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Analyses of del(GJB6-D13S1830) and del(GJB6-D13S1834) deletions in a large cohort with hearing loss: Caveats to interpretation of molecular test results in multiplex families

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Abstract

Background: Mutations involving the closely linked *GJB2* and *GJB6* at the DFNB1 locus are a common genetic cause of profound congenital hearing loss in many populations. In some deaf *GJB2* heterozygotes, a 309 kb deletion involving the *GJB6* has been found to be the cause for hearing loss when inherited in trans to a *GJB2* mutation.

Methods: We screened 2,376 probands from a National DNA Repository of deaf individuals.

Results: Fifty-two of 318 heterozygous probands with pathogenic *GJB2* sequence variants had a *GJB6* deletion. Additionally, eight probands had an isolated heterozygous *GJB6* deletion that did not explain their hearing loss. In two deaf subjects, including one proband, a homozygous *GJB6* deletion was the cause for their hearing loss, a rare occurrence not reported to date.

Conclusion: This study represents the largest US cohort of deaf individuals harboring *GJB2* and *GJB6* variants, including unique subsets of families with deaf parents. Testing additional members to clarify the phase of GJB2/*GJB6* variants in multiplex families was crucial in interpreting clinical significance of the variants in the proband. It highlights the importance of determining the phase of *GJB2*/*GJB6* variants when interpreting molecular test results especially in multiplex families with assortative mating.

KEYWORDS

GJB2/*GJB6* variant, hearing loss, interpretation of results, unique family structure

1 | INTRODUCTION

Deafness is an etiologically heterogeneous trait with many recognized genetic and environmental causes (Kremer, 2019; Nance, 2003; Tekin, Arnos, & Pandya, 2001). The DFNB1 locus on 13q12 harbors two homologous genes, *GJB2* (OMIM: 121011) and *GJB6* (OMIM: 604418) that code for the connexin 26 and 30 subunits of gap junction proteins, respectively. Although initially these proteins were thought to participate in the recycling of potassium ions from the hair cells back into the cochlear endolymph (Steel & Bussoli, 1999), recent literature suggests the digenic mutations reduce

endocochlear potential and/or result in cochlear developmental disorder (Mei et al., 2017; Wingard & Zhao, 2015; Zhao, 2017). DFNB1 mutations are the most common genetic cause of severe to profound congenital hearing loss (HL) in many populations (Chan & Chang, 2014; Kenneson, Van Naarden Braun, & Boyle, 2002). The carrier frequency for *GJB2* mutation in North Americans has been reported to be as high as 3.5% (Green et al., 1999). The *GJB6* was first implicated as a cause for deafness in a single family demonstrating dominant inheritance segregating a one base pair substitution, designated DFNA3 (Grifa et al., 1999). In 2002, del Castillo et al. along with other groups (Lerer et al., 2001; Pallares-Ruiz, Blanchet, Mondain, Claustres, & Roux, 2002) reported a large deletion involving the 5′ noncoding region of *GJB6* that extended into coding region of the gene, which when present in trans with a *GJB2* mutation, explained the HL in 67% of their deaf *GJB2* heterozygotes. This deletion known as del(GJB6-D13S1830) was initially described as a 342 kb deletion, however, it is currently estimated at 309 kb (del Castillo et al., 2005). The reported frequency of this deletion among deaf individuals has ranged from as high as 15% in Southern France to between 9% and 5% in Spain, Israel, and United Kingdom, to as low as 1.4% in Italy and Belgium (Marlin et al., 2005). Its incidence in Eastern Europe and other nations is reported to be lower or not present (Adhikary et al., 2015; Frei et al., 2004; Wonkam, Ngo, & Ngogang, 2015). A second smaller deletion in the 5′ untranslated region of *GJB6* [del(GJB6-D13S1854)] was subsequently identified in other deaf *GJB2* heterozygotes, mainly in Spain and the UK (del Castillo et al., 2005).

