexin 30 deletions, del(GJB6-D13S1830) and del(GJB6-D13S1854), shown on occasions to be associated with *GJB2*-related deafness, were carried out as described previously [del Castillo I et al., 2003; del Castillo FJ et al., 2005]. The mutation c.1555A>G in *MTRNR1* was ana-

Table 1. Variations in *GJB2* and *GJB6* identified in 252 unrelated patients

Protein change	Nucleotide change	Amino acid substitution	Domain	Patients n	Alleles n	Classification	Type of mutation
–	c.–23+1G>A	–	none	1	1	AR	Splice site
Thr8Met	c.23 C>T	P > NP	IC1	1	1	AR	Missense
Leu10Pro	c.29 T>C	NP > NP	IC1	1	1	NEW	Missense
Gly12Val	c.35 G>T	NP > NP	IC1	1	1	AR	Missense
Val13X	c.35delG	Frameshift	IC1	32	46	AR	Deletion/frameshift
Val27Ile	c.79 G>A	NP > NP	TM1	29	32	Benign variant	Missense
Met34Thr	c.101 T>C	NP > P	TM1	6	7	AR	Missense
Val37Ile	c.109 G>A	NP > NP	TM1	2	2	AR	Missense
Glu47X	c.139 G>T	Acid > stop	EC1	2	2	AR	Nonsense
Leu56ArgfsX26	c.167delT	Frameshift	EC1	2	2	AR	Deletion/frameshift
Arg75Trp	c.223 C>T	Basic > NP	EC1	1	1	AD	Missense
Trp77Arg	c.229 T>C	NP > basic	TM2	1	1	AR	Missense
Ile82Met	c.246 C>G	NP > NP	TM2	1	1	AR	Missense
Phe83Leu	c.249 C>G	NP > NP	TM2	1	1	Benign variant	Missense
Leu90Pro	c.269 T>C	NP > NP	TM2	2	2	AR	Missense
Val91SerfsX11	c.269insT	Frameshift	TM2	1	1	AR	Insertion/frameshift
Val95Met	c.283 G>A	NP > NP	IC2	1	1	AR	Missense
Gly109Val	c.326 G>T	NP > NP	IC2	1	1	NEW	Missense
Arg127His	c.380 G>A	Basic > basic	IC2	1	1	Benign variant	Missense
Glu129Lys	c.385 G>A	Acid > basic	IC2	1	1	AR	Missense
Lys112GlufsX2	c.334_335delAA	Frameshift	IC2	1	1	AR	Deletion/frameshift
Arg143Trp	c.427 C>T	Basic > NP	TM3	1	1	AR	Missense
Val153Ile	c.457 G>A	NP > NP	TM3	2	2	Benign variant	Missense
Gly160Ser	c.468 G>A	NP > P	EC2	1	1	Benign variant	Missense
Met163Val	c.487 A>G	NP > NP	EC2	2	2	Benign variant	Missense
Mel163Leu	c.487 A>C	NP > NP	EC2	1	1	AD	Missense
Lys168Arg	c.503 A>G	Basic > basic	EC2	3	3	AR	Missense
Arg184Pro	c.551 G>C	Basic > basic	EC2	2	2	AR	Missense
–	c.682 C>T	–	None	1	1	Benign variant	3′UTR
–	del(GJB6-D13S1830)	–		2	2	AR	Deletion
–	del(GJB6-D13S1854)	–		2	2	AR	Deletion
Total				106	124		

Numbering of *GJB2* nucleotides starts with the A of the ATG initiation codon in exon 2 as position +1. 'c.' designates the mutated nucleotide numbering with the complementary DNA reference sequence. UTR = Untranslated region; IC = intracellular domain; TM = transmembrane domain; EC = extracellular domain; AR = autosomal recessive; AD = autosomal dominant; NP = non-polar; P = polar. The classification given above is based on the Connexin-deafness homepage [http://davinci.crg.es/deafness].

lyzed by RFLP following the protocol published by Prezant et al. [1993] with modifications. A 499-bp fragment was amplified with the primers mt12SF 5′GCAAACCCTGATGAAGGCTA and mt12SR 5′GCGCCAGGTTTCAATTTCTA using the same PCR conditions as for *GJB2*. The amplification product was then digested with *BsmA*I restriction endonuclease according to the manufacturer's instructions (New England Biolabs, Beverly, Mass., USA), and the digestion fragments were resolved by electrophoresis on a 2% agarose gel. In the wild-type allele there is one *BsmA*I site, resulting in two fragments of 278 and 221 bp. This restriction site disappears in the mutated allele. The Q829X mutation in *OTOF* was analyzed by RFLP as described previously [Migliosi et al., 2002].

Results

Mutation Analysis

Mutations in *GJB2*, del(GJB6-D13S1830) and del(GJB6-D13S1854) in *GJB6*, Q829X in *OTOF*, and c.1555A>G in *MTRNR1* were analyzed in 252 Argentinean patients with sensorineural HI. A total of 31 different mutations in *GJB2* and *GJB6* were identified in 86 of the 252 (34%) unrelated patients (61 familial cases and 191 sporadic). The localization of mutations spanned the entire length of Cx26 and involved different protein domains. As

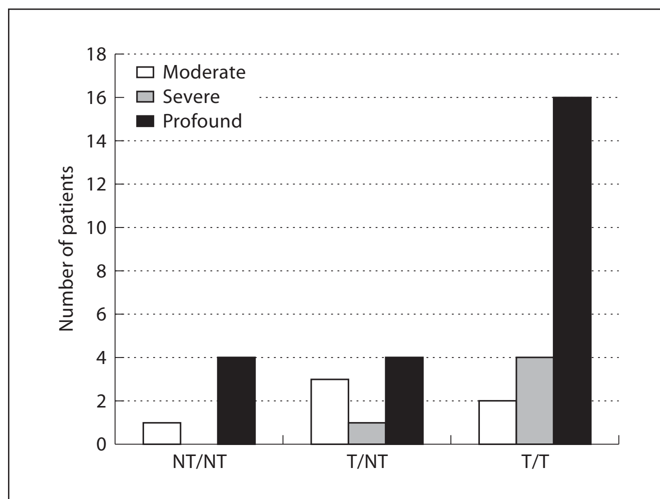

Fig. 1. Distribution of degree of hearing loss among patients with biallelic DFNB1 mutations (patients homozygous p.Val27Ile were excluded). A total of 35 different genotypes were found: 22 were homozygous truncating (T/T), 5 homozygous nontruncating (NT/NT), and 8 compound heterozygous truncating/nontruncating (T/NT). Patients with biallelic truncating mutations had a worse degree of hearing impairment (HI), since the T/T phenotype was observed in 73% of patients with profound HI, 18% with severe and 9% with moderate HI.

Table 2. Frequency *GJB2* mutations found in 50 healthy control samples

Sequence variation	Patients, n	Classification
c.–23+15C>T	1	Polymorphism 5′UTR
c.35delG	2	AR
p.Val27Ile	6	Polymorphism
p.Met34Thr	1	AR
p.Glu129Lys	1	AR
p.Val27Ile/p.Met34Thr	1	Polymorphism/AR
Total	12	

The classification given above is based on the Connexin-deafness homepage [http://davinci.crg.es/deafness]. AR = Autosomal recessive; UTR = untranslated region.

shown in table 1 and, according to the Connexin-deafness homepage [http://davinci.crg.es/deafness], the classification of variants found was: 20 (65%) of autosomal recessive inheritance (c.–23+1G>A, p.Thr8Met, p.Gly12Val, c.35delG, p.Met34Thr, p.Val37Ile, p.Glu47X, c.167delT, p.Trp77Arg, p.Ile82Met, p.Leu90Pro, c.269insT, p.Val95Met, c.333delAA, p.Glu129Lys, p.Arg143Trp, p.Lys168Arg, p.Arg184Pro, del(GJB6-D13S1830), del(GJB6-D13S1854)); 2 (6%) of autosomal dominant inheritance (p.Met163Leu, p.Arg75Trp), and 7 (23%) benign variants (p.Val27Ile, p.Phe83Leu, p.Arg127His, p.Val153Ile, p.Val160Ser, p.Met163Val, c.682 C>T (3′UTR)). Two new mutations were identified: c.29 T>C (p.Leu10Pro) and c.326 G>T (p.Gly109Val). None of the new mutations were detected in 100 samples obtained from the general healthy population.

The c.35delG mutation in *GJB2* was the most frequent mutation found in the cohort: 32 of the 252 (13%) patients and 46 of the 124 (37%) mutated alleles (table 1). The carrier frequency for this mutation analyzed in 50 normal hearing adults was 4% (table 2). The second most common mutation found in our cohort was p.Val27Ile, detected in 29 of the 252 (12%) patients and 32 of the 124 (26%) mutated alleles, with a carrier frequency of 7/50 (14%) in the healthy population (table 2). It was followed by p.Met34Thr found in 6 of the 252 (2%) patients and 6% of the mutated alleles, with a carrier frequency of 2/50 (4%) in the control population, and finally p.Lys168Arg in 3 (1%) patients representing 2% of the mutated alleles. All other mutations were identified only once or twice. Mutations p.Glu129Lys, c.–23+15C>T in the 5′UTR and p.Val37Ile were detected once in 50 control subjects analyzed (table 2).

None of the 252 patients carried the mutations p.Q829X in *OTOF* or c.1555A>G in *MTRNR1*.

Genotypes

The novel mutations identified in this work were present in sporadic cases: c.29 T>C (p.Leu10Pro) associated in *trans* with p.Val27Ile and c.326 G>T (p.Gly109Val) in *trans* with the nonsense mutation p.Glu47X.

Eighty-six of the 252 (34%) patients were genotyped: 38 with biallelic mutations, 18 with heterozygous mutations, and 30 with heterozygous sequence variations reported as polymorphisms (table 3). Mutation c.35delG was found in 32 patients: 14 (43%) c.35delG homozygous, 13 (41%) compound heterozygous for c.35delG and a different *GJB2* or *GJB6* mutation, and the remaining 5 (16%) were heterozygous c.35delG with no other mutation identified. Mutation c.35delG was by far the most frequent in biallelic genotyped patients, 27 of 38 (71%). Remaining 11 biallelic genotyped patients were either homozygous or compound heterozygous for 2 non-c.35delG mutations (29%).

Identified allele variants were classified as truncating or nontruncating mutations [Azaiez et al., 2004; Snoeckx et al., 2005]. The group of truncating mutations contained nonsense mutations and deletions, insertions, and duplications that introduced a shift in the reading frame. The splice-site mutation (c.–23+1G>A) and large deletions, del(GJB6-D13S1830) and del(GJB6-D13S1854), were also classified as truncating. Missense mutations were classified as nontruncating, although it has been reported [Azaiez et al., 2004; Snoeckx et al., 2005] that for some amino acid substitutions functional activity of the protein might be lost. Of the 31 different mutations identified, 23 were nontruncating, and 8 were truncating (table 1). A total of 35 different genotypes were found (excluding homozygous p.Val27Ile): 22 homozygous truncating (T/T), 5 homozygous nontruncating (NT/NT), and 8 compound heterozygous truncating/nontruncating (T/NT).

Phenotypic Analysis

All degrees of HI were observed in our cohort. Patients genotyped with biallelic mutations (n = 35, excluding homozygous p.Val27Ile) had mostly profound deafness, 24 profound versus 11 moderate-severe (table 2). Biallelic truncating mutations were associated with a worse degree of HI, since the T/T phenotype was observed in 73% of patients with profound HI, 18% with severe and 9% with moderate HI (fig. 1).

Prelingual deafness was observed in most of the cases (212/252). Eighty-two of these 212 (39%) patients with prelingual deafness carried mutations in *GJB2* or *GJB6*. Only 4 of 40 (10%) patients with postlingual deafness had mutations in *GJB2*, their genotypes being p.Val27Ile/p.Val27Ile and either p.Val27Ile, p.Val153Ile or c.35delG, with no mutation identified in the other allele. Since both p.Val27Ile and p.Val153Ile are considered as benign polymorphisms and c.35delG is found in a high proportion of healthy subjects, these mutations are most likely not the underlying cause of the postlingual deafness.

The patient that had the c.223C>T (p.Arg75Trp) mutation did not present any other symptoms and did not report a familial history. Although it is tempting to speculate the appearance of a de novo mutation, samples from the family were not available at the time of the study in order to corroborate this.

Audiometric curve shapes showed no pathognomonic pattern associated with a particular mutation, since flat, U-shaped and sloping curves were present (data not shown). In some cases different phenotypes were detected in siblings with the same genotype, i.e. audiological parameters showed progressive hearing loss in only one of the affected. Moreover, as previously reported [Cohn and Kelley, 1999], asymmetrical HI was found in some but not in all siblings.

Both patients carrying the new mutations (p.Leu10Pro and p.Gly109Val) had congenital bilateral profound HI and were cochlear implanted with a good outcome.

Discussion

Hearing loss is the most frequent inherited sensory disorder in humans [Kudo et al., 2004]. A high heterogeneity of ARNSHL has been reported among different populations or even within a certain country, in particular with respect to *GJB2* mutations [Dalamon et al., 2005; Esmaeili et al., 2007; Liu et al., 2002; Morell et al., 1998; Neocleous et al., 2006; Samanich et al., 2007; Snoeckx et al., 2005]. Therefore, a systematic study of different *GJB* mutations must be established for each population. The data presented in this study substantially extend our previous report [Dalamon et al., 2005] and indicate that mutations in the *GJB2* gene are prevalent in Argentinean patients with non-syndromic sensorineural HI. The high frequency of the c.35delG mutation in our group correlates with our previous published data; the carrier frequency detected in the healthy population (1/25) being higher than that reported in recent work (1/65) [Gravina et al., 2007]. This difference might arise from variations in the demographic characteristics of the control sam-

Table 3. *GJB2/GJB6* genotypes and phenotypes in the 252 unrelated patients

Genotype	Number of subjects with this genotype	Phenotype			Onset
		moderate	severe	profound	
Biallelic mutations	38				
p.Thr8Met/p.Val153Ile	1			1	Prelingual
c.35delG/p.Ile82Met	1		1		Prelingual
c.35delG/c.167delT	2	1		1	Prelingual
c.35delG/p.Val37Ile	1	1			Prelingual
c.35delG/del(GJB6-D13S1830)	1			1	Prelingual
c.35delG/del(GJB6-D13S1854)	2		1	1	Prelingual
c.35delG/p.Arg143Trp	1			1	Prelingual
c.35delG/p.Trp77Arg	1			1	Prelingual
c.35delG/c.–23+1G>A	1			1	Prelingual
c.35delG/c.35delG	14	1	2	11	Prelingual
c.35delG/p.Arg184Pro	2	1		1	Prelingual
c.35delG/c.269insT	1		1		Prelingual
p.Glu47X/del(GJB6-D13S1830)	1			1	Prelingual
p.Glu47X/p.Gly109Val	1			1	Prelingual
p.Met34Thr/p.Met34Thr	1			1	Prelingual
p.Val27Ile/p.Leu10Pro	1			1	Prelingual
p.Val27Ile/c.333delAA	1			1	Prelingual
p.Val27Ile/p.Arg75Trp	1			1	Prelingual
p.Val27Ile/p.Val27Ile	3	1		2	Post (1), pre (2)
p.Val95Met/p.Gly12Val	1	1			Prelingual
Heterozygous mutations	18				
c.35delG	5	1	1	3	Post (1), pre (4)
p.Met34Thr	5	1		4	Prelingual
p.Val37Ile	1		1		Prelingual
p.Leu90Pro	2		1	1	Prelingual
p.Glu129Lys	1			1	Prelingual
p.Met163Leu	1			1	Prelingual
p.Lys168Arg	3		1	2	Prelingual
Polymorphism only	30				
p.Val27Ile	23	2	1	20	Post (1), pre (22)
p.Phe83Leu	1			1	Prelingual
p.Arg127His	1			1	Prelingual
p.Val153Ile	1	1			Postlingual
p.Gly160Ser	1			1	Prelingual
p.Met163Val	2	1		1	Postlingual
c.*1C>T	1			1	Prelingual
No mutation detected	166	32	32	102	
Total	252				

ples. Mutation c.35delG is the most frequent reported mutation in most European populations [Azaiez et al., 2004; Denoyelle et al., 1997; Snoeckx et al., 2005]. The ethnic data collected in the anamnesis of our patients showed that European ancestors were the most prevalent in our cohort, clearly derived from the emigrational waves from Europe in the early and middle 20th century. The second most frequent sequence variation observed in patients and healthy carriers in the present work was p.Val27Ile, which has been considered as a polymorphism not leading to hearing loss [Connexin-deafness homepage]. The fact that p.Met34Thr was the third most observed mutation in patients is in accordance with a carrier frequency of 1/43 (2.3%) in the normal population

observed in France [Roux et al., 2004] and with the 4% carrier frequency reported in the present study. In spite of the fact that c.167delT is the most frequent mutated allele in Ashkenazi Jews and Palestinians [Morell et al., 1998; Shahin et al., 2002; Sobe et al., 1999], none of those ethnic backgrounds were referred by the affected families in our cohort.

The novel c.29 T>C mutation produces a proline for leucine substitution (p.Leu10Pro) in the N terminal domain of Cx26. This residue is evolutionarily highly conserved in all species studied and between different connexins [ConSeqServer: http://conseq.tau.ac.il/]. When analyzing the mutation using the PolyPhen prediction tool [http://genetics.bwh.harvard.edu/pph/], one can suggest that this variation would probably be damaging for the protein. A substitution of a proline for the highly conserved hydrophobic residue leucine, most likely changes the three-dimensional arrangement of the α-helix structure in the N terminal domain, leading to a defective protein and associated deafness. However, since this mutation was associated in *trans* with p.Val27Ile considered as a polymorphism, further studies would be needed to unequivocally relate the mutation to the HI in this patient.

The other novel mutation detected, c.326 G>T, produces a valine for glycine substitution (p.Gly109Val) in the intracytoplasmic domain of the protein. This location within the *GJB* genes is conserved in all species analyzed [ConSeqServer]. Moreover, the PolyPhen prediction tool indicates that this mutation would probably be damaging for the protein conformation. The fact that this mutation was associated in *trans* with the nonsense mutation p.Glu47X most likely indicates that it is the underlying cause of the HI in the patient.

An allele frequency of 1.6%, as observed in the present study for del(GJB6-D13S1830), is similar to the frequency reported for Italy and Belgium [del Castillo I et al., 2003; Gualandi et al., 2004]. This mutation occurs much more frequently in Spain (7.6–9.7%), Israel (6–7.1%), United Kingdom (5.9%) and the south of France (15%) [Marlin et al., 2005]; it is less frequent in the USA, Belgium, and Australia (1.3–4.5%) and it has not been detected in Iran [Esmaeili et al., 2007], Cyprus [Neocleous et al., 2006], Taiwan [Yang et al., 2007] and China [Yuan et al., 2008]. Analysis of haplotypes associated with the deletion suggest a common founder effect for Western European countries [del Castillo I et al., 2003].

The del(GJB6-D13S1854) mutation was found in our cohort in 1.6% of the mutated alleles, resulting in 2.4% of *GJB2* heterozygous who were unresolved after screening for del(GJB6-D13S1830). A multicenter study has shown that it accounts for 22.2% of affected *GJB2* heterozygotes in the United Kingdom, 6.3% in Brazil, and 1.9% in northern Italy. It was not found in screenings carried out on samples from France, Belgium, Israel, USA, or Australia [del Castillo FJ et al., 2005]. Different studies reveal significant differences in the frequency of each of the deletions, and also different patterns of geographical distribution. The del(*GJB6*-D13S1830) mutation, found in many populations over the world, seems to be much more frequent than del(*GJB6*-D13S1854). These differences between countries further illustrate the complexity of the genetic epidemiology of non-syndromic HI.

The proportion of biallelic mutations was significantly higher in familial cases than in sporadic cases (12 of 61 (20%) vs. 22 of 191 (12%)). In addition, in agreement with published data [Azaiez et al., 2004; Cryns et al., 2004; Snoeckx et al., 2005], the degree of HI associated with biallelic truncating mutations in *GJB2* was significantly more severe. The fact that in several families phenotypes were different between siblings is in accordance with the finding that the DFNB1 phenotype is determined not only by the *GJB2/GJB6* genotype but also by environmental factors and genetic background [Cryns and Van Camp, 2004; Marlin et al., 2005; Stinckens et al., 2004]. Thus, genotype-phenotype correlations in DFNB1 deafness should be cautiously interpreted during clinical genetic counseling.

Forty-eight genotyped patients revealed heterozygous mutations in *GJB2*. Further analysis or linkage studies are necessary in order to rule out if they are merely *GJB2*-mutation carriers and if the pathogenic mutations related to the pathology are located in a different locus. Worth mentioning is the relatively high frequency found for the p.Met34Thr mutation. Based on the high incidence of p.Met34Thr found in the normal population and other reports, it was originally considered a polymorphism not leading to hearing loss [Connexin-deafness homepage]. However, functional experiments have shown that p.Met34Thr is a pathological variant of Cx26 associated with HI. Thus, expression experiments of the Cx26 protein carrying p.Met34Thr in transiently transfected HeLa cells indicate that the protein is correctly synthesized and targeted to the plasma membrane, but that intercellular channels are inefficiently formed [Bicego et al., 2006]. Moreover, mutated channels fail to support the intercellular diffusion of Lucifer Yellow and the spreading of mechanically induced intercellular Ca^{2+} waves. In addition, as derived from the structure of the connexin 26 channel at 3.5A resolution, Met34 is fundamental for the gating

process [Maeda et al., 2009]. Taken together, these results suggest that Met34Thr is most likely a pathogenic mutation and not merely a benign variant as currently proposed in the Connexin-deafness homepage.

The present results confirm the importance of genetic screening to provide etiological diagnosis of HI. Moreover, it strengthens the importance of routine screening for *GJB2* mutations, resulting in more efficient and useful genetic counseling for affected individuals.

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