

Frequency and distribution of GJB2 (connexin 26) and GJB6 (connexin 30) mutations in a large North American repository of deaf probands

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Purpose: Profound hearing loss occurs with a frequency of 1 in 1000 live births, half of which is genetic in etiology. The past decade has witnessed rapid advances in determining the pathogenesis of both syndromic and nonsyndromic deafness. The most significant clinical finding to date has been the discovery that mutations of GJB2 at the DFNB1 locus are the major cause of profound prelingual deafness in many countries.¹ More recently, GJB2 mutations have been shown to cause deafness when present with a deletion of the GJB6 gene. We report on the prevalence of GJB2 and GJB6 mutations in a large North American Repository of DNA from deaf probands and document the profound effects of familial ethnicity and parental mating types on the frequency of these mutations in the population. **Methods:** Deaf probands were ascertained through the Annual Survey of Deaf and Hard of Hearing Children and Youth, conducted at the Research Institute of Gallaudet University. Educational, etiologic, and audiologic information was collected after obtaining informed consent. DNA studies were performed for the GJB2 and GJB6 loci by sequencing and PCR methods. **Results:** GJB2 mutations accounted for 22.2% of deafness in the overall sample but differed significantly among Asians, African-Americans and Hispanics and for probands from deaf by deaf and deaf by hearing matings, as well as probands from simplex and multiplex sibships of hearing parents. In our sample, the overall incidence of GJB2/GJB6 deafness was 2.57%. **Conclusion:** GJB2 mutations account for a large proportion of deafness in the US, with certain mutations having a high ethnic predilection. Heterozygotes at the GJB2 locus should be screened for the GJB6 deletion as a cause of deafness. Molecular testing for GJB2 and GJB6 should be offered to all patients with nonsyndromic hearing loss. *Genet Med* 2003;5(4):295–303.

Key Words: GJB2 (connexin 26), GJB6 (connexin 30), genetic hearing loss, national DNA repository, prevalence

Approximately 1 in 1000 children is born with a hearing loss severe enough to require special education services and another 1 to 2 in 1000 have a lesser but clinically significant hearing loss. The deafness in at least half of all infants with profound hearing loss can be attributed to genetic factors,^{2,3} and it is estimated that more than 400 loci may contribute to syndromic and/or nonsyndromic deafness. Nonsyndromic forms account for approximately 67% of genetic deafness, whereas a specific syndrome can be identified in about 33% of cases.²

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Autosomal recessive transmission is found in 77% and autosomal dominant in 22% of genetic deafness. X-linked and mitochondrial forms are much less common in most populations.⁴ Recently, significant progress has been made in documenting the extreme degree of locus heterogeneity through the mapping and cloning of several dozen genes for syndromic and nonsyndromic deafness. As of 2002, nearly 70 genes for nonsyndromic hearing loss have been localized and the protein product has been identified for approximately one-third of these.⁵ The pace at which additional genes are discovered is expected to increase in the coming years because of the availability of cochlea-specific cDNA libraries and completion of the sequencing of the human and mouse genomes.⁶

In addition to confirming locus heterogeneity, molecular studies of hereditary deafness have revealed a more complex pattern of inheritance in some cases. Examples of digenic deafness,^{7–11} deafness secondary to gene-environment interactions,^{12,13} or deafness suppressed by specific modifier genes¹⁴ have been documented. Future studies are likely to uncover additional examples of oligogenic transmission where the

deafness is attributable to the combined effects of genes at more than one locus and/or specific environmental influences.

Perhaps the most remarkable and clinically significant discovery to date has been the finding that mutations involving a single gene, GJB2 (also known as connexin 26), are the most common cause of congenital hereditary deafness in many populations.^{1,15,16} The GJB2 gene encodes connexin 26, a component of gap junctions. Gap junctions are widely expressed in the cochlea and are thought to participate in the recycling of potassium ions from hair cells to the cochlear endolymph.¹⁷ Mutations of GJB2 have been estimated to account for 30% to 40% of all cases of profound, prelingual hereditary deafness in the United States, with a carrier frequency of 2.5% in a Midwestern US population.¹⁸ Testing in many other populations has shown that mutations of GJB2 explain 50% to 80% of nonsyndromic recessive deafness^{16,19} and 10% to 37% of deafness of unknown cause.^{20,21} One particular mutation, 35delG, accounts for approximately 70% of all recessive mutations of the gene.¹⁶ The 167delT mutation has a high prevalence in the Ashkenazi Jewish population,⁸ with a carrier frequency of approximately 3% to 4%. GJB2 screening has become widely available, in part because the small size of the single coding exon facilitates gene sequencing. PCR-based sequence analysis has been shown to be an efficient method for identifying pathogenic mutations in this gene and is rapidly emerging as the standard of care for the evaluation of newborn infants as well as older children and adults with nonsyndromic deafness of uncertain cause.^{22,23} Using this and other methods, more widespread use of screening on a clinical basis, particularly in newborns and young children who are identified with hearing loss, will doubtless become more common in the future.

Recently, it has been shown that GJB2 mutations, when present with mutations in other nonallelic, functionally related genes, cause deafness. A 342-kb deletion including GJB6, which encodes gap junction protein connexin 30 (Cx30), fails to complement in trans with GJB2 mutations to cause deafness.¹¹

We have established a nationwide DNA Repository of samples from deaf probands to pursue a sequential screening strategy for the identification of new genes for deafness. As a by-product, this strategy yields estimates of the prevalence of known mutations for deafness. Here, we describe the results of sequential screening of this Repository for mutations in the GJB2 and GJB6 genes.

MATERIALS AND METHODS

Patient ascertainment

The majority of the deaf probands and their family members were ascertained through the Annual Survey of Deaf and Hard of Hearing Children and Youth, conducted at the Research Institute of Gallaudet University, which is an institution of higher education for the deaf and hard of hearing. The Annual Survey collects educational, etiologic and audiologic data, as well as demographic information such as race, parental mating type, and hearing status of siblings on a very large nationwide

sample of nearly 50,000 deaf and hard of hearing students who receive special education services because of their hearing loss. In 1997, a brief family history questionnaire was sent to the parents of about 30,000 of these students. Approximately 6,000 (20%) returned the questionnaire and gave permission for a genetic counselor to contact them by phone. The process was repeated in 2001 for students who were new to the Annual Survey, resulting in additional responses. Each proband is counted only once. In families with two or more probands, all probands are counted. Allowing for multiple probands in a family adjusts for variation in family size and segregation ratio among families. Study participants were also recruited from the student body at Gallaudet University, as well as the genetics clinics at the Medical College of Virginia and the Instituto de la Comunicacion Humana in Lomas de Plateros, Mexico.

Interviews to obtain family and medical history information from deaf participants or their parents were conducted by genetic counselors by phone (voice or telecommunication device for the deaf) or in person. Open-ended interviews were conducted to explore relevant medical information and pedigree data. Systematic data were collected on the age at onset, severity and progression of the hearing loss, and potentially important factors such as viral infections, antibiotic administration, surgical procedures, etc. Records were collected to document the type, dose, and duration of relevant antibiotics use. A pregnancy and birth history was also obtained. Copies of audiometric studies were requested on all affected individuals in the family. When there was evidence for progression, serial audiograms were requested. A structured systems review was used to elicit evidence for syndromic forms of hearing loss such as Waardenburg syndrome, Pendred syndrome, Jervell and Lange-Nielsen syndrome, Branchio-Oto-Renal syndrome, Alport syndrome, Norrie disease, Usher syndrome, and X-linked congenital fixation of the stapes. When the pedigree data could be extended to relatives living before 1900, an attempt was made to link the data to the family history records collected by EA Fay.²⁴ For any deaf or hard of hearing individuals identified in the family, information on parents, siblings (number and hearing status of each), spouse and children (number and hearing status) was obtained. Information about ethnicity was also collected. Blood samples on the proband, parents and siblings were drawn and shipped to the molecular genetics laboratory at the Medical College of Virginia.

Molecular testing

DNA was extracted from peripheral blood samples using standard nonorganic protocols. Samples from all probands were screened for mutations in exon 1 and 2 of the GJB2 by cycle sequencing. The primers for exon 1 were as follows: F, 5'-CCCTCCGTAACCTTCCAGT-3'; R, 5'-CCAAGGACGTGTGTTGGTC-3'. A 363-bp product was obtained after amplification. The same primers were used for cycle sequencing of exon 1. The primers for exon 2 are as follows: F4, 5'-GCTTACCAGACTCAGAGAAG-3'; R1, 5'-CTACAGGGGTTTCAATGGTTGC-3', which yield a 920-bp product. The forward and reverse strand primers for sequencing exon 2 are as fol-