Both proteins are expressed in the inner ear where they form homomeric or heteromeric connexons in largely overlapping regions of the supporting cells of the cochlea (Chen, Chen, Zhu, Liang, & Zhao, 2014; Forge, Marziano, Casalotti, Becker, & Jagger, 2003; Wang et al., 2009; Wingard & Zhao, 2015; Zhao & Santos-Sacchi, 2000; Zhao & Yu, 2006). Connexin 26 (Cx26) plays a role in the normal development of the auditory sensory epithelium and Connexin 30 (Cx30) is essential for normal repair after sensory hair cell loss (Jagger & Forge, 2015). The deletions in the coding portion of *GJB6*, and less so the deletion in the 5′ untranslated region of *GJB6* result in HL in a substantial proportion of the deaf *GJB2* heterozygotes. Studies suggest this phenotype to be secondary to the disruption of a cis-acting regulatory element for *GJB2*, in contrast to the prior hypothesis suggesting the contribution of *GJB6* haploinsufficiency (Boulay et al., 2013; Rodriguez-Paris & Schrijver, 2009).

We report on results from screening 2,376 deaf probands from a North American National Repository, the largest sample cohort studied to date (Pandya et al., 2003), and present the frequency of pathogenic variants involving the two genes, and the reported audiologic phenotype in individuals with sequence changes in both the *GJB2* and *GJB6*. We also report some unique pedigrees with the *GJB6* deletion that highlight pitfalls and caution needed in interpreting results on deaf probands tested in isolation, especially when they have both parents with hearing loss.

2 | METHODS AND MATERIALS

2.1 | Patient ascertainment

Deaf probands and their family members were ascertained for this study through several sources, including the Annual Survey of Deaf and Hard of Hearing Children and Youth, conducted at the Gallaudet Research Institute of Gallaudet University (GU), an institution of higher education for the deaf and hard of hearing. The Annual Survey collected educational, etiologic and audiologic data, as well as demographic information such as race, parental mating type, and hearing status of siblings on a nationwide sample of nearly 50,000 deaf and hard of hearing students who receive special education services because of their HL (Pandya et al., 2003). Participants were also recruited through the Gallaudet University Alumni Association (Arnos et al., 2008) and the genetics clinics held at GU and the Virginia Commonwealth University (VCU). All participants completed the informed consent process approved by the VCU and GU Institutional Review Board.

2.2 | Molecular testing

DNA was extracted from peripheral blood samples using Pure Gene (Gentra Systems) protocols. Samples from all probands were screened for mutations in exon 1 and 2 of *GJB2* by cycle sequencing as described in Pandya et al., 2003. The product was subjected to cycle sequencing with the ABI PRISM Big Dye Terminator cycle sequencing kit. Forward and reverse sequences were analyzed for mutations using Phred, Phrap, and Consed software suite (Gordon, Abajian, & Green, 1998).

The del(GJB6-D13S1830) deletion was tested using primers described by del Castillo et al. (2003). Briefly, the breakpoint junction and an internal control sequence outside the deletion were amplified simultaneously to test for the deletion in all deaf probands. Individuals heterozygous for the deletion yielded a 460 bp product (breakpoint junction) along with a 360 bp fragment (internal control) from the nondeleted chromosome. Individuals who did not carry the deletion showed only the 360 bp band.

To confirm the presence of a homozygous deletion, all samples showing the breakpoint junction product were tested further using primers for exon 1 of *GJB6* along with an internal control. The primers used for *GJB6* exon 1 were; Forward E1: 5′-ATG GAT TGG GGG ACG CTG CA

TABLE 1 Frequency of GJB2 and GJB6 mutations in deaf probands

	GJB6 (*Cx30*)			
GJB2 (*Cx26*)	wt/wt	wt/del	del/del	Totals
wt/wt	1507	7	1	1515
mut/wt	266	52	0	318
mut/mut	542	1	0	543
Totals	2,315	60	1	2,376

Note: Genotypic distribution of del(GJB6-D13S1830) and pathogenic *GJB2* variants in our cohort of deaf probands.

-3′. Reverse E1 5′ CCA CCA CTA GGA TCA TGA CTC GGA- 3′. Individuals homozygous for the deletion showed no amplification product for exon 1 of *GJB6*. Amplification of the internal control verified that the PCR reaction was working.

The del(GJB6-D13S1853) deletion was also tested using primers described by del Castillo et al. (2005). The probands were tested and were negative for changes in the *MT-RNR1* for the m.1555A>G variant, and *MT-TS1* for the m.7445A>C/T/G variant.

2.3 | Audiologic studies

Audiometric data were extracted from copies of available audiograms. Only the results of audiometric testing performed in soundproof booths according to ANSI 1969 standards were used for the analysis. Coded data included pure tone averages for all available frequencies, speech reception thresholds, and word recognition scores. A determination was also made about the bilateral symmetry of the audiograms based on the criteria of Liu and Xu (1994).

3 | RESULTS

DNA samples from 2,376 deaf probands were screened for the *GJB2* and *GJB6* mutations as described above. Overall, 22.85% probands carried two pathogenic *GJB2* variants that are most likely the cause for their HL. An additional 13.38% of the probands were heterozygous for a single pathogenic *GJB2* variant, and 16.35% (52/318) of these apparent heterozygotes carried the del(GJB6-D13S1830) deletion (Table 1). Seven other probands who were not heterozygous for a *GJB2* variant carried the del(GJB6-D13S1830) deletion, one proband had the GJB6-D13S1830 deletion in addition to two pathogenic variants in the *GJB2* and one proband was homozygous only for the *GJB6* deletion. Among individuals with a *GJB2* mutation and the del(GJB6-D13S1830) deletion, 38 (71.7%) were from multiplex families and more than half had deaf parents. Although the overwhelming majority of them were Caucasians (79%), we also observed the *GJB6* deletion in probands who were Hispanic (4%), African American (2%), Filipino (2%), and East Indian (2%) (Table 2). In 69.81% (37/53) of the digenic compound heterozygotes the *GJB2* mutation was the common c.35delG allele, but the *GJB6* deletion was also observed in combination with p.V37I, p.V84M, p.S85Y, p.R143W, c.167delT, c.312del14, and the p.R184P mutant alleles.

Figure 1a–c illustrate a few families from the study cohort with several unusual features. In addition to a deaf Caucasian proband homozygous for the del(GJB6-D13S1830) deletion, we also identified a deaf Caucasian parent in one of our families with the homozygous *GJB6* deletion (Figure 1a- NSDF 27-202). Of note, both parents were deaf in this family, and all their children were deaf (noncomplementary mating). This raised the suspicion that a second deafness gene was segregating in the family. Figure 1a - NSDF 2024 illustrates a second family with a few deaf individuals that were initially identified

TABLE 2 Proband characteristics

	Cx30 del/wt				Gender		Family mating type		
Ethnicity	Cx26	mut/wt	mut/mut	wt/wt	M	F	DxD	DxH	HxH
Caucasian		43	1	6	21	29	25	2	23
Ashkenazi Jew		4	—	—	2	2	1	—	3
South Asian		2	—	—	1	1	—	1	1
African		1	—	—	1	—	—	—	1
Eastern European		1	—	—	1	—	1	—	—
Hispanic		1	—	1	—	2	1	—	1
Totals		52	1	7	26	34	28	3	29

Note: Characteristics of the 60 probands with the Cx30 deletion del(GJB6-D13S1830).

Mut: Pathogenic sequence variant.

DxD: Both parents of the proband have hearing loss.

DxH: Only one parent of the proband has hearing loss.

HxH: Neither parent of the proband has hearing loss.

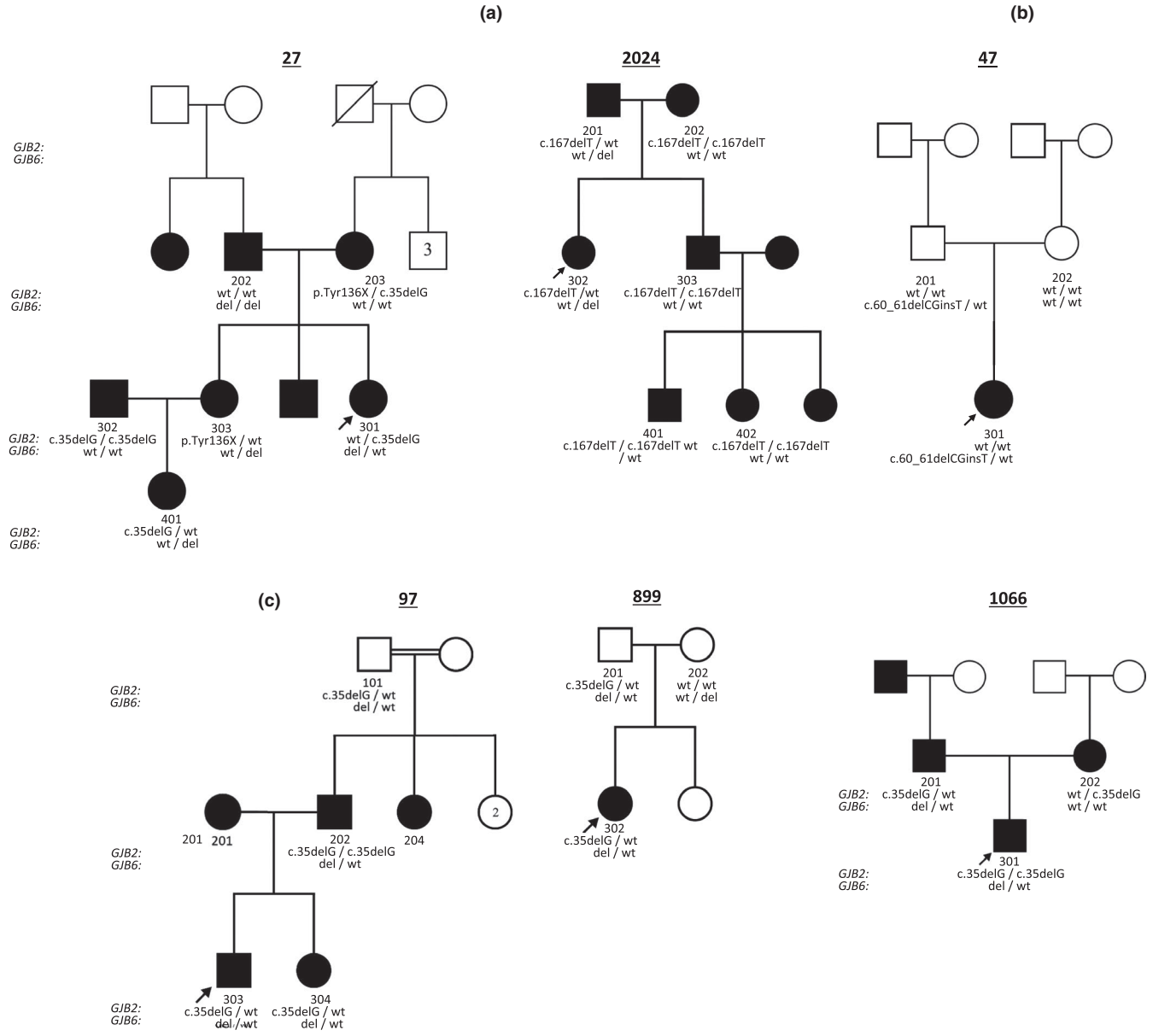

FIGURE 1 Representative Families from cohort with the digenic variants in both *GJB2* and *GJB6*. (a) Compound heterozygous state in simplex and multiplex families with HL. (b) Simplex family with a 2bp frameshift deletion without *GJB2* mutations that may not be the cause of HL. (c) Families requiring testing of additional members for result interpretation in proband. GJB6 del = del(GJB6-D13S1830)

as heterozygous carriers for the c.167delT variant in *GJB2* until we could test them for the *GJB6* deletion to determine the etiology for the HL.

In three probands (97-303, 899-302, 1066-301) who were heterozygous or homozygous for variants in both *GJB2* and *GJB6*, studying additional members in the family provided valuable information on the phase of these two variants (Figure 1c). Additional members tested including 97-101 and 899-201 had variants in *GJB2* and *GJB6* with normal hearing. In contrast, 1066-201 has HL but it appears that the *GJB2* and *GJB6* variants are in cis and his hearing loss may be from a different etiology. We hypothesize that 97-303 and 97-304 could either have inherited the 35delG variant from their father and the *GJB6* deletion from their mother (97-201 not available for testing), with DFNB1 as an etiology for HL or they inherited the 35delG and*GJB6* deletion in cis from their father and the deafness is due to an unrelated etiology yet to be determined. Individual 899-302 most likely inherited the DFNB1 changes in cis from his father that would not explain his HL.

A 5-year-old child with mild-to-moderate progressive hearing loss (Figure 1b) had negative deletion testing for *GJB6* but had a frameshift deletion involving a single guanine residue at position 61 resulting in a truncated protein with the introduction of a stop at codon 34. Her father carried the same deletion but had normal hearing by testing. The initial interpretation was to ascribe the moderate HL in the child to the frameshift mutation and postulate reduced penetrance in her hearing father.

However further testing by NGS for additional HL genes has identified a more plausible explanation for her phenotype that is being investigated as the etiology for her HL.

No proband was found to carry the shorter del(GJB6-D13S1854) deletion reported by del Castillo et al. (2005).

The average hearing thresholds in 38 probands with digenic pathogenic variants in the *GJB2* and *GJB6*, and six probands heterozygous for the *GJB6* deletion alone, are shown across various frequencies in Figure 2. The average degree of HL in all the probands is in the severe range with no statistically significant difference among those with a c.35delG variant as compared to those with a different *GJB2* pathogenic variant. The average thresholds in probands with digenic change are consistently in severe to profound range across most frequencies.

4 | DISCUSSION

In 2002, del Castillo et al. reported compelling evidence that deafness can result from the presence of a 342 kb (revised as 309 kb) deletion that included the coding region of *GJB6*, when present *in trans* with a pathogenic *GJB2* allele. The coinheritance of this deletion was shown to be the apparent cause of the HL in 67% of deaf *GJB2* heterozygotes in Spain (del Castillo et al., 2002), and was reported to be the second most frequent mutation apart from the c.35delG allele in patients with DFNB1 deafness (Marlin et al., 2005).

Our study of *GJB6* deletion represents one of the largest series of deaf probands studied to date in North America. Digenic compound heterozygotes for the del(GJB6-D13S1830) deletion explained HL in 16.35% of deaf *GJB2* heterozygotes, which is lower compared to reports from other Western European countries (Cama et al., 2009; Gualandi et al., 2004; Feldmann et al., 2004; Santos et al., 2005). This difference is likely due to the smaller cohort size of deaf individuals studied in comparison to the present study. The del(GJB6-D13S1830) deletion occurred most commonly with the c.35delG mutation in the *GJB2*, although it is reported in association with other *GJB2* pathogenic alleles.

The probands with digenic changes in the present study were often from multiplex families, many times with both parents having HL, which is in contrast to the other large studies (Feldmann et al., 2004; Marlin et al., 2005). In our sample, 71.6% of the digenic probands were from a multiplex family and a unique finding was that 65.7% of these probands had both deaf parents.

In the United States, 80%–90% of individuals with profound prelingual deafness who communicate by sign language select similarly affected marriage partners (Nance, 2003). This linguistic homogamy has created a substantially separate mating pool in which, the frequency of genes for deafness may be amplified. Nance and Kearsey (2004) showed that when combined with relaxed selection among the deaf, this mechanism will preferentially amplify mutations at the commonest locus for recessive deafness and could have doubled the frequency of connexin deafness in this country during the past two centuries.

Families in which both parents are deaf also pose a challenge with interpretation of molecular test results in deaf probands. When the results for the proband are interpreted out of context to the parental genotype (Figure 1c) it was misleading to assume the digenic variant as the etiology for HL. These families have clearly highlighted that the two variants when present in cis tend to be of little clinical significance. The corollary is that

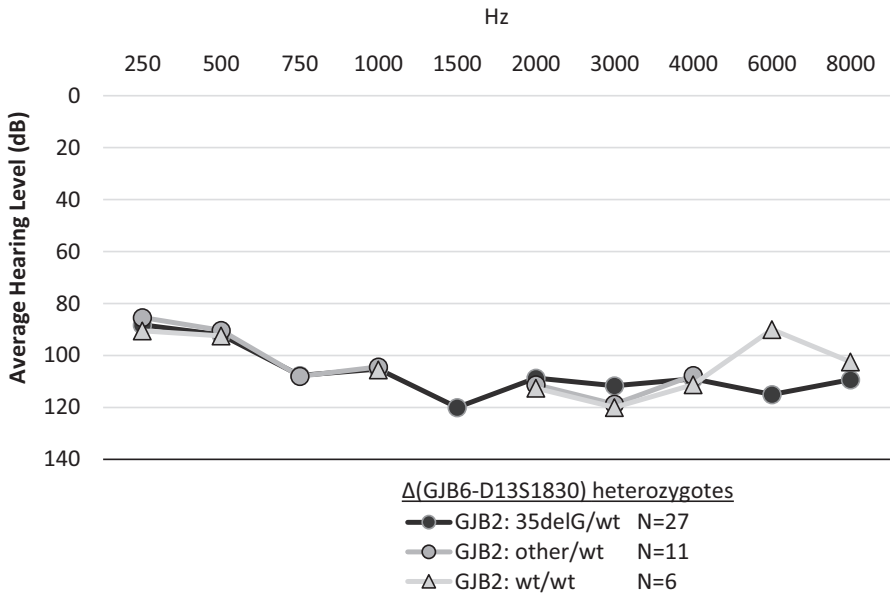

FIGURE 2 Average hearing threshold in deaf probands with del(GJB6-D13S1830) deletion in trans with GJB2 variant, or GJB6 deletion by itself

TABLE 3 Review of del(GJB6-D13S1830) frequency reported in literature

Country	Subjects (No.)	Total no. of subjects with a *GJB6* deletion	Total no. of GJB2 heterozygotes tested for a *GJB6* deletion	Freq. of *GJB6* deletion		Study
				Freq. of *GJB6* deletions among *GJB2* heterozygotes	In all deaf probands	
Czechia	13	1	13	8%	8%	Seeman et al. (2005)
Belgium	15	7	9	78%	47%	Stinckens et al. (2004)[a]
Germany	25	2	4	50%	8%	Bolz et al. (2004)
Russia	30	2	2	7%	0%	Bliznets et al. (2012)
Switzerland	32	1	8	13%	3%	Gürtler et al. (2008)
Brazil	33	3	6	50%	9%	Piatto et al. (2004)
Russia	35	1	1	3%	3%	Pshennikova et al. (2017)
Spain	38	1	7	14%	3%	Gallo-Teran et al. (2005)
Venezuela	40	1	9	11%	3%	Utrera et al. (2007)
Syria	41	1	2	0%	2%	Zaidieh et al. (2015)
Argentina	46	4	17	18%	9%	Dalamón et al. (2005)
Hungary	47	2	2	4%	4%	Tóth et al. (2007)
Italy	59	2	9	22%	3%	Gualandi et al. (2004)
U.S	68	2	27	7%	3%	Erbe et al. (2004)
North America	95	2	9	22%	2%	Schimmenti et al. (2008)
U.S	108	2	30	7%	2%	Wu et al. (2003)
Denmark	165	2	9	22%	1%	Grønskov et al. (2004)
Belarus	213	3	3	1%	1%	Danilenko et al. (2012)
Netherlands	222	6	14	29%	3%	Santos et al. (2005)
France	255	16	29	52%	6%	Feldmann et al. (2004)
France	256	25	NR	NR	-	Marlin et al. (2005)[b]
Brazil	300	3	31	10%	1%	Batissoco et al. (2009)
Italy	376	6	30	20%	2%	Cama et al. (2009)
Spain	422	45	33	67%	5%	del Castillo et al. (2002)
Argentina	476	5	9	1%	2%	Dalamón et al. (2013)
Brazil	600	2	46	4%	0%	da Silva-Costa et al. (2011)
North America	888	9	NA	NA	1%	Putcha et al. (2007)
North America	2,376	61	318	22%	3%	Current study

Note: Literature review spanning 2003—present, reporting subjects with a del(GJB6-D13S1830) presented by ascending cohort size

Abbreviation: NR, not reported.

[a]Single family.

[b]Reported on the basis of the presence of compound heterozygous mutations in GJB2, which includes the association of one GJB2 mutation with a GJB6 deletion.

the identification of *GJB2/GJB6* variants in a deaf individual should not be assumed to be in *trans* or the cause of their HL.

Overall, the del(GJB6-D13S1830) deletion allele was present in 2.1% of all deaf probands, which is similar to the frequency reported by several other studies (Table 3). There also appears to be notable differences in the frequency of the del(GJB6-D13S1830) deletion, as depicted in studies in different populations. Although the frequency of the del(GJB6-D13S1830) deletion in Brazil, France, Czechia, Russia, and Argentina is high (Bliznets et al., 2012; Dalamón et al., 2005; Marlin et al., 2005; Piatto, Bertollo, Sartorato, & Maniglia, 2004; Seeman et al., 2005), it is rare or infrequently observed in Austria and Morocco and Syria (Abidi et al., 2007; Frei et al., 2004; Gazzaz et al., 2005; Zaidieh, Habbal, & Monem, 2015). In contrast, the *GJB6* deletion was not identified in many regions including India, Taiwan, Cameroon, Sicily, Mexico, Iran, Macedonia, and Turkey (Adhikary et al., 2015; Amorini et al., 2015; Bhalla, Sharma, Khandelwal, Panda, & Khular, 2009, 2011; Bosch et al., 2014; Esmaeili, Bonyandi, & Nejadkazem, 2007; Hernandez-Juarez et al., 2014; Naddafnia, Noormohammadi, Irani, & Salahshoorifar, 2019; Sirmaci, Akcayoz-Duman, & Tekin, 2006; Sukarova

Stefanovska, Cakar, Filipce, & Plaseska Karanfilska, 2012; Wonkam et al., 2015; Yang et al., 2007).

Most studies from the United States, including our report, have identified deaf individuals with the *GJB6* deletion in trans in heterozygous carriers for the *GJB2* variant. The frequency of the *GJB6* deletion in the general population is not well characterized. Fitzgerald et al. (2004) did not identify the *GJB6* deletion in nearly 2,000 blood spots from a diverse ethnic group of newborns presumably with normal hearing, screened in New York State. These data correlate with the reported allele frequency of 0.0001863 in the gnomAD database. It also explains the rare occurrence of individuals carrying the deletion in a homozygous state, as the expected frequency would be ~1 in 28,500,000. In some individuals, the *GJB6* deletion would be in *cis* with a *GJB2* variant.

The mechanism by which the del(GJB6-D13S1830) deletion causes HL, when present in trans with a *GJB2* mutation, remains uncertain and could result from an alteration of the expression of the normal *GJB2* located in cis to the deletion (del Castillo et al., 2005, 2003), or from haplo-insufficiency of the *GJB6* product or both. The expression of *GJB2* was shown to be affected by the del(GJB6-D13S1830) deletion in a cell specific manner in sweat glands by immuno-histochemical staining, suggesting the disruption of *GJB2 cis*-regulatory elements located within the deletion that control expression of Cx26 in cells (Common et al., 2005). Wilch et al. (2006) have also reported an interesting family with HL segregating a novel DFNB1 allele characterized by significant reduction in expression of both *GJB2* and *GJB6*, suggesting the presence of as-yet unidentified *cis*-regulatory element. Other studies demonstrating abolished *GJB2* expression due to an in cis *GJB6* deletion (Rodriguez-Paris & Schrijver, 2009) and the finding of normal hearing prior to postnatal day 30 in a *Cx30* knock-out mouse model ($Cx30^{\Delta/\Delta}$) (Boulay et al., 2013) favors the mechanistic hypothesis that the del(GJB6-D13S1830) eliminates a putative cis-regulatory element in the deleted region. At present, based on our results of the frameshift variant in *GJB6* not being the etiology of HL, caution needs to be exercised with results obtained by targeted sequencing of *GJB6* or whole exome sequencing in individuals with HL.

The more severe audiologic findings in digenic probands is difficult to reconcile with the sole theory of a putative cis-regulatory element in the deleted region, and it could suggest that both the *GJB6* and *GJB2* product may make a contribution to the HL. The degree of HL in individuals with a pathogenic *GJB2* sequence variant and a *GJB6* deletion is more profound than those with bi-allelic change in *GJB2* as previously observed (Pandya et al., 2003), and as reported in other studies (Marlin et al., 2005; Santos et al., 2005; Snoeckx et al., 2005). Homozygous deletion of *GJB6*, although rare, was found in one proband and another relative of a proband in our study which results in severe to profound HL, similar to the mouse model (Chen et al., 2014; Marziano, Casalotti, Portelli, Becker, & Forge, 2003; Mei et al., 2017). Unlike in humans, the *GJB2/GJB6* double heterozygous mice also show decreased endocochlear potential but have moderate HL (Mei et al., 2017; Teubner et al., 2003).

Whether individuals with the *GJB2* and *GJB6* changes in cis could have a milder HL remains to be determined, as we have identified family members (Figure 1c) with this molecular complement with both normal hearing and HL.

In conclusion, we report the del(GJB6-D13S1830) deletion in 16.35% of all *GJB2* heterozygotes as a cause for their HL. The overall frequency of the digenic mutations was 2.1% among the 2,376 deaf probands studied. There was one proband and a relative of another proband with the homozygous del(GJB6-D13S1830) deletion not reported previously. The audiologic profile of digenic probands revealed a profound HL. This body of evidence therefore suggests that the HL in digenic heterozygotes may result at least in part from a deficiency of the *GJB6* product in addition to any effect the deletion has on the regulation of the adjacent normal *GJB2* locus.

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CONFLICT OF INTEREST

Arti Pandya, Alexander O'Brien, Michael Kovasala, Guney Bademci, Mustafa Tekin, and Kathleen S. Arnos have nothing to declare.

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