article

A multicenter study of the frequency and distribution of *GJB2* and *GJB6* mutations in a large North American cohort

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Purpose: The aim of the study was to determine the actual *GJB2* and *GJB6* mutation frequencies in North America after several years of generalized testing for autosomal recessive nonsyndromic sensorineural hearing loss to help guide diagnostic testing algorithms, especially in light of molecular diagnostic follow-up to universal newborn hearing screening. **Methods:** Mutation types, frequencies, ethnic distributions, and genotype-phenotype correlations for *GJB2* and *GJB6* were assessed in a very large North American cohort. **Results:** *GJB2* variants were identified in 1796 (24.3%) of the 7401 individuals examined, with 399 (5.4%) homozygous and 429 (5.8%) compound heterozygous. *GJB6* deletion testing was performed in 12.0% (888/7401) of all cases. The >300-kb deletion was identified in only nine individuals (1.0%), all of whom were compound heterozygous for mutations in *GJB2* and *GJB6*. Among a total of 139 *GJB2* variants identified, 53 (38.1%) were previously unreported, presumably representing novel pathogenic or benign variants. **Conclusions:** The frequency and distribution of sequence changes in *GJB2* and *GJB6* in North America differ from those previously reported, suggesting a considerable role for loci other than *GJB2* and *GJB6* in the etiology of autosomal recessive nonsyndromic sensorineural hearing loss, with minimal prevalence of the *GJB6* deletion. ***Genet Med* 2007:9(7):413–426.**

Key Words: *hearing loss, deafness, connexin, GJB2, GJB6*

Hearing loss is one of the most common inherited disorders present at birth and is the most common congenital sensory impairment. In developed countries, approximately 1 in 1000 children is born deaf, and 1 in 300 has hearing loss significant enough to affect development.[1] Moreover, an additional 1 in 1000 children develops severe to profound hearing loss in the first two decades of life.[2,3] At least 50% of prelingual hearing loss is thought to be genetic in etiology, and approximately 70% of this genetic hearing loss is nonsyndromic, whereas the remaining 30% is associated with one of the >400 clinical syndromes (e.g., Pendred and Usher) in which hearing loss is a component. Finally, although nonsyndromic sensorineural hearing loss (NSNHL) is associated with a variety of inheritance patterns (e.g., autosomal dominant [AD] in 15–20%, X-linked in 2–3%, and mitochondrial in 1%), approximately 80% of NSNHL exhibits autosomal recessive (AR) inheritance.

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To date, 67 different loci and 23 different genes have been reported to be causally associated with AR-NSNHL (http://webhost.ua.ac.be/hhh/). Despite this significant heterogeneity, up to 50% of AR-NSNHL is associated with mutations in the locus DFNB1 (MIM 220290) on chromosome 13q12, which contains the *GJB2* gene (MIM 121011) encoding connexin 26 (Cx26) and the *GJB6* gene (MIM 604418), which encodes connexin 30 (Cx30).[4] Cx26 and Cx30 belong to a family of transmembrane proteins, which, as homo- and heteromeric hexamers, form connexons. Docking between two connexons on adjacent cells forms intercellular gap junctions,[5] which are permeable to ions and small metabolites $\leq$1.2 kDa.[6] In the cochlea, Cx26- and Cx30-containing gap junctions are proposed to maintain K^+ homeostasis,[7] thereby contributing to the efficient generation of action potentials in response to sound. Among individuals with DFNB1-associated AR-NSNHL, 98% is estimated to carry two identifiable mutations in *GJB2*, whereas 2% are reported to have mutations in both *GJB2* and *GJB6* (Genetests DFNB1, http://www.genetests.org).

The spectrum of sequence variants in *GJB2* varies significantly with ethnicity. For example, 35delG is common among white individuals (carrier rate of 2–4%),[8–10] 235delC in the Japanese (carrier rate of 1–2%),[11,12] 167delT in the Ashkenazi Jewish population (carrier rate of 4.0%),[13] and V37I in Taiwanese (carrier rate of 11.6%).[14] Nonetheless, the cumulative frequency of all *GJB2* mutations is sufficiently high in most populations to warrant clinical testing, as evidenced by its inclusion in clinical practice guidelines.[15] Perhaps more controversial, however, is the role of clinical testing for *GJB6* mutations in *GJB2* heterozygotes. Although preliminary studies reported that the del(*GJB6-D13S1830*) mutation explained hearing loss in as many as 30% to 70% of affected *GJB2* heterozygotes,[16] subsequent studies in other populations clearly suggest that other mutations, both within DFNB1 and elsewhere, contribute significantly to AR-NSNHL.[17] To examine actual mutation frequencies in North America after several years of generalized diagnostic and research testing and to help guide diagnostic testing algorithms, especially in light of molecular diagnostic follow-up to universal newborn screening, we conducted a large collaborative study with participation from laboratories in the United States and Canada. This study includes only probands with hearing loss tested in the consortium laboratories and provides a realistic overview of clinical practice and molecular diagnostic testing results in North America.

MATERIALS AND METHODS

Study description

Questionnaires were distributed to 16 facilities participating in the North American Connexin Study Consortium in the United States and Canada. Responding facilities included Children's Hospital of Eastern Ontario, University of Alberta, Case Western Reserve University, Emory University, University of Chicago, University of North Carolina Hospitals, University of Utah School of Medicine, Sacred Heart Medical Center, Columbus Children's Hospital, Cincinnati Children's Hospital, Stanford University Medical Center, Harvard-Partners Center for Genetics & Genomics, Children's Hospital Boston, University of Colorado School of Medicine, Chapman Institute (St. Francis), and Athena Diagnostics. Results from DNA analysis (by polymerase chain reaction [PCR] and/or sequencing) of *GJB2* and *GJB6* were collected from these facilities. In total, 7401 individuals with hearing loss were evaluated for *GJB2* mutations. Among these, 888 (12.0%) were also evaluated for *GJB6* mutations (Fig. 1). Although all 16 laboratories performed comprehensive sequencing of *GJB2* exon 2, four also sequenced the noncoding exon 1 in a total of 2256 individuals. In addition, 11 of 16 laboratories performed *GJB6* testing, in each case testing for the large deletion (*GJB6-D13S1830*) as previously described,[18,19] with one laboratory also sequencing the single coding exon in 112 individuals. Testing for the recently identified 232-kb deletion, del(*GJB6-D13S1854*),[20] was not performed on any patients in this cohort before collection of the study data. Moreover, information about phase was not explicitly provided for any subject in this study. Therefore, we assumed that individuals carrying more than one *GJB2* variant

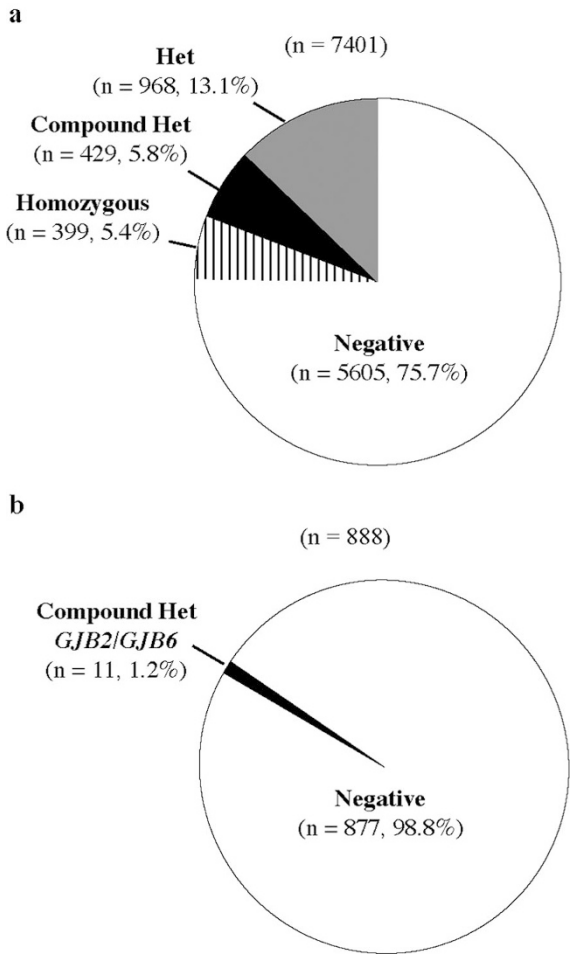

Fig. 1. *GJB2* and *GJB6* testing results. (a) *GJB2* results. Het refers to heterozygous individuals with only a single *GJB2* variant identified. (b) *GJB6* results. Individuals with any *GJB6* variant were counted. Nine had the *GJB6-D13S1830* deletion, and two had novel variants of unknown relation to hearing loss. All individuals with *GJB6* variants were also compound heterozygous for *GJB2* variants .

were biallelic. In addition to molecular genetic testing results, ethnic and phenotypic information was obtained when available to the participating laboratories. All samples were made anonymous before the data were sent to the Stanford investigators to preclude the ability to retrospectively link a given genotype and/or phenotype to a particular individual. Institutional approval for this study was obtained from Stanford University.

Statistical analysis

Fisher exact tests were performed to determine the statistical significance of differences in prevalence rates between the current and previous studies. Cochran-Mantel-Haenszel tests were performed to evaluate the differences among mutation types (i.e., 35delG versus non-35delG and truncating [T] versus nontruncating [NT]) with respect to the severity of hearing loss (Figs. 2 and 3). Post hoc analysis was performed using the Wilcoxon rank sum test with a Bonferroni correction.

RESULTS

Demographic and phenotypic information about the study sample is provided in Table 1. In addition to DNA sequencing of the coding exon 2, which was performed by all participating laboratories, four sequenced exon 1 as well. One laboratory, which contributed 112 cases, only reported previously characterized mutations, but not known benign variants. *GJB2* variants were identified in 1796 of the 7401 individuals examined (24.3%). Biallelic *GJB2* variants were identified in 828 (11.2%) of the 7401 cases, with 399 of these homozygous (48.2%) and 429 compound heterozygous (51.8%) (Fig. 1, a). Among the 828 individuals with biallelic variants, phenotypic information was provided for 215 (26.0%). Seventy-nine of these were homozygous for the 35delG mutation, another 79 (36.7%) were compound heterozygous for 35delG and a second mutation, and 57 (26.5%) had two changes other than 35delG, with severity nonrandomly distributed among these three genotypic classes (Fig. 2; $P = 0.0069$, Cochran-Mantel-Haenszel test). In 2003, Lim et al.[21] reported that the presence of biallelic nonsense mutations correlated with severe to profound hearing loss in the vast majority of cases. Recently, in their study of genotype-phenotype correlations in *GJB2*-associated hearing loss, Snoeckx et al.[22] classified *GJB2* variants as either T or NT, concluding that biallelic T mutations were associated with more severe hearing loss. Similar classification in our cohort demonstrated that 59.1% were T/T, 23.7% were T/NT, and 17.2% were NT/NT (vs. 36.7%, 43.8%, and 19.6%, respectively, in the Snoeckx et al. report). Consistent with those studies,[21,22] the severity of hearing loss was nonrandomly distributed among these

Variants	Number of Cases with Known Phenotype	Mild (20–39 dB)	Moderate (40–69 dB)	Severe (70–89 dB)	Profound (>90 dB)	Unclassifiable
35delG/35delG	79 (36.7%)	1 (1.3%)	20 (25.3%)	21 (26.6%)	37 (46.8%)	0
35delG/other	79 (36.7%)	17 (21.5%)	17 (21.5%)	9 (11.4%)	33 (41.8%)	3 (3.8%)
2 non-35delG variants	57 (26.5%)	20 (35.1%)	11 (19.3%)	4 (7.0%)	22 (38.6%)	0
Total	215 (100%)	38 (17.7%)	48 (22.3%)	34 (15.8%)	92 (42.8%)	3 (1.4%)

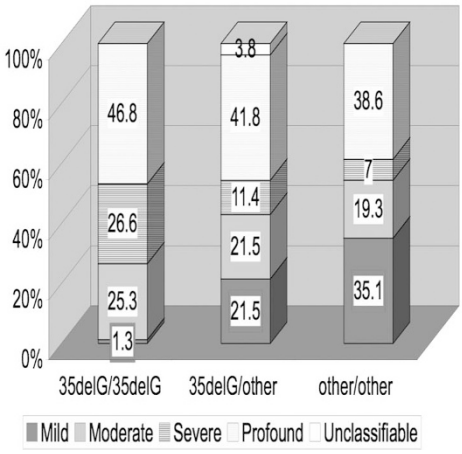

Fig. 2. Phenotypes of biallelic *GJB2* genotypes, based on presence or absence of 35delG. Individuals were classified according to their reported genotypes (35delG homozygous, 35delG compound heterozygous with a second *GJB2* variant, and compound heterozygous with two non-35delG *GJB2* variants) and their reported phenotype. Unclassifiable refers to individuals for whom phenotypic information was available but was difficult to categorize. If hearing loss was reported as consecutive ranges (such as mild to moderate), the more severe phenotype was reported. The severity of hearing loss was nonrandomly distributed among the three genotype classes ($P = 0.0069$, Cochran-Mantel-Haenszel test).

Mutations	Number of Cases with Known Phenotype	Mild (20–39 dB)	Moderate (40–69 dB)	Severe (70–89 dB)	Profound (>90 dB)	Unclassifiable
Truncating (T)/ Truncating (T)	127 (59.1%)	3 (2.4%)	30 (23.6%)	29 (22.8%)	63 (49.6%)	2 (1.6%)
Truncating (T)/ Non-truncating (NT)	51 (23.7%)	18 (35.3%)	12 (23.5%)	1 (2.0%)	20 (39.2%)	0
Non-truncating (NT)/ Non-truncating (NT)	37 (17.2%)	17 (45.9%)	6 (16.2%)	4 (10.8%)	9 (24.3%)	1 (2.7%)
Total	215 (100%)	38 (17.7%)	48 (22.3%)	34 (15.8%)	92 (42.8%)	3 (1.4%)

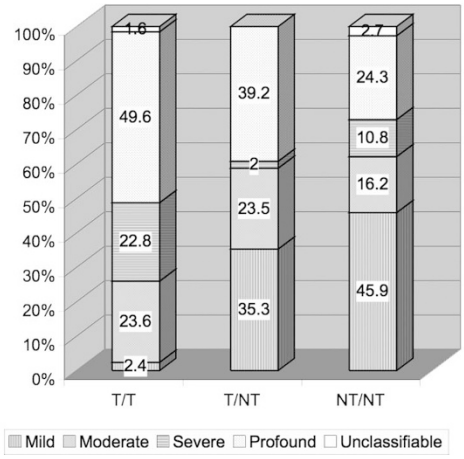

Fig. 3. Phenotypes of biallelic *GJB2* genotypes. Individuals were classified according to their reported mutation type (two T variants, one T, and one NT variant, or two NT variants) and their reported phenotypes. Unclassifiable refers individuals for whom phenotypic information was available but was difficult to categorize. If hearing loss was reported as consecutive ranges (such as mild to moderate), the more severe phenotype was reported. The degree of hearing loss was nonrandomly distributed among the three genotype classes ($P < 0.0001$, Cochran-Mantel-Haenszel test).

three genotype classes (Fig. 3; $P < 0.0001$, Cochran-Mantel-Haenszel test), with hearing impairment being more severe in the biallelic T/T class than in the T/NT class, which in turn was more severe than in the NT/NT class.

GJB6 deletion testing was performed in 12.0% (888/7419) of all cases. Of the 888 subjects tested for *GJB6*, 319 (35.9%) were *GJB2* heterozygotes, 115 (13.0%) were *GJB2* compound heterozygotes, and 105 (12.0%) were *GJB2* homozygotes. No *GJB2* variant was identified in 349 (39.3%) of subjects tested for del(*GJB6-D13S1830*). The >300-kb deletion was identified in only nine individuals (1.0%), all of whom were compound heterozygous for mutations in *GJB2* and *GJB6* (Fig. 1, b and Table 2). Two novel variants in the *GJB6* coding region (689insA and 631T>G) of unclear clinical significance were each reported in one individual. Although the severity of hearing loss among *GJB2-GJB6* double heterozygotes ranged from mild to profound, the two cases of del(*GJB6-D13S1830*) heterozygosity combined with a T *GJB2* mutation (frameshift 35delG or W77X) were associated with severe and profound hearing loss, respectively. Unfortunately, the small sample size severely limits the possibility of meaningful genotype-phenotype correlations for *GJB6* mutations.

Of the 139 *GJB2* variants identified (Table 3), 61 (43.9%) were AR mutations, 5 (3.6%) were AD, 9 (6.5%) represented likely or known benign variants, 11 (7.9%) were reported to be variants with unclear relation to phenotype, and 53 (38.1%) were previously unreported, presumably representing novel mutations or benign variants based on comparison with mutations listed at the Connexin Deafness Homepage (http://davinci.crg.es/deafness) and the Human Gene Mutation Database (www.hgmd.org), in conjunction with PubMed searches (www.pubmed.gov). Overall, the most common mutations across all ethnic groups were 35delG, 101T>C (M34T), 109G>A (V37I), and 167delT, which represented 42.8%, 10.9%, 7.8%, and 3.6% of *GJB2* allelic variants, respectively (Table 3). The most common benign variant across all ethnic groups was and 79G>A (V27I, 6.3%). However, as previously reported, the frequency of allelic variants varies significantly with ethnicity (Tables 4 and 5). For example, although 35delG was highly prevalent in white and Hispanic individuals, this variant was not even among the "top 10" in Asians, for whom V37I (109G>A), V27I (79G>A), and E114G (341A>G) predominate. Unfortunately, a relative lack of nonwhite individuals in the study sample preclude stronger conclusions regarding allelic frequencies for these ethnicities. Previously reported dominant mutations were identified in 11 heterozygous individuals (0.1%): four with R75Q (224G>A), three with R184Q (551G>A), two with R75W (223C>T), one with R143Q

Table 1
Demographics of subject population[a]

	GJB2	GJB6
Probands tested	7401	888
Gender		
Known	778 (10.5%)	221 (24.9%)
Unknown	6623 (89.5%)	667 (75.1%)
Male	386 (49.6%)	96 (43.4%)
Female	392 (50.4%)	125 (56.6%)
Ethnicity		
Known	1313 (17.7%)	155 (17.5%)
Unknown	6088 (82.3%)	733 (82.5%)
White	1033 (78.7%)	98 (63.2%)
African	92 (7.0%)	5 (3.2%)
Hispanic	57 (4.3%)	22 (14.2%)
Asian	65 (5.0%)	17 (11.0%)
Native American	5 (0.4%)	3 (1.9%)
Ashkenazi Jew	3 (0.2%)	1 (0.6%)
Arab	7 (0.5%)	3 (1.9%)
Mixed	49 (3.7%)	6 (3.9%)
Other	4 (0.3%)	0
Level of hearing loss[b]		
Known	422 (5.7%)	189 (21.3%)
Unknown	6979 (94.3%)	699 (78.7%)
Mild	73 (17.3%)	46 (24.3%)
Moderate	102 (24.2%)	42 (22.2%)
Severe	83 (19.7%)	20 (10.6%)
Profound	141 (33.4%)	71 (37.6%)
Unilateral	6 (1.4%)	2 (1.1%)
Unclassifiable[c]	17 (4.0%)	8 (4.2%)

[a]All percentages are relative to the total number of subjects with a known result for each demographic category (e.g., 778 for the gender of subjects tested for GJB2).
[b]Phenotypic information based on reported audiometric testing from the submitting facility was classified as mild (20–39 dB), moderate (40–69 dB), severe (70–89 dB), or profound (>90 dB).
[c]Refers to individuals for whom phenotypic information was available but difficult to classify for various reasons, such as different hearing loss in each ear and range of hearing loss reported as normal to profound.

Table 2
GJB2-GJB6 compound heterozygotes

GJB2 variant(s)	*GJB6* variant(s)	Phenotype	Ethnicity
35delG	GJB6-D13S1830	Severe	White
35delG	689insA	Moderate	White
79G>A/341A>G (V27I/E114G)	GJB6-D13S1830	Mild	White
231G>A (W77X)	GJB6-D13S1830	Profound	White
1A>G (M1V)	GJB6-D13S1830	Profound	White
110T>C (V37A)	631T>G (C211G)	Moderate	Canadian aboriginal
312del14	GJB6-D13S1830	Unknown	White
312del14	GJB6-D13S1830	Unknown	Unknown
35delG	GJB6-D13S1830	Unknown	Unknown
139G>T (E47K)	GJB6-D13S1830	Unknown	Unknown
167delT	GJB6-D13S1830	Unknown	Unknown

(428G>A), and one with D50N (148G>A). Concurrent V27I/E114G (79G>A and 341A>G) substitutions, which may have a pathogenic effect when present together in *cis*,[17] were observed in 39 cases (0.5%) with variable degrees of hearing loss. In five of these, a third mutation in *GJB2* was reported. (Note that for this study, 79G>A (V27I) and 341A>G (E114G), when present together regardless of their *cis/trans* relation, were considered a disease-causing mutation, but when present separately, each variant was considered a benign variant.) Of the 828 subjects with biallelic variants, 46 (5.6%) carried at least one variant that was classified as benign, novel, or of unclear clinical significance. Furthermore, the breakdown among the 968 heterozygotes seen in this study according to the classification of the variants shown in Table 3 was 0.9% AD, 74.8% AR, 17.0% benign variant, 3.1% with unknown relationship to disease, and 4.1% novel (data not shown). Finally, unilateral hearing loss was reported in six (0.08%) instances. In three such cases, no *GJB2* variant was detected, and *GJB6* was not tested. In two instances, the individuals were heterozygous for 35delG and del(*GJB6-D13S1830*) was not detected. In the final case, the study participant, whose *GJB6* status was unknown, was heterozygous for V27I (79G>A).

Among the 53 putatively novel variants (Tables 3, 6, and 7), 40 are nucleotide substitutions (with 36 missense mutations and four synonymous mutations), 7 are frameshift mutations, 4 occur upstream of the transcription initiation site and therefore are of unclear significance, 1 is a nonsense mutation, and 1 is an in-frame deletion. Among the 36 nonsynonymous variants (Tables 6 and 7), 33 occur at residues conserved across the species examined (chimpanzee, mouse, rat, oxen, sheep, and guinea pigs). No obvious ethnic bias was identified among these variants.

DISCUSSION

We present a large study of the frequency and distribution of sequence variations in *GJB2* and *GJB6* in various ethnic groups in the largest North American study of hearing impaired probands reported to date. The principal weakness of this study was the limited availability of information related to demographics, phenotype, and family history, especially for patients who lacked *GJB2* and/or *GJB6* variants by current molecular testing methods. More detailed information was uniformly available for patients with *GJB2* and/or *GJB6* variants. Another obvious limitation is lack of *GJB6* results in all patients, but espe-

Table 3
Frequency of *GJB2* alleles[a]

Classification	Nucleotide change	Protein change (if applicable)	Total	Percentage of all Cx26 mutations
Autosomal dominant	224G>A	*R75Q*	4	0.17
	551G>A	*R184Q*	3	0.13
	223C>T	*R75W*	2	0.09
	428G>A	*R143Q*	1	0.04
	148G>A	D50N	1	0.04
Autosomal recessive	35delG	Frameshift	1001	42.76
	101T>C[b]	*M34T*[d]	256	10.94
	109G>A	*V37I*	183	7.82
	167delT	Frameshift	83	3.55
	269T>C	*L90P*	46	1.96
	79G>A/341A>G	V27I/E114G	39	1.67
	235delC	Frameshift	36	1.54
	380G>A	R127H	36	1.54
	71G>A	W24X	33	1.41
	312_325del	Frameshift	28	1.20
	139G>T	E47X	22	0.94
	416G>A	S139N	17	0.73
	427C>T	*R143W*	16	0.68
	250G>C	*V84L*	12	0.51
	44A>C	K15T	12	0.51
	169C>T	Q57X	10	0.43
	1A>G	M1V	10	0.43
	−3172G>A		10	0.43
	35G>T	*G12V*	10	0.43
	283G>A	V95M	9	0.38
	365A>T	K122I	9	0.38
	617A>G	N206S	9	0.38
	358_360delGAG	delE120	8	0.34
	94C>T	*R32C*	8	0.34
	551G>C	*R184P*	7	0.30
	298C>T	H100Y	6	0.26
	269insT	Frameshift	6	0.26
	596C>T	S199F	6	0.26
	229T>C	*W77R*	5	0.21
	334_335del	Frameshift	5	0.21
	439G>A	E147X	5	0.21
	632_633del	Frameshift	5	0.21
	645_648del	Frameshift	4	0.18
	231G>A	*W77X*	4	0.17
	132G>A	W44X	3	0.13
	299_300del	Frameshift	3	0.13

(*Continued*)

Table 3
Continued

Classification	Nucleotide change	Protein change (if applicable)	Total	Percentage of all Cx26 mutations
	493C>T	R165W	3	0.13
	176_191del	Frameshift	2	0.09
	23C>T	T8M	2	0.09
	246C>G	I82M	2	0.09
	268C>G	*L90V*	2	0.09
	314_327del14[c]	Frameshift	2	0.09
	370C>T	Q124X	2	0.09
	51_62del12insA	Frameshift	2	0.09
	56G>C	S19T	2	0.09
	95G>A	*R32H*	2	0.09
	134G>A	G45E	1	0.04
	195C>G	Y65X	1	0.04
	238C>T	*Q80X*	1	0.04
	239A>C	*Q80P*	1	0.04
	279G>A	*M93I*	1	0.04
	290insA	Frameshift	1	0.04
	310_323del[c]	Frameshift	1	0.04
	31_68del	Frameshift	1	0.04
	35insG	Frameshift	1	0.04
	408C>A	Y136X	1	0.04
	516G>A	W172X	1	0.04
	550C>T	*R184W*	1	0.04
	592_600del9ins17	Frameshift	1	0.04
Benign variant	79G>A	V27I	147	6.28
	457G>A	V153I	34	1.45
	249C>G	F83L	15	0.64
	608T>C	I203T	11	0.47
	478G>A	G160S	7	0.30
	−15C>T		5	0.21
	341A>G	E114G	2	0.09
	682C>T	(3'UTR)	2	0.09
	468C>A	V156V	2	0.09
Unknown relationship to disease	−34C>T		11	0.47
	34G>T	*G12C*	4	0.17
	11G>A	G4D	3	0.13
	40A>G	N14D	2	0.09
	385G>A	E129K	2	0.09
	511G>A	A171T	2	0.09
	−12C>T		2	0.09
	218A>G	H73R	1	0.04
	368C>A	T123N	1	0.04

(*Continued*)

Table 3
Continued

Classification	Nucleotide change	Protein change (if applicable)	Total	Percentage of all Cx26 mutations
	−6T>A		1	0.04
	670A>C	K224Q	1	0.04
Novel	503A>G	K168R	7	0.30
	109G>C	*V37L*	5	0.21
	314A>G	K105R	4	0.17
	−3287C>G		3	0.13
	355G>A	E119K	3	0.13
	107T>C	L36P	2	0.09
	139G>C	E47Q	2	0.09
	209C>T	P70L	2	0.09
	233delC	Frameshift	2	0.09
	250G>T	*V84L*	2	0.09
	428G>T	*R143L*	2	0.09
	563A>G	K188R	2	0.09
	−6T>C		2	0.09
	488T>C	M163T	2	0.09
	101T>G	*M34R*	1	0.04
	104T>G	I35S	1	0.04
	110T>C	*V37A*	1	0.04
	11delG	Frameshift	1	0.04
	161A>G	N54S	1	0.04
	167T>C	L56P	1	0.04
	186C>T	N26N	1	0.04
	187G>A	V63M	1	0.04
	200A>G	H67R	1	0.04
	227T>C	L76P	1	0.04
	232insG	Frameshift	1	0.04
	241C>G	L81V	1	0.04
	264G>C	A88A	1	0.04
	278T>C	*M93T*	1	0.04
	−3187C>T		1	0.04
	358G>A	E120K	1	0.04
	389G>A	G130D	1	0.04
	39G>T	*V13V*	1	0.04
	401delG	Frameshift	1	0.04
	433_435del	delI145	1	0.04
	434_435del	Frameshift	1	0.04
	444C>G	A148A	1	0.04
	456C>A	V152X	1	0.04
	458_475dup	Frameshift	1	0.04
	557C>A	T186K	1	0.04

(*Continued*)

Table 3
Continued

Classification	Nucleotide change	Protein change (if applicable)	Total	Percentage of all Cx26 mutations
	576delA	Frameshift	1	0.04
	60T>G	I20M	1	0.04
	653G>A	C218Y	1	0.04
	677T>G	V226G	1	0.04
	91T>A	F31I	1	0.04
	175G>C	G59R	1	0.04
	475G>A	D159N	1	0.04
	−24A>C		1	0.04
	191G>A	C64Y	1	0.04
	473A>G	Y158C	1	0.04
	37G>A	*V13M*	1	0.04
	650A>G	Y217C	1	0.04
	499G>A	V167M	1	0.04
	17T>C	L6P	1	0.04
Total			2341	100.00

[a]Five AD, 61 AR, 10 benign variants, 53 novel variants, and 11 variants of unclear clinical significance were identified. Within each classification, variants are listed in decreasing order of incidence within the study sample. The reference sequences for human *GJB2* were BC017048 and U43932. Numbering of *GJB2* nucleotides starts with the A of the ATG initiation codon in exon 2 as position +1.
[b]Classification of several variants, including specifically 101T>C (M34T), 380G>A (R127H), and 109G>A (V37I), remains controversial. The classification provided above is based on various online resources, such as the Connexin Deafness Homepage, and a review of the literature (see references). Where appropriate, and where our data provided some potentially useful, albeit indirect, insight, we have attempted to address such controversies. The pathogenicity of 101T>C (M34T) is mentioned in the discussion. All 11 subjects homozygous for V37I lacked other *GJB2* or *GJB6* variants and all demonstrated mild to moderate NSHL. Similarly, of the nine subjects compound heterozygous for *V37I* and another *GJB2* variant (three with 235delC, two with 35delG, two with M34T, one with I203T, and one with V27I), all had mild to profound NSNHL.
[c]In the absence of access to the primary sequence tracings, we cannot confirm with certainty the distinctness of the 310_323del and 314_327del mutations.
[d]Those nucleotide or amino acid positions at which multiple changes were identified in this study are italicized.

Table 4
Ethnic distributions of the most common *GJB2* mutations[a]

Mutation	No. of variant alleles	Frequency (%)	White (%)[b]	African (%)[b]	Hispanic (%)[b]	Asian (%)[b]	Ashkenazi Jewish (%)[b]	Other (%)[b]	Ethnicity unknown (%)
35delG	1001	42.8	31.3	0	0.5	0.1	0.1	1.4	66.6
101T>C (M34T)	256	10.9	24.6	1.2	0.4	0	0	1.6	72.3
109G>A (V37I)	183	7.8	5.5	0	1.1	15.3	0	0	78.1
167delT	83	3.6	24.1	0	0	0	7.2	2.4	66.3
269T>C (L90P)	46	2.0	43.5	0	0	0	0	0	56.5
79G>A/341A>G (V27I/E114G)	39	1.7	15.8	0	0	33.3	0	5.1	46.2
235delC	36	1.5	5.6	0	0	27.8	0	0	66.7

[a]Frequency refers to the frequency of the particular variant allele among all variant alleles in the study.
[b]The number of instances that a particular variant allele was observed within a specific ethnic group divided by the total number of instances that the allele was observed among all ethnic groups, expressed as a percentage. Other includes individuals of Native American, Arab, or mixed ethnicity.

cially in *GJB2* heterozygotes, which may result in an inaccurate estimate of the prevalence of the del(*GJB6-D13S1830*) mutation in our study population. While both are common problems with retrospective, multicenter studies such as ours, much valuable information can still be obtained from such studies.

First and foremost, our findings suggest that the frequency and distribution of sequence changes in *GJB2* and *GJB6* in North America differ from those previously reported in other studies, although various studies have reported a wide range for the prevalence of DFNB1-associated AR NSNHL,[8,9,17,21,23–31] most likely related to ethnic differences between the populations studied. Nonetheless, our results suggest a more prominent role for other loci in the etiology of AR-NSNHL in this patient population. For example, variations in *GJB2* were

Table 5
Most frequent *GJB2* variants within each ethnic group[a,b]

	White	African	Hispanic	Asian	Ashkenazi Jewish
Total no. of variant alleles	526	21	21	82	7
Variant alleles (in decreasing order of frequency) M, mutation, B, benign variant, U, unclear significance, N, novel	35delG (M, 59.5%)	−34C>T (U, 38.1%)	35delG (M, 23.8%)	109G>A (V37I) (M, 34.2%)	167delT (M, 85.7%)
	101T>C (M34T) (M, 12.0%)	101T>C (M34T) (M, 14.3%)	79G>A (V27I) (B, 19.1%)	79G>A/341A>G (V27I/E114G) (M, 15.9%)	35delG (M, 14.3%)
	167delT (M, 3.8%)	94C>T (R32C) (M, 9.5%)	109G>A (V37I) (M, 9.5%)	235delC (M, 12.2%)	
	269T>C (L90P) (M, 3.8%)	249C>G (F83L) (B, 9.5%)	71G>A (W24X) (M, 9.5%)	71G>A (W24X) (M, 7.3%)	
	79G>A (V27I) (B, 2.5%)	79G>A (V27I) (B, 4.8%)	488T>C (M163T) (N, 9.5%)	1A>G (M1V) (M, 6.1%)	
	109G>A (V37I) (M, 1.9%)	132G>A (W44X) (M, 4.8%)	427C>T (R143W) (M, 4.8%)	79G>A (V27I) (B, 4.9%)	
	79G>A/341A>G (V27I/E114G) (M, 1.1%)c	187G>A (V63M) (N, 4.8%)	35G>T (G12V) (M, 4.8%)	608T>C (I203T) (B, 3.7%)	
	71G>A (W24X) (M, 1.0%)	241C>G (L81V) (N, 4.8%)	365A>T (K122I) (M, 4.8%)	176-191del16 (M, 2.4%)	

[a]Data for all nonwhite ethnic groups suffer from low numbers.
[b]Frequency of the particular variant allele among all variant alleles in the specific ethnic group.
[c]Counted as a mutation when 79G>A (V27I) and 341A>G (E114G) were present together, even though *cis/trans* status could not usually be confirmed. When found individually, each was considered a benign variant.

present in individuals with NSNHL in only 11.2% of cases (Fig. 1) in our large North American cohort, even taking into account both AR and AD mutations, as well as those thought to be benign or of unknown clinical significance. This is significantly lower than earlier estimates for the United States, ranging from 21.7%[17] to 40%.[9] Similarly, the prevalence of *GJB6-D13S1830*-associated hearing loss (1.0%), which in our study occurred solely in the form of *GJB2/GJB6* compound heterozygosity, was far below that previously reported in other North American populations ($P = 0.02$ compared with Pandya et al.[17] and $P = 0.0002$ compared with del Castillo et al.[16]). Indeed, a potentially digenic etiology for AR-NSNHL based on concurrent *GJB2* and del(*GJB6-D13S1830*) mutations accounted for hearing loss in only 3.4% of *GJB2* heterozygotes tested for del(*GJB6-D13S1830*) in our study, well below previous estimates ranging from 16% to 21% in two different international studies ($P < 0.0001$ compared with del Castillo et al.[16] and $P = 0.0002$ compared with Pandya et al.[17]), including one in North America in which 737 deaf probands were ascertained from the United States and Mexico.[17] Although study participants were not tested for the recently described del(*GJB6-D13S1854*) mutation, the absence of this deletion in 180 *GJB2* heterozygotes in Virginia and Iowa suggests that del(*GJB6-D13S1854*) is unlikely to explain the hearing loss observed in *GJB2* heterozygotes, at least in certain populations (such as in the United States, France, and Australia, among others).[20] Thus, whereas the frequency of a *GJB2* and/or *GJB6* etiology may be higher in very well characterized cases of AR-NSNHL, the frequencies reported in this study more accurately reflect the result of clinical practice and diagnostic testing. In addition, the frequency of heterozygosity in this study was 13.1% (Fig. 1). This is markedly above the carrier frequency in a predominantly white population, which has previously been reported to be approximately 3%.[9] However, this study consisted solely of probands with NSNHL. Furthermore, the higher frequency of heterozygosity may reflect some previously unclassified autosomal dominant mutations or, perhaps more likely, the presence of as yet unidentified mutations in *GJB2* or *GJB6*. Finally, this finding may be associated with mutations in other genes whose protein products physiologically interact with or influence the function of cochlear gap junctions.

The ethnic distributions of variants were generally similar to published reports.[16,17] As shown in Tables 4 and 5, the most frequently observed variants were 35delG in whites and Hispanics, 109G>A (V37I) in Asians, −34C>T in African Americans, and 167delT in Ashkenazi Jews. However, the small number of nonwhites in our study cohort limits our ability to generalize our findings to any other ethnicities. As previously reported in Pandya et al.,[17] 79G>A (V27I) was observed with a greater frequency in Asians and Hispanics, representing 20.8% and 19.1% of variant alleles seen in these populations, respectively (Table 5). In addition, the 341A>G (E114G) variant was

Table 6
Novel missense mutations and their sequence conservation in other species: protein changes[a]

Human variant		Predicted			Predicted		Guinea
Nucleotide	Protein	chimpanzee	Mouse	Rat	oxen	Sheep	pig
17T>C	L6P	L	L	L	L	L	L
37G>A	V13M	V	V	V	V	V	V
60T>G	I20M	I	I	I	I	I	I
91T>A	F31I	F	F	F	F	F	F
101T>G	M34R	M	M	M	M	M	M
104T>G	I35S	I	I	I	I	I	I
107T>C	L36P	L	L	L	L	L	L
109G>C	V37L	V	V	V	V	V	V
110T>C	V37A	V	V	V	V	V	V
139G>C	E47Q	E	E	E	E	E	E
161A>G	N54S	N	N	N	N	N	N
167T>C	L56P	L	L	L	L	L	L
175G>C	G59R	G	G	G	G	G	G
187G>A	V63M	V	V	V	V	V	V
191G>A	C64Y	C	C	C	C	C	C
200A>G	H67R	H	H	H	H	H	H
209C>T	P70L	P	P	P	P	P	P
227T>C	L76P	L	L	L	L	L	L
241C>G	L81V	L	L	L	L	L	L
250G>T	V84L	V	V	V	V	V	V
278T>C	M93T	M	M	M	M	M	M
314A>G	K105R	K	K	K	K	K	**R**
355G>A	E119K	E	E	E	E	E	E
358G>A	E120K	E	E	E	E	E	E
389G>A	G130D	G	G	G	G	G	G
428G>T	R143L	R	R	R	R	R	R
473A>G	Y158C	Y	Y	Y	Y	Y	Y
475G>A	D159N	D	**N**	**N**	D	D	**N**
488T>C	M163T	M	M	M	M	M	M
499G>A	V167M	V	V	V	V	V	V
503A>G	K168R	K	K	K	K	K	K
557C>A	T186K	T	T	T	T	T	T
563A>G	K188R	K	K	K	K	K	K
650A>G	Y217C	Y	Y	Y	**F**	**F**	Y
653G>A	C218Y	C	C	C	C	C	C
677T>G	V226G	V	V	V	V	V	V

[a]The 36 novel missense variants from this study were compared with the corresponding residues in sequences from chimpanzee (XP_522618), human (BC017048), mouse (NM_008125), rat (NM_001004099), oxen (XP_592125), sheep (NP_001009780), and guinea pig (BAC07264) using ClustalW 1.8 (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Nonconserved residues are shown in bold and are underlined.

observed most frequently among Asians, representing 17.1% of all variant alleles seen in this population (Table 5 and data not shown). Although the *cis/trans* relationship was only known for four patients with biallelic V27I/E114G, including one Asian patient with profound hearing loss (ethnicities and phenotypes unknown for the other three), concurrent presence of V27I and E114G (79G>A/341A>G) was observed in 39 instances (0.5% of the 7401 subjects) with a slight Asian predominance and variable degrees of hearing loss (5 profound, 3 severe, 2 moderate, 4 mild, and 3 unclassifiable of the 17 with phenotypic information). In this study, concurrent presence of the V27I and E114G (79G>A/341A>G) variants was considered pathogenic regardless of the confirmed *cis/trans* status. However, the pathogenicity of V27I/E114G remains controversial, at least in part because V27I/E114G in *cis* and homozygous V27I/E114G have been reported in unaffected individuals.[32] Finally, the 101T>C (M34T) variant, which was observed more frequently than any variant other than 35delG in our study and accounted for 10.9% of all variant alleles across all ethnic groups, was particularly frequent among whites and African Americans. It was homozygous in eight study participants, with variable degrees of hearing loss (three profound, five mild). Taken together with the findings of Snoeckx et al.,[22] which demonstrated NSNHL in 38 individuals with the 35delG/M34T genotype and in 16 persons homozygous for M34T, our findings indicate that this variant may *not* be an innocent variant but rather a pathogenic mutation.

With respect to genotype-phenotype correlations, phenotypic information (by report from the submitting institution) was available for 422 subjects (5.7%), 215 (2.9%) of whom had biallelic *GJB2* variants. Analysis of this limited subset demonstrated the following: consistent with recent reports,[21,22] genotypic classification of mutations as either T/NT or 35delG/non-35delG were nonrandomly associated with the severity of hearing loss (respectively, $P < 0.0001$ and $P = 0.0069$ by Cochran-Mantel-Haenszel test). Moreover, hearing loss was more severe in individuals with biallelic T mutations (T/T) versus those heterozygous for both T and NT mutations (T/NT; $P < 0.001$ post hoc Wilcoxon rank sum test with Bonferroni correction); in turn, individuals compound heterozygous for T and NT mutations had more severe hearing loss than those with biallelic NT mutations (NT/NT; Fig. 3). However, unlike the study by Snoeckx et al.,[22] the difference in the degree of hearing loss between the T/NT and NT/NT classes did not achieve statistical significance in this study ($P = 0.71$ vs. $P < 0.005$ in Snoeckx et al. (by post hoc Wilcoxon rank sum test with Bonferroni correction), presumably reflecting the larger sample size in the Snoeckx et al. study.

The classification of mutations (Table 3) is challenging in the absence of functional studies that mimic the in vivo environment. Although nonsense mutations and frameshifts, which often lead to premature termination of translation, are generally accepted as pathogenic due to their obvious effect on the protein, the impact of missense mutations and especially noncoding variants is more difficult to predict. Nevertheless, genotype-phenotype correlations are beginning to emerge.[19,22,33] Novel missense mu-

Table 7
Novel GJB2 alleles[a]

Nucleotide change	Protein change (if applicable)	Total	Autosomal dominant[b]	Autosomal recessive[b]	Benign variant[b]	Unclear significance[b]	Novel[b]
503A>G	K168R	7					
109G>C	V37L	5					
314A>G	K105R	4					
					79G>A		
−3287C>G		3			79G>A		
					79G>A		
355G>A	E119K	3					
107T>C	L36P	2					107T>C
139G>C	E47Q	2					
209C>T	P70L	2					
233delC	Frameshift	2		109G>A			
				109G>A			
250G>T	V84L	2		35delG			
				35delG			
428G>T	R143L	2		101T>C			
563A>G	K188R	2		35delG			
−6T>C		2					
488T>C	M163T	2					
101T>G	M34R	1					
104T>G	I35S	1					
110T>C	V37A	1					
11delG	Frameshift	1					
161A>G	N54S	1					186C>T (U)
167T>C	L56P	1					
186C>T	N26N	1					161A>G (U)
187G>A	V63M	1					475G>A (Mo)
200A>G	H67R	1					
227T>C	L76P	1		645_648del			
232insG	Frameshift	1					
241C>G	L81V	1 (M)					
264G>C	A88A	1					
278T>C	M93T	1					
−3187C>T		1					
358G>A	E120K	1					
389G>A	G130D	1					
39G>T	V13V	1					
401delG	Frameshift	1					
433-435delATC	delI145	1					
434delTC	Frameshift	1					
444C>G	A148A	1					

(*Continued*)

Table 7
Continued

Nucleotide change	Protein change (if applicable)	Total	Autosomal dominant[b]	Autosomal recessive[b]	Benign variant[b]	Unclear significance[b]	Novel[b]
456C>A	V152X	1		365A>T			
458-475dup	Frameshift	1		94C>T		−34C>T	
557C>A	T186K	1					
576delA	Frameshift	1		35delG			
60T>G	I20M	1					
653G>A	C218Y	1					
677T>G	V226G	1					
91T>A	F31I	1					
175G>C	G59R	1					
475G>A	D159N	1					187G>A (Mo)
−24A>C		1					
191G>A	C64Y	1		109G>A			
473A>G	Y158C	1					
37G>A	V13M	1		109G>A			
650A>G	Y217C	1					
499G>A	V167M	1					
17T>C	L6P	1					

[a]Phenotype if known: profound (P), severe (S), moderate (Mo), mild (Mi), and unclassifiable (U).
[b]If the novel allele was present with a second variant allele, the identity of this second allele was provided and categorized below.

tations can be evaluated for possible pathogenic protein effects by prediction tools such as SIFT (http://blocks.fhcrc.org/sift/SIFT_BLink_submit.html) and PolyPhen (http://coot.embl.de/PolyPhen/). However, these programs are not always in agreement and should be used with caution.[32] Our categorization was largely guided by the entries on the Connexin Deafness Homepage and literature searches. However, many variants are rare and have been reported only once or a few times, precluding a general assessment of pathogenicity. For some very recently described sequence variants, specifically 34G>T (G12C) and 40A>G (N14D),[32] we adhered to a relatively conservative classification, categorizing these as variants of unclear clinical significance.

Fifty-three putatively novel *GJB2* variants with no apparent ethnic biases were identified in the current study (Tables 3, 6, and 7), representing a surprisingly high 38.1% of all variants identified. This illustrates that characterization of variants in this gene is still in progress, although they are often not reported in the literature or in the Connexin Deafness Homepage. It is, therefore, imperative for the development of our understanding of the *GJB2* gene, including assessment of pathogenicity and genotype-phenotype correlations, that novel variants be reported to a mutation database and ultimately in the literature, even when they are identified in a clinical molecular diagnostic laboratory rather than in the research setting. Seven of the novel *GJB2* variants resulted in frameshifts, 1 produced a premature termination codon, 36 were missense mutations, 4 were synonymous changes, 4 occurred upstream of the transcription initiation site, and 1 was an in-frame deletion of 3 nucleotides. Compared with the nucleotide (data not shown) and protein sequences from other species (Table 6), strict sequence conservation existed at 33 of the 36 residues affected by missense variants. The pathogenic significance of these variants, however, remains to be elucidated by functional studies. To our knowledge, no other mutations or variants have been described at the nonconserved residues (amino acids 105, 159, and 217). The effects of variants upstream of the initiating methionine are unclear, as is the effect of the in-frame deletion. Synonymous changes are unlikely to be pathogenic. Any previously unreported change was classified as novel, including those that involve changes at the nucleotide or amino acid positions of formerly reported variants and those nucleotide changes that result in a previously described amino acid change (Table 3, italics). In such instances, pathogenicity may be similar to that of the change reported earlier, although the replacement of an amino acid by a small or bulky residue may have very different effects on protein function. In the absence of functional studies, missense mutations can be investigated by multiple complementary means: a database and literature search, an evaluation of conservation across species, family studies, and research tools such as SIFT and PolyPhen.

Taken together, our findings suggest that loci other than *GJB2* and *GJB6* contribute to the pathogenesis of AR-NSNHL and that the full spectrum of *GJB2* sequence changes is not yet

fully elucidated. In light of our findings, we concur with previous reports that *GJB2* testing is warranted in patients with NSNHL. However, the steady decline in the reported prevalence of the del(*GJB6-D13S1830*) in *GJB6* since the initial report from del Castillo et al.[16] should give the clinical community in North America pause regarding the utility of screening for this and the recently reported del(*GJB6-D13S1854*) mutation, even in *GJB2* heterozygotes. Nonetheless, given the nominal incremental expense and relative ease of testing for one or both mutations, testing may be appropriate in some *GJB2* heterozygotes but should only be considered as a second-tier test. Although nearly all (six of seven with reported ethnic information) *GJB6* variants in this study were reported in individuals of white descent, the limited ethnic diversity of the population tested for *GJB6* (63.2% were white) prevents us from recommending any stratification of populations for *GJB6* testing based solely on ethnicity. The higher frequency of this deletion in Hispanics described in previous publications, however, may guide clinical testing.[16] Last, we suggest that consideration be given to testing for *SLC26A4*, the gene encoding Pendrin and responsible for both NSNHL and syndromic hearing loss (Pendred syndrome), and, depending on clinical and family history, perhaps to a mitochondrial mutation panel in patients with hearing loss for whom no molecular etiology can be defined by testing for the preceding genes.[1,34,35] Looking ahead, the advent of universal newborn hearing screening, as well as our growing understanding of the molecular genetics of NSNHL, should facilitate more detailed genotype-phenotype correlations and thereby contribute to improved genetic counseling, prognosis, and management of NSNHL.

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