lows: F4', 5'-CTGTCCTAGCTATGTTCC-3'; and R1, 5'-TGAGCACGGGTTGCCCTCATC-3'. Approximately 200 ng of PCR product was incubated with 20 U of exonuclease I and 4 U of alkaline phosphatase at 37°C for 30 minutes followed by heat inactivation at 80°C for 15 minutes. The product was subjected to cycle sequencing with the ABI PRISM Big Dye Terminator cycle sequencing kit. The sequenced product was purified using a spin-50 column (Biomax-In) and dried. Four microliters of loading dye was added to the sample and a 1.5 microliter aliquot was loaded onto a 5% Long Ranger gel and electrophoresed on an ABI- 377. Forward and reverse sequences were analyzed for mutations using phred, phrap, and consed software suite.^{25,26}

The GJB6 deletion was screened for using the method and primers described by del Castillo et al.¹¹

Audiologic analysis

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.²⁷ Pure tone averages (PTA) were determined by calculating the mean of tested thresholds at 500, 1000, and 2000 Hz. PTA in the better ear was used for the analysis. For subjects with evidence of progressive loss, serial audiograms were requested and coded into the database.

Demographic data

Data were recorded on the racial classification and family structure of the probands. For the purposes of the present study, the data were cross-classified into four racial categories (Caucasian, Hispanic, African American, and Asian); as well as four family structure categories [hearing by hearing (HxH): simplex; hearing by hearing (HxH): multiplex; deaf by hearing (DxH), and deaf by deaf (DxD)] to determine the observed number of probands in each of the 16 resulting cells resulting from cross-classification of the data by race and structure.

RESULTS

We report on the analysis of 737 deaf probands ascertained from 608 families for mutations in GJB2 and GJB6. Table 1 gives a distribution of the probands in our Repository by ethnicity and family structure. Seventy three percent were Caucasian, 16.4% were Hispanic, 6.8% were African Americans and 3.8% were of Asian origin. Except for the African Americans where 86% of the probands were simplex cases, the simplex cases accounted for slightly greater than half of the probands. Multiplex sibships with hearing (HxH) parents represented a quarter of the cases and deaf by deaf (DxD) parental matings ranged between 5% to 17% in the different ethnic groups. In Table 2, the frequency of GJB2-associated deafness in the 16 racial and family structure subsets of the data are shown.

Table 1

Distribution of probands in repository by race and family structure

Race	Probands		Parental mating types, %			
	No.	%	HXH Simplex	HXH Multiplex	HXD	DXD
Caucasian	538	73.0	46.5	29.0	7.8	16.7
Hispanic	121	16.4	59.5	27.3	8.3	4.9
African American	50	6.8	86.0	4.0	4.0	6.0
Asian	28	3.8	60.7	25.0	3.6	10.7
Total	737	100	51.8	26.9	7.5	13.8

Table 2

Frequency of Cx26 deafness by race and family structure

Race	No. of probands	Total	Frequency of Cx26 deafness			
			HXH Simplex	HXH Multiplex	HXD	DXD
Caucasian	538	0.263	0.184	0.353	0.095	0.411
Hispanic	121	0.115	0.042	0.212	0.000	0.667
African American	50	0.040	0.046	0.000	0.000	0.000
Asian	28	0.038	0.582	0.286	0.000	0.667
Total	737	0.221	0.136	0.323	0.073	0.422

Heterogeneity among races: $\chi^2 = 18.19$, 3df, $P < 0.001$.

Heterogeneity among family structure: $\chi^2 = 45.88$, 3df, $P < 0.001$.

Among the total of 737 probands, biallelic GJB2 mutations were found in 22.2%. Of these, the majority (69.5%) were homozygous for the 35delG mutation, 18.4% were compound heterozygous for 35delG and a second mutation, and 12.8% carried two mutations other than 35delG. Of the 164 probands with biallelic GJB2 mutations, 32% were from simplex families and 68% had at least one other affected first degree relative.

The types, distribution, and frequency of the 38 different alleles in the four racial groups are shown in Table 3. The majority of allelic variants ($n = 29$) were seen in the Caucasians, whereas only 10, 7, and 4 different alleles were noted in the Hispanic, Asian, and African American probands, respectively. The number of subjects who carried the GJB6 deletion are shown in parenthesis next to the normal or pathologic GJB2 allele with which they were paired. Only three of the 22 GJB6 deletion alleles occurred in non-Caucasian probands. We detected seven novel changes not previously reported in the connexin mutation database.⁵ Data on these newly recognized variants are summarized in Table 4. Of the remaining 31 alleles, four are of uncertain pathogenicity (V27I+E114G, M34T,V84A, and K224Q), two are known dominant mutations (W44C and R184Q), and three are previously described polymorphisms (V27I, F83L, and R127H). The frequency of the most common pathogenic allele, 35delG, ranged from 25.4% in the Caucasians, to $< 4\%$ in the Asian subpopulations. In the Caucasians, the 167delT and M34T were the next most common alleles with a frequency of two and one percent respectively.

Table 3
Frequency of Cx26 alleles by ethnicity and their co-occurrence with Cx30 deletion

Mutation			Caucasian		Hispanic		African-American		Asians	
Nucleotide change	Protein change		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
IVS1 + 1 GtoA		^b P	1	0.1						
19 C → T	Q7X	P/N			1					
29 T → C	L10P	P/N	1	0.1						
31del14		P	1	0.1						
30delG		P/V	265 (12) ^a	25.4	30 (1)	12.3	7	6.8	2	3.7
44 A → C	K15T	P	3	0.29						
79 G → A	V27I	V	3	0.29	28	11.5			2	3.7
(" + 341 A → G)	V27I + E114G	U	1	0.1					3	5.5
95 G → A	R32C	P	1	0.1	1	0.4				
101 T → C	M34T	U	11	1.05						
109 G → A	V37I	P			3	1.23			2	3.7
132 G → A	W44C	D	2	0.38						
139 G → T	E47X	P	3	0.29	1	0.4				
167delT		P	24 (2)	2.2						
208 C → G	P70A	P/N	1	0.1						
208 C → T	P70S	P/N	1	0.1						
235delC		P							1	1.85
246 C → G	182M	P	2	0.2						
249 C → G	F83L	V					1	0.98		
250 G → A	V84M	NU	2	0.2						
251 T → C	V84A	U	1	0.1						
269 T → C	L90P	P	3	0.29						
269InsT		P	3	0.29						
283 G → A	V95M	P	1	0.1						
298 C → T	H100Y	P	3	0.29						
299-300delAT		P	1	0.1						
312del14		P	3	0.29						
delE120		P	1	0.1						
365 A → T	K122I	P			1	0.4				
370 C → T	Q124X	P								
379 G → A	R127H	V	1	0.1					2	3.7
408InsA		P/N							1	1.85
427 C → T	R143W	P	1	0.1	2	0.82	1	0.98		
439 G → A	E147K	P	1	0.1						
456 C → A	Y152X	P/N			1	0.4				
551 G → A	R184Q	P/D	1	0.1						
617 A → G	N206S	P			2	0.82				
670 A → C	K224Q	U	1	0.1						
Total No. of mutant alleles			343 (14)	.329	70 (1)	.289	9	.089	13	.241
Total No. of wt alleles			699 (4)	.671	172 (2)		92		41	
Total alleles			1042		242		101		54	

^aNumber in parenthesis indicates co-occurrence with Cx30 deletion allele; ^bP, Pathologic change, N, New mutation reported for the first time, D, Dominant, U, Unknown pathogenicity, V, Polymorphic Variant.

Table 4
Novel Cx26 mutations found in 737 deaf probands

Mutation	Base pair change	Structural domain	Genotype	Family structure	Race/country
Q7X	<u>C</u> AG to <u>T</u> AG	IC1	Q7X/wt	S	Hispanic/Equador
L10P	CTG to <u>C</u> CG	IC1	L10P/wt	M, HxH	Caucasian/Various European
P70A	<u>C</u> CC to <u>G</u> CC	EC1	P70A/35delG	S, HxH	Caucasian, Asian/Various European, India
P70S	<u>C</u> CC to <u>T</u> CC	EC1	P70S/35delG	M, HxH	Caucasian/Italy
V84M	<u>G</u> TG to <u>A</u> TG	TM2	V84M/wt	S, HxH	Caucasian/Various European
V84M			V84M/wt	M, DxH	Caucasian (Jewish)/Various European
408InsA	—	IC2	408InsA/35delG	M, HxH	Asian/India
Y152X	TAC to <u>TAA</u>	TM3	Y152X/wt	S	Hispanic, Caucasian/Mexico, Mixed European

wt, wild type allele; S, HxH simplex; M, multiplex; HxH, hearing by hearing; DxH, deaf by hearing.

The mutation screening identified 87 deaf probands or 11.8% of the total sample who were heterozygous carriers of a single sequence variation of GJB2. Of these, 55.2% had a 35delG allele, and 44.8% another pathologic sequence change including 9 (1.2%) with the M34T allele. The V27I polymorphism occurred with a much higher frequency among the Hispanic and Asian subjects.

We also screened all the probands for the common GJB6 deletion and found 19 subjects who were heterozygous and one who was homozygous, giving an overall gene frequency of 0.0149. Fourteen of the 19 GJB6 deletion heterozygotes carried a single GJB2 mutation, thus accounting for the deafness in 15.9% of the GJB2 heterozygotes. Four GJB6 deletion carriers had no mutation identified in the GJB2 gene.

Audiologic data were available for analysis on 421 probands and family members. Our analysis includes the audiograms from 94 individuals with biallelic mutations, 24 probands with a single change, 9 probands with a digenic deafness, and 303 deaf probands in whom GJB2 gene was not the cause of their deafness. Fig. 1 shows the pure tone average in the better ear for frequencies at 500 Hz, 1000 Hz, and 2000 Hz. Individual pure tone averages in the better ear were combined into the six separate categories shown in the figure, ranging from mild (< 30 dB) to profound (> 90 dB) hearing losses. Probands with Cx26 deafness had a more severe hearing loss overall than those with other etiologies for their deafness. Some variability in the severity of hearing loss was apparent in probands with Cx26 deafness, with 68% having profound and 20% having moderate to severe loss. However, we did not detect a significant difference in the severity of hearing loss when biallelic probands with at least one 35delG allele were compared with biallelic probands who lacked a 35delG allele. When we compared the average hearing at different frequencies in our sample, two interesting observations were noted (Fig. 2). We observed a less severe hearing loss in a small sample ($n = 6$) of deaf relatives with the same mutation as the probands who were compound heterozygotes for GJB2 mutations. Second, we noted a greatly increased severity of the hearing loss in nine subjects with digenic GJB2 and GJB6 deafness.

DISCUSSION

We present the frequency of GJB2 and GJB6 hearing loss in the largest data set of probands ascertained to date, representing a range of ethnic groups. The overall frequency of GJB2 hearing loss is 21.7% when calculated independently of the ethnic background and family structure. This is substantially lower than some published estimates from the US of 36% to 40%; however, Kenneson et al.¹ also report a lower frequency of 18.2% in a clinic population of deaf children in Boston. The frequency of GJB2 deafness in probands from multiplex sibships of hearing parents was 32.3%, whereas the frequency in probands from simplex sibships was 14.4%, reflecting the inclusion of sporadic, nongenetic causes in this group along with chance isolated genetic cases. Clearly, a negative family history does not preclude GJB2 deafness. The identification of a genetic etiology in these simplex cases permits more definitive counseling and avoids their misclassification as sporadic, nongenetic cases. When probands with biallelic GJB2 mutations were classified by ethnic group and family structure, we found highly significant evidence for variation in the frequency of GJB2 deafness with respect to both variables. Considering only the marginal totals, our data suggest that the observed frequency of GJB2 deafness could vary from 0.05 to 0.42 depending on the composition of the sample. To make meaningful comparisons, it would be essential to contrast groups that were similar with respect to relevant demographic variables. Until data become available on the molecular screening of all newborn infants for GJB2 deafness, estimating the contribution of GJB2 mutations to deafness in the US population will remain a challenging exercise, which would require a knowledge of the distribution of the relevant demographic variables in the deaf population of our country. Although our data on minority groups is still limited, our findings support the low frequency of GJB2 deafness reported in African Americans¹ and the moderately high frequency in ethnic Chinese (Liu et al., unpublished data, 2002). One would expect the frequency of GJB2 deafness to be higher in families where there are one or more affected first degree relatives (parents or siblings). The only

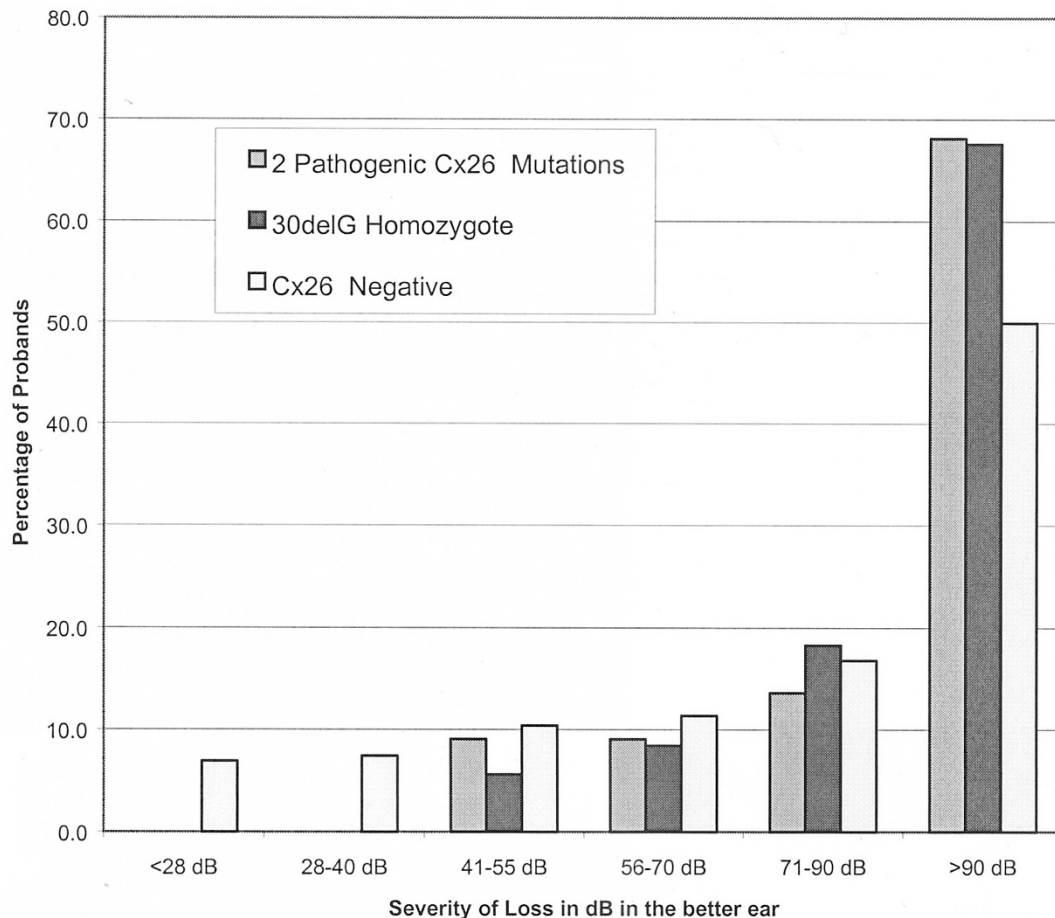


Fig.1 Clinical severity of hearing loss in probands.

exception to the rule are the deaf offspring of deaf by hearing matings, where the deafness is likely to reflect genetic causes that show dominant transmission. Probands from simplex sibships have a lower frequency of GJB2 deafness because they include sporadic, nongenetic causes of deafness in addition to chance isolated genetic cases.

More than 90 mutations of Cx26 have been reported in the connexin database²⁸ and include common alleles in several populations, such as 35delG in Caucasians, 167delT in Ashkenazi Jews, 235delC in Asians, and R143W in Ghana. We found a total of 38 variants, seven of which are novel and have not previously been reported. The Gln7Term or Q7X mutation was found in a 7-year-old male from Ecuador who had profound congenital hearing loss and was the product of close consanguinity. This boy also has developmental and cognitive delays, and has a possible diagnosis of oculocutaneous albinism, suggesting a syndromic form of deafness. The Tyr152Term or Y152X mutation occurred in a 18-year-old female who also had a profound congenital hearing loss. Both of these mutations occurred in the heterozygous state, but because the protein is truncated in both situations, these changes are most likely pathogenic. Whether these changes are dominant remains debatable. The insertion of an “A” at position 408

also leads to a truncated protein resulting from a frame shift and is almost certainly pathogenic. It occurred in trans with a 35delG allele in both members of a pair of 18-year-old female, deaf, monozygotic (MZ) twins and their profoundly deaf sister. A second set of female, MZ twins in the same sibship were carriers of the mutation and had normal hearing. In addition, we observed single examples of four missense changes (L10P, P70A, P70S, and V84M) not previously reported in the literature. The leucine to proline substitution at position 10 replaces a highly conserved amino acid, not only among all the β connexins, but also in almost all alpha connexin molecules and across several species. However this substitution was the only change identified in a 32-year-old male with profound sensorineural hