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Identification of four novel connexin 26 mutations in non-syndromic deaf patients: genotype–phenotype analysis in moderate cases

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Abstract This paper presents a mutation as well as a genotype–phenotype analysis of the *GJB2* and *GJB6* genes in 476 samples from non-syndromic unrelated Argentinean deaf patients (104 familial and 372 sporadic cases). Most of them were of prelingual onset (82 %) and 27 % were cochlear implanted. Variation of sequences was detected in 171 of the 474 patients (36 %). Overall, 43 different sequence variations were identified in *GJB2* and *GJB6*. Four of them are reported for the first time in *GJB2*: c.233dupG, p.Ala78Ser, p.Val190Asp and p.Cys211Tyr. Mutations in *GJB6* were detected in 3 % of patients [nine del(GJB6-D13S1830) and three del(GJB6-D13S1854)]. Of the 43 different variations identified in *GJB2*, 6 were polymorphisms and of the others, 10 (27 %) were truncating and 27 (73 %) were nontruncating. Patients with two truncating mutations had significantly worse hearing impairment than all other groups. Moderate phenotypes were observed in a group of patients carrying biallelic mutations (23 %). This work shows the high prevalence of *GJB2* mutations in the Argentinean population and presents an analysis of moderate phenotypes in our cohort.

Keywords *GJB2* · Connexin 26 · *GJB6* · Deafness · Novel mutations · Hearing loss · Molecular study · Autosomal recessive deafness · DFNB1 · Moderate 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 and incidence of 1:1,000 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 [1]. The pattern of inheritance of non-syndromic cases is autosomal recessive (ARNSHL) in about 80 %, resulting the most prevalent form of deafness [2, 3]. To date over 70 non-redundant loci have been characterized for ARNSHL (http://hereditaryhearingloss.org) and the most predominant cause is related to mutations in the DFNB1 locus, containing the gap junction beta 2 gene (*GJB2*) that encodes connexin 26 (Cx 26), and the gap junction beta 6 gene (*GJB6*) that encodes connexin 30 (Cx 30). More than 100 mutations have been described in *GJB2* (http://davinci.crg.es/deafness/), but the c.35delG accounts for the majority of mutations in deaf Caucasians [4–6]. We and others have shown that c.35delG is very frequent in the Argentinean population [7, 8]. 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, named del(GJB6-D13S1830) and del(GJB6-D13S1854) respectively, involving the 5′-portion of the *GJB6* gene may be common mutations causing ARNSHL.

DFNB1 HI is generally 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, symmetric and affects all frequencies and audiometric curves are either flat or sloping [9, 10]. However, it is now well recognized that some cases have delayed onset, are occasionally progressive, and phenotypes are associated with milder hearing loss [11]. Identifying mutations that lead to these milder phenotypes are thus of importance for a better genetic counseling of the family. In earlier studies, we found that mutations in the *GJB2* gene, alone or associated with the del(GJB6-D13S1830) or del(GJB6-D13S1854) are present in the Argentinean population [7, 12]. In this study, we broad our previous report for the prevalence of mutations in *GJB2* and *GJB6*, and we analyze the correlation with the phenotype in a series of non-syndromic sensorineural hearing impaired patients. In addition, we report for the first time four novel apparently recessive mutations in *GJB2,* which add to the complexity of already known mutations in the DFNB1 locus. Moreover, we describe a novel compound heterozygous *GJB2/GJB6* mutation associated with moderate hearing loss of late onset, and we study the genotype–phenotype relationship in moderate cases.

Materials and methods

Subjects and selection criteria

Clinical data and samples were obtained from a prospective collection compiled between 2004 and 2010. 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. Four hundred and seventy-six unrelated patients with non-syndromic bilateral sensorineural HI were included [390 (82 %) congenital-prelingual, and 86 (18 %) postlingual]. Patients with HI related to environmental causes (for example, infectious diseases and ototoxic drugs) were excluded during interview. Written informed consents were 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 372 cases (78 %) and familial (2 or more affected members) in 104 families. The degrees of HI varied from 82 moderate (17 %), 99 severe (21 %) to 295 profound (62 %). In addition 100 Argentinean individuals with normal hearing were recruited as a control group. Part of the results (252 patients), were previously reported in Dalamon et al. (2010).

Audiological assessment

All patients underwent audiometric examination using age appropriate methods according to current clinical standards. The severity of deafness was considered as: mild (20 to $\leq$39 dB), moderate (40 to $\leq$69 dB), severe (70 to $\leq$89 dB) and profound ($\geq$90 dB). The severity of deafness in each patient was defined by the degree of hearing loss in the best ear.

Molecular genetic analysis

DNA from the 476 patients and 381 relatives was extracted from peripheral blood according to standard techniques. Mutations in *GJB2* were analyzed by direct sequencing of the coding (exon 2) and the non-coding regions (exon 1). A 783 bp-PCR product was amplified using primers CxP1F: 5′GAAGTCTCCCTGTTCTGTCCT and CxP4R: 5′TCTA ACAACTGGGCAATGC spanning the entire coding region of exon 2 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, CA, USA). The sequence obtained was

aligned to the wild-type sequence of the *GJB2* gene (accession number NM_004004.5) 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, Sao Paulo, Brazil) in a final volume of 25 μl. A Bio-Rad PTC200 thermal cycler (Hercules, CA, USA) was used. The PCR protocol was: 30 cycles with annealing at 60° for 30 s, extension at 72° for 30 s, denaturation at 94° for 40 s, with an initial denaturing step at 95° for 5 min and a final extension step at 72° 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 by direct sequencing. Untranslated exon 1, including surrounding splice sites was amplified by PCR using the primers: Cx 26E1F 5′CAGTCTCCGAGGGAAGAGG and Cx 26E1R 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 in all samples as described previously [13, 14].

All mutations detected in *GJB2* and *GJB6* were classified as truncating or nontruncating. Truncating mutations include nonsense mutations, deletions and insertions that introduce 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. Nontruncating mutations contain inframe deletions as well as aminoacid substitutions, although it is known that for some of these functional activity of the protein is lost.

Sequence aligment and Molecular modeling

The evolutionary conservation of the Cx26 novel mutated residues identified was assessed by performing a multiple sequence alignment of different mammalian connexin 26 sequences, which were retrieved from the Ensembl database (http://www.ensembl.org). The Mega5 version5 (http://www.megasoftware.net/) software was used for generating the sequence alignment. Mutation c.233dupG (p.Ala78Glyfs*101) was adjusted by hand, to deduce its effect in the protein sequence.

Structure/function analysis was performed by modeling the p.Ala78Ser, p.Val190Asp and p.CysC211Tyr mutants with programs Spdbviewer [15] (http://www.expasy.org/spdbv/), SCWRL [16, 17] (http://www1.jcsg.org/scripts/prod/scwrl/serve.cgi) and FOLDX [18] (http://foldx.crg.es/). The figure of the structural model was made with the program VDM (http://www.ks.uiuc.edu/Research/vdm/) [19].

Results

Mutation Analysis

Mutations in *GJB2*, del(GJB6-D13S1830) and del(GJB6-D13S1854) in *GJB6,* were analyzed in 476 Argentinean patients with sensorineural HI. A total of 43 different variations of sequence were identified in 171 of the 476 (36 %) unrelated patients (130 sporadic and 41 familial cases) in *GJB2* and *GJB6,* and are listed with their frequencies in Table 1. Four of them are novel and are reported for the first time in *GJB2*. The localization of detected variations spanned the entire length of Cx 26 and involved different protein domains.

The four novel mutations in *GJB2* identified in this study were: c.233dupG (p.Ala78Glyfs*101), c.232G>T (p.Ala78Ser), c.569T>A (p.Val190Asp) and c.632G>A (p.Cys211Tyr). None of the novel mutations were detected in 100 samples obtained from the general healthy population and have not been reported in the 1,000 genomes database (http://www.1000genomes.org/).

A total of 74 patients had biallelic pathogenic mutations, 7 had one pathogenic mutation and a benign "in trans" variant, 29 had only one pathogenic mutation, and 61 only benign variants (genotypes of patients with biallelic pathogenic mutations and their phenotypes are shown in Table 2). Overall 36 different biallelic genotypes were established. The c.35delG mutation in *GJB2* was the most frequent variation found in the cohort: 59 of 476 patients (representing 12 % of patients). Mutation c.167delT was found in 10 (2 %) patients representing 6 % of the mutated alleles. Mutations in *GJB6* gene were detected in 2 % of patients [nine del(GJB6-D13S1830) and three del(GJB6-D13S1854)], resulting in 7 % of the detected mutations. All of them presented a second mutation in *GJB2*. One patient presented a mutation in the splice site of the non-coding exon1 of *GJB2* (c.−23+1G>A) and c.35delG as the second mutation in the other allele. There were 30 patients with homozygous mutations: c.35delG (26), p.Met34Thr (2), p.Asn206Ser (1) and c.167delT (1).

Protein analysis

The evolutionary conservation of the three Cx 26 novel mutated residues identified was assessed by performing a multiple sequence alignment of different mammalian connexin 26 sequences, Ala78, Val190 and Cys211 have a complete conservation among the mammalian sequences analyzed (Fig. 1).

Mutation p.Ala78Ser is a c.232G>T transversion in the DNA sequence and it is located in transmembrane domain 2 of Cx 26. The aspartic acid 190 for valine substitution introduced by the c.569T>A mutation (p.Val190Asp) and

Table 1 Variations in *GJB2* and *GJB6* identified in 171 of the 476 unrelated patients

Nucleotide change	Protein change	Amino acid substitution	Domain	Number of patients	Number of alleles	Classification	Type of mutation
Presumably pathogenic variants							
−3172 G>A	c.−23+1G>A	None	None	1	1	AR	Splice site
c.23 C>T	p.Thr8Met	P > NP	IC1	1	1	AR	Missense
c.29 T>C	p.Leu10Pro	NP > NP	IC1	1	1	NEW* (AD)	Missense
c.35 G>T	p.Gly12Val	NP > NP	IC1	2	2	AR	Missense
c.35delG	p.Gly12Valfs*2	Frameshift	IC1	59	85	AR	Deletion/frameshift
c.56 G>C	p.Ser19Thr	P > P	IC1	1	1	AR	Missense
c.101 T>C	p.Met34Thr	NP > P	TM1	11	13	AR	Missense
c.109 G>A	p.Val37Ile	NP > NP	TM1	7	7	AR	Missense
c.139 G>T	p.Glu47*	Acid > stop	EC1	5	5	AR	Nonsense
c.167delT	p.Leu56Argfs*81	Frameshift	EC1	10	11	AR	Deletion/frameshift
c.223 C>T	p.Arg75Trp	Basic > NP	EC1	1	1	AD	Missense Syndromic
c.224 G>A	p.Arg75Gln	Basic > basic	EC1	1	1	AD	Missense Syndromic
c.229 T>C	p.Trp77Arg	NP > basic	TM2	1	1	AR	Missense
c.233dupG	p.Ala78Glyfs*101	Frameshift	TM2	1	1	NEW (AR)	Insertion/frameshift
c.232 G>T	p.Ala78Ser	NP > P	TM2	1	1	NEW (AR)	Missense
c.246 C>G	p.Ile82Met	NP > NP	TM2	1	1	AR	Missense
c.250 G>C	p.Val84Leu	NP > NP	TM2	1	1	AR	Missense
c.269 T>C	p.Leu90Pro	NP > NP	TM2	3	3	AR	Missense
c.269dupT	p.Leu90Leufs*101	Frameshift	TM2	1	1	AR	Insertion/frameshift
c.283 G>A	p.Val95Met	NP > NP	IC2	2	2	AR	Missense
c.313_326del14	p.Gly109 fs*111	Frameshift	IC2	2	2	AR	Deletion/frameshift
c.326 G>T	p.Gly109Val	NP > NP	IC2	1	1	NEW* (AR)	Missense
c.334_335delAA	p.Lys112Glufs*113	Frameshift	IC2	1	1	AR	Deletion/frameshift
c.358_360delGAG	p.Glu120del	Deletion	IC2	2	2	AR	Deletion
c.385 G>A	p.Glu129Lys	Acid > basic	IC2	1	1	AR	Missense
c.427 C>T	p.Arg143Trp	Basic > NP	TM3	6	6	AR	Missense
c.487 A>C	p.Met163Leu	NP > NP	EC2	1	1	AD	Missense
c.503 A>G	p.Lys168Arg	Basic > basic	EC2	4	4	AR	Missense
c.551 G>C	p.Arg184Pro	Basic > basic	EC2	3	3	AR	Missense
c.569 T>A	p.Val190Asp	NP > acid	TM4	1	1	NEW (AR)	Missense
c.617 A>G	p.Asn206Ser	NP > P	TM4	2	3	AR	Missense
c.632 G>A	p.Cys211Tyr	NP > P	TM4	1	1	NEW (AR)	Missense
del(GJB6-D13S1830)				9	9	AR	Deletion
del(GJB6-D13S1854)				3	3	AR	Deletion
Presumably polymorphisms							
c.24 G>A	p.Thr8=	P > P	IC1	1	1	Benign variant	Synonimous
c.79 G>A	p.Val27Ile	NP > NP	TM1	62	70	Benign variant	Missense
c.249 C>G	p.Phe83Leu	NP > NP	TM2	1	1	Benign variant	Missense
c.380 G>A	p.Arg127his	Basic > basic	IC2	1	1	Benign variant	Missense
c.384 C>T	p.Ile128=	NP > NP	IC2	1	1	Benign variant	Synonimous
c.457 G>A	p.Val153Ile	NP > NP	TM3	3	3	Benign variant	Missense
c.468 G>A	p.Gly160Ser	NP > P	EC2	2	2	Benign variant	Missense
c.487 A>G	p.Met163Val	NP > NP	EC2	2	2	Benign variant	Missense
c.*682 C>T	3′UTR	None	None	1	1	Benign variant	3′UTR

Numbering of *GJB2* 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

IVS intervening sequence/intron, *UTR* untranslated region, *IC* intracellular domain, *TM* transmembrane domain, *EC* extracellular domain, *AR* autosomal recessive, *AD* autosomal dominant, *NP* non polar, *P* polar, *NEW** indicates mutations previously detected by our laboratory and still not included in databases. The classification given above is based on the Connexin and Deafness Homepage (http://www.davinci.crg.es/deafness) and on reference sequences from ncbi: NP_003995.2 and NM_004004.5

Table 2 *GJB2/GJB6* genotypes and phenotypes in the 74 unrelated patients with biallelic mutations (patients with monoallelic variations or with polymorphisms were excluded)

Genotype	Severity of deafness			Total number
	Moderate	Severe	Profound	
Biallelic pathogenic mutations				
c.35delG/c.35delG	3	3	20	26
c.35delG/p.Val37Ile	1			1
c.35delG/p.Glu47*			1	1
c.35delG/p.Trp77Arg			1	1
c.35delG/p.Ile82Met		1		1
c.35delG/p.Leu90Pro	1			1
c.35delG/p.Val95Met			1	1
c.35delG/p.Val190Asp/p.Val27Ile			1	1
c.35delG/c.−23+1G>A			1	1
c.35delG/p.Arg143Trp/p.Val27Ile		1	1	2
c.35delG/p.Arg143Trp			1	1
c.35delG/c.167delT	1		3	4
c.35delG/c.167delT/p.Ile128Ile			1	1
c.35delG/p.Arg184Pro	1		1	2
c.35delG/c.269dupT		1		1
c.35delG/del(GJB6-D13S1854)		1	2	3
c.35delG/del(GJB6-D13S1830)			4	4
p.Glu47*/del(GJB6-D13S1830)			2	2
p.Met34Thr/p.Met34Thr	2			2
p.Met34Thr/p.Ser19Thr	1			1
p.Arg143Trp/c.167delT			2	2
p.Tre8Met/p.Val153Ile	1			1
p.Arg143Trp/c.233dupG/p.Val27Ile			1	1
p.Val37Ile/c.167delT	1			1
p.Val37Ile/del(GJB6-D13S1830)	1			1
p.Val37Ile/p.Gly12Val			1	1
p.Val37Ile/p.Glu47*	1			1
c.167delT/c.167delT			1	1
c.167delT/p.Arg184Pro		1		1
p.Val84Leu/c.313_326del14	1			1
p.Val95Met/p.Gly12Val	1			1
p.Asn206Ser/p.Asn206Ser			1	1
p.Glu47*/p.Glu120del			1	1
p.Cys211Tyr/del(GJB6-D13S1830)	1			1
p.Asn206Ser/del(GJB6-D13S1830)			1	1
c.313_326del14/p.Glu120del	1			1
Grand total	18	8	48	74

mutation p.Cys211Tyr, produced by the c.632G>A transition, are located in the transmembrane domain 4 of the protein.

Structure/function analysis performed by modeling the p.Ala78Ser, p.Val190Asp and p.Cys211Tyr mutants with three different programs gave similar results. Figure 2a, b show the location of Ala78 (red), Val190 (green), and Cys211 (yellow) in the structure of the channel. c, d and e show the theoretical structural models of the p.Ala78Ser, p.Val190Asp, and p.Cys211Tyr mutants, respectively.

Within transmembrane region 2, the hydroxyl (OH) group of the serine,that replaces the alanine residue (p.Ala78Ser), might converge in one of the two hydrophobic pockets, perturbing interactions with Leu36, Arg75 and Ile82 of the same chain and with Val38 of the contiguous chain in the structure of the channel (Fig. 2c). The mutation p.Val190Asp also produces the perturbation of intra-chain interactions with Phe191 and Phe194, and inter-chain interactions with Ile71 (Fig. 2d). Mutation p.Cys211Tyr perturbs the interaction with Tyr212 and

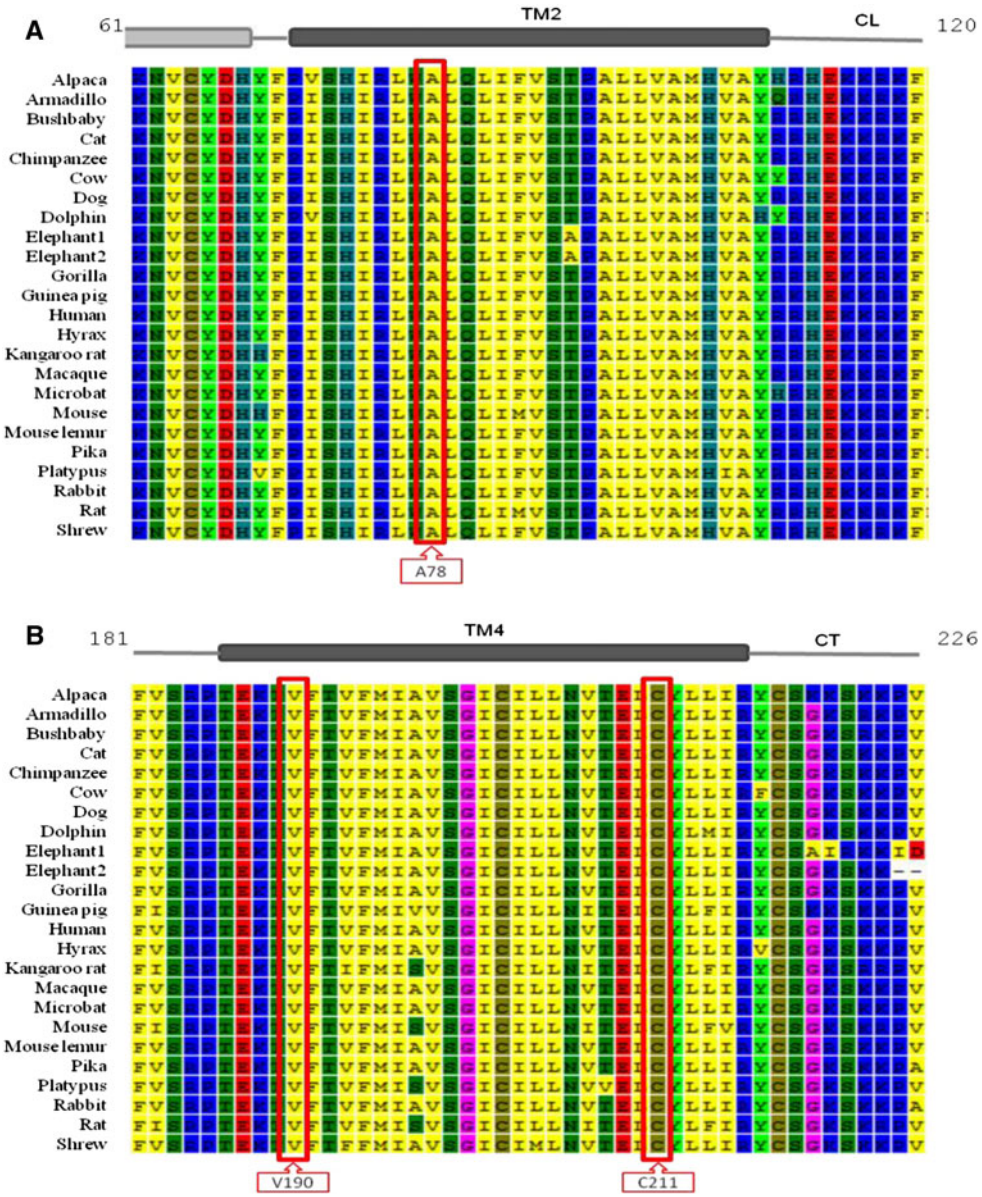

Fig. 1 Aligment of mammalian connexin 26 sequences. The evolutionary conservation of the Cx26 novel mutated residues identified was assessed by performing a multiple sequence alignment of different mammalian connexin 26 sequences. Positions A78, V190 and C211 are completely conserved

Ile215 of the same chain in transmembrane region 4 and the intracellular domains (Fig. 2e). Therefore, these mutations might have an effect on the oligomerization of the Cx 26 subunits, which might lead to alteration of protein function.

Genotypic/Phenotypic analysis

Three of the novel mutations reported in this work, c.233dupG, p.Ala78Ser and p.Val190Asp, were present in sporadic cases with congenital, bilateral profound HI, which were cochlear implanted with a good outcome. Parents of all patients were healthy carriers, supporting the idea that they are probably of recessive inheritance. Genotype of those patients was: p.Val190Asp/c.35delG with p.Val27Ile in cis, c.233dupG/p.Arg143Trp with p.Val27Ile in cis, and p.Ala78Ser/p.Val27Ile in *trans*.

The novel mutation p.Cys211Tyr was identified in a familial case with postlingual (third decade), bilateral, moderate deafness, being the genotype in the patient p.Cys211Tyr/del(GJB6-D13S1830). The mutation resulted ligated to the pathology according to its segregation in the family. One affected brother of the patient showing the same biallelic mutations presented a similar audiogram at all frequencies, meanwhile, another brother carrying only the del(GJB6-13S1830) mutation, showed a normal audiogram. Both parents resulted heterozygous for one of the mutations and presented no symptoms at all frequencies, supporting the idea of the recessive inheritance of the mutation (Fig. 3).

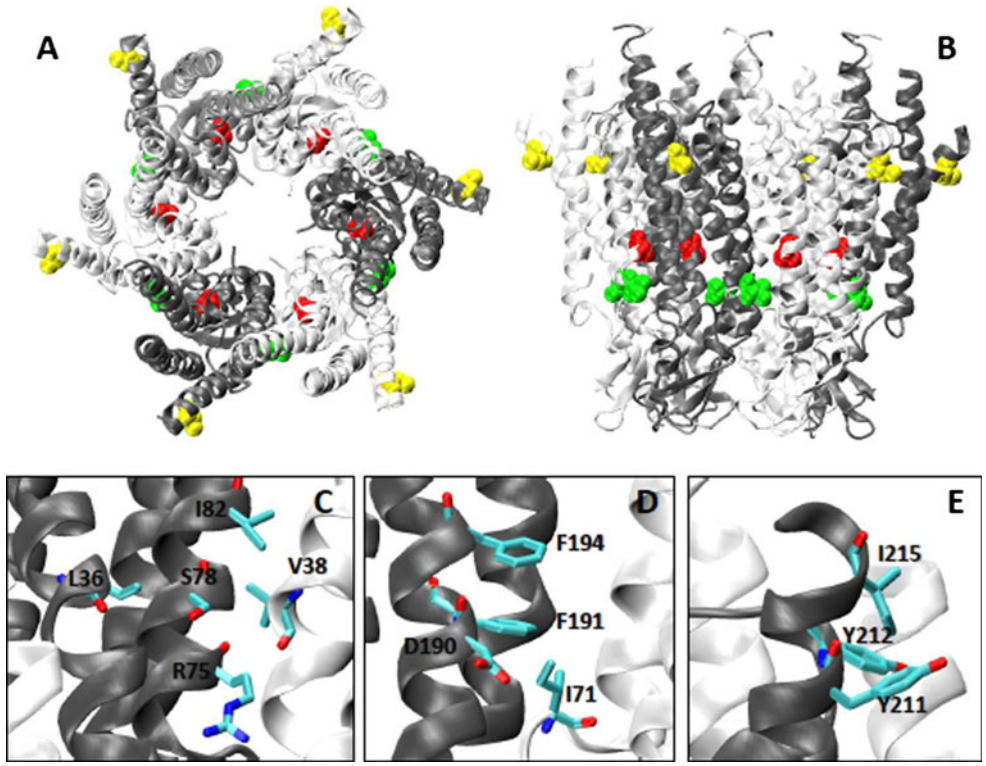

Fig. 2 Structural model of the p.Ala78Ser, p.Val190Asp and p.Cys211Tyr mutants. **a**, **b** show the location of Ala78 (*red*), Val190 (*green*), and Cys211 (*yellow*) in the structure of the channel. **c–e** show the theoretical structural models of the p.Ala78Ser, p.Val190Asp, and p.Cys211Tyr mutants, respectively. The direct contact interactions on a radial distribution of 5 Å for each mutant are shown in the corresponding *panels*. The figure was made with the program VMD (http://www.ks.uiuc.edu/Research/vmd/). **c** The visualization work shows that the –OH form the serine that replaces the alanine residue (p.Ala78Ser) might converge in one of the two hydrophobic pockets, perturbing interactions with Leu36, Arg75, and Ile82 of the same chain and with Val38 of the contiguous chain in the structure of the channel. **d** The mutation p.Val190Asp also produces the perturbation of intra-chain interactions with Phe191 and Phe194, and inter-chain interactions with Ile71. **e** Mutation p.Cys211Tyr perturbs the interaction with Tyr212 and Ile215 of the same chain in TM4 and IC domains

In order to analyze a phenotypic correlation, all of the 43 types of *GJB2/GJB6* variations detected were classified as truncating (T) or nontruncating (NT): 10 (27 %) were truncating and 27 (73 %) were nontruncating (6 were not considered since they have been reported as polymorphisms). When phenotypes were compared, the degree of hearing loss was nonrandomly distributed among patients. Patients with biallelic T/T mutations (46) were more likely to have a worse degree of HI, since 78 % of patients had profound deafness, in contrast to 22 % with severe or moderate phenotypes. Moreover, most patients with NT/NT and NT/T mutations (28) had moderate or severe phenotypes (61 %) (Fig. 4a). These results strongly suggest that there would be a bias towards a T/T genotype and the observed profound phenotype ($p = 0.002$ Chi square test). Additionally, individuals with profound HI were more likely to have biallelic T/T mutations (75 %) than T/NT or NT/NT mutations (21 and 4 % respectively) (Fig. 4b).

Most of the audiograms were flat for profound congenital deafness. In the case of moderate deafness, including the patient bearing the novel p.Cys211Tyr mutation, audiograms were slightly sloping and in general were the consequence of NT mutations (Fig. 5). There were only 4 patients with moderate deafness involving T/T mutations, with genotypes c.35delG/c.35delG and c.35delG/c.167delT. These have already been reported to exhibit phenotypic variability, most likely due to the action of modifier genes [20].

Figure 6, shows the audiograms of patients bearing *GJB6* mutations [nine with del(GJB6-D13S1830) and three with del(GJB6-D13S1854)]. In most of them, del(*GJB6*) was accompanied by another truncating mutation (c.35delG or p.Glu47*) and the phenotype was a profound hearing loss. Meanwhile, when *GJB6* mutations were accompanied with a non-truncating mutation (p.Val37Ile, p.Cys211Tyr) the phenotype resulted moderate. However, del(GJB6-D13S1830)/p.Asn206Ser (a non-truncating

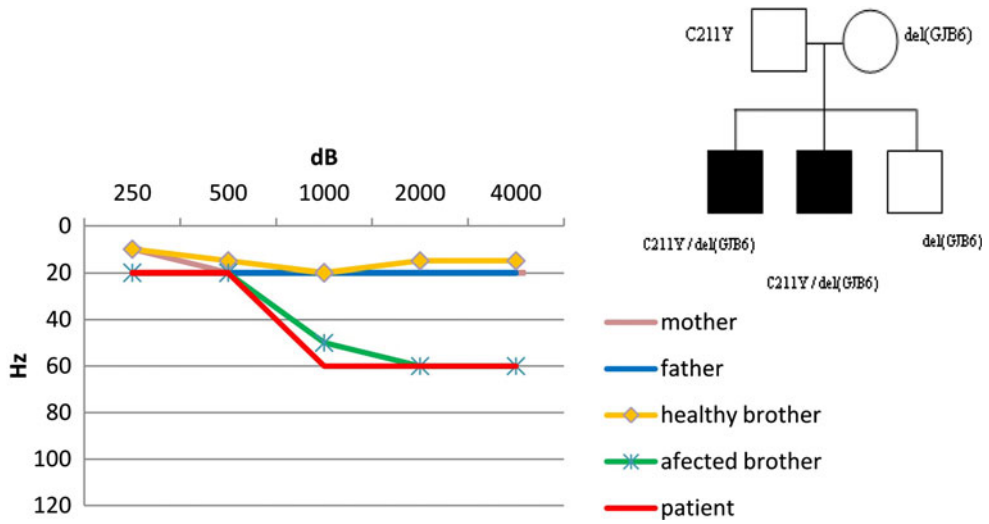

Fig. 3 Audiogram of a patient bearing mutations p.Cys211Tyr and del(GJB6-D13S1830) and his family. The novel mutation p.Cys211Tyr was identified in a familial case with postlingual (third decade), bilateral, moderate deafness, being the genotype in the patient p.Cys211Tyr/del(GJB6-D13S1830). The mutation resulted ligated to the pathology according to its segregation in the family. One affected brother of the patient showing the same biallelic mutations presented a similar audiogram at all frequencies, meanwhile, another brother carrying only the del(GJB6-13S1830) mutation showed a normal audiogram. Both parents resulted heterozygous for one of the mutations and presented no symptoms at all frequencies, supporting the idea of the recessive inheritance of the mutation. The figure represents the mean for both ears. The pedigree shows the segregation in the family

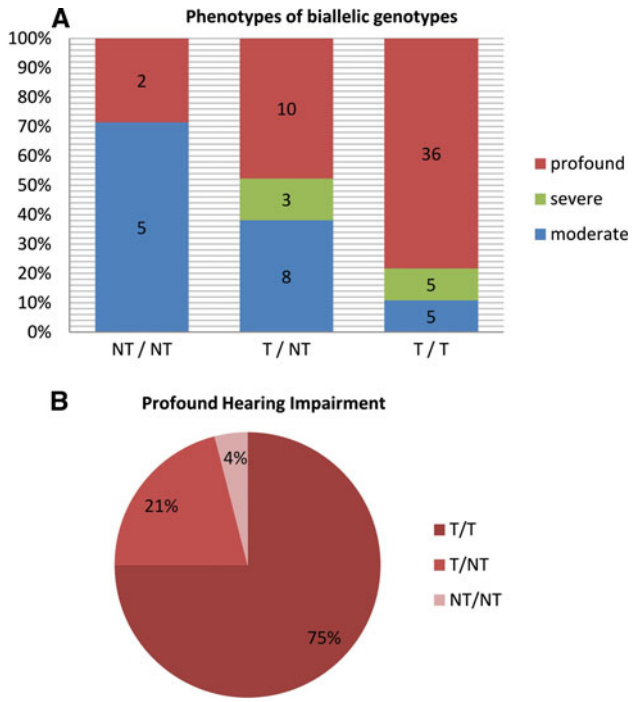

Fig. 4 Phenotype of 74 patients with biallelic pathogenic mutations were analysed according to their effect in the protein. In order to analyze a phenotypic correlation, individuals were classified according to their reported mutation type and their reported phenotype. Mutations detected in *GJB2* were classified as truncating (T) when they produced a frameshift or nonsense mutations, and nontruncating (NT) when they were missense mutations (polymorphisms were not included). **a** Phenotypic comparisons for biallelic genotypes. **b** Distribution of genotypes within profound hearing impairment

mutation) resulted in congenital profound deafness. Residue Asn206 in Cx 26 is proposed to perform a hydrogen bond or salt bridge interaction needed for intra-protomer stabilization, which could explain its functional behavior [21].

Discussion

A high heterogeneity of ARNSHL has been reported among different populations or even within a certain country, in particular with respect to *GJB2* mutations [9, 12, 22–26]. Therefore, a systematic study of different *GJB2* mutations must be established for each population. The data presented in this study extends our previous reports [7, 12] and indicates 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 of the carrier frequency detected in the healthy population (1/25) [7] and with that of most European populations [9, 27, 28].

We have identified four novel, apparently recessive, Cx 26 mutations: p.Ala78Ser, c.233dupG, p.Val190Asp and p.Cys211Tyr. The fact that these sequence variations were not found in 100 healthy Argentineans and have not been reported in the 1,000 genomes database, most likely indicates that they are pathogenic mutations. This hypothesis is further supported by the residue conservation analysis and/

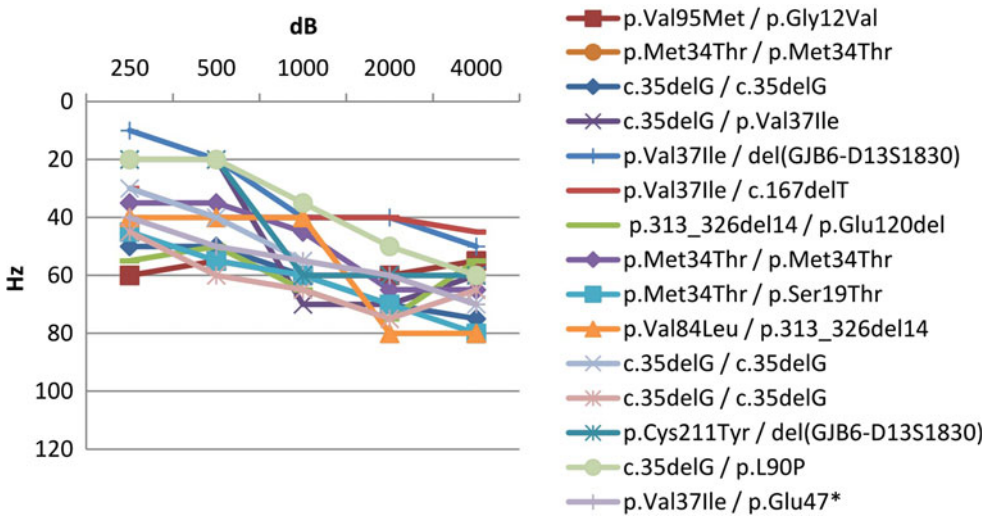

Fig. 5 Audiograms and genotypes for patients with moderate hearing loss. In the case of moderate deafness, including the patient bearing the novel p.Cys211Tyr mutation, audiograms were slightly sloping and in general were the consequence of non-truncating mutations. The figure represents the mean for both ears in each patient

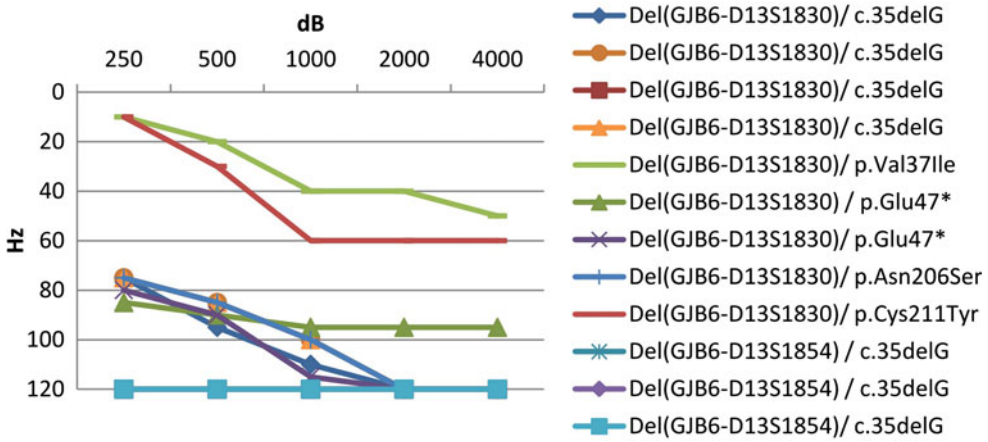

Fig. 6 Audiograms of patients with genotypes involving *GJB6* deletions. The figure represents the mean for both ears in each patient

or protein modeling, suggesting that they are important for the function of the protein and that when mutated they might alter protein function.

In addition to previous knowledge based on functional experiments, the elucidation of the atomic structure of the human Cx 26 gap junction channel has determined which residues of the protein are presumed to be associated with non-syndromic hereditary deafness based on their involvement in intra-or intermolecular interactions, conformational structure or their influence in channel gating [21]. It has been proposed that there are two hydrophobic cores that would stabilize the protomer structure, one of them involving residues Trp77, Phe154, and Met195. In this context, as shown by our protein modeling, the substitution of a neutral alanine by a hydrophilic residue like serine in position Ala78 perturbs residue interactions within the same chain and contiguous chain in the structure of the channel, altering the appropriate distance of folding of the protomer. As mutation p.Ala78Ser has been detected in the patient in *trans* with a benign variant, its pathogenic potential according to dominant or recessive inheritance can only be ruled out by functional experiments.

Mutation c.233dupG, produces a frameshift leading to a truncated protein (p.Ala78Glyfs*101) from transmembrane domain 2 of the protein onwards. This would result unequivocally in a non-functional protein and consequently a pathogenic mutation. The fact that this mutation was associated in *trans* with recessive mutation p.Arg143Trp, most likely indicates that it is the underlying cause of the HI in the patient and that it is of recessive inheritance.

Mutation c.569T>A produces a hydrophilic aspartic acid for a hydrophobic valine substitution (p.Val190Asp) in transmembrane domain 4 of Cx 26. This residue is evolutionarily conserved in Cx 26 across mammals, as well as most of the residues in region. It has been described that residues Arg184, Tht186 and Glu187 in transmembrane domain 4 are important for forming the core of the inter-protomer interactions in the hexameric connexon [21]. The proximity of Val190 to those residues indicates that the mutation might change the proper folding or accurate

distance for oligomerization of connexins, thus resulting in defective channels and associated deafness. Since this mutation was associated in *trans* with c.35delG, it could be considered as a recessive mutation.

The novel mutation p.Cys211Tyr was identified in a familial case with postlingual (third decade), bilateral, moderate deafness, being the genotype in the patient p.Cys211Tyr/del(GJB6- D13S1830). The mutation was ligated to the pathology in the family and all affected members shared the biallelic genotype. Position Cys211 is completely conserved between different forms of Cx 26 in mammals and protein modeling proved that the mutation detected perturbs the interaction with residues of other domains, further suggesting that mutations at this position might be functionally deleterious.

All degrees of HI were observed in our cohort of 476 patients, however they were predominantly congenital-prelingual (83 %). Patients genotyped with biallelic mutations had mostly profound or severe deafness (56/74 = 76 %), nevertheless 18 patients (24 %) showed a moderate phenotype.

Collectively, the results of this study clearly demonstrate a statistically significant association between a T/T genotype and the profound phenotype observed in our cohort. It is interesting to note that NT missense mutations p.Val37Ile, p.Met34Thr or p.Leu90Pro, that in our cohort are associated with a moderate phenotype have been proved, using in vitro approaches, to have a moderate effect in channel permeability or conductivity [29–33], thus correlating with the degree of phenotype. Initially, hearing loss related to *GJB2/GJB6* mutations was reported as being only severe or profound, but later observations have shown that certain mutations may also be found in cases of hearing loss of mild and moderate severity. In this regard, a large multicentric study has established genotype–phenotype correlations for connexin 26-related hearing loss, based on audiometric data of 1,531 patients from 26 laboratories [9]. All patients suffered from non-syndromic mild-to-profound hearing loss due to biallelic *GJB2* mutations. The study clearly demonstrated that inactivating mutations of *GJB2* cause a more severe phenotype than non-inactivating mutations [20]. Our results are in accordance with those observations, since most NT mutations are associated with a more moderate phenotype. In this regard, one could speculate that the NT missense novel mutation p.Cys211Tyr might be the cause of the moderate hearing loss in the patient, where it is found together with del(GJB6-D13S1830) and had a sloping curve audiogram. Thus, the genotype–phenotype analysis reported in this paper, adds to increasing reports, showing that many of the moderate phenotypes might also be explained by mutations in *GJB2* and *GJB6* genes.

Mutations del(GJB6-D13S1830) and del(GJB6-D13S1854) represented 7 % of genotyped patients, being del(GJB6-D13S1830) more frequent than del(*GJB6*-D13S1854), in accordance with published data [13]. Ninety-seven 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 the pathogenic mutations related to the pathology are located in a different locus or if second mutations are still in the DFNB1 locus. Although more than 200 mutations in *GJB2* have been described, population screening commonly yields an excess of individuals with hearing loss who carry only a single identified DFNB1 mutation. This finding strongly suggests that additional mutations that lie outside of the analyzed regions remain to be studied.

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. This is particularly important in light of the accumulating evidence suggesting that children with *GJB2* mutations do well with cochlear implants, having a better speech performance after implant and achieving faster and greater benefits on language expression tests and comprehension than non-DFNB1 patients [34–36].

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Conflict of interest The authors declare no conflicts of interest.

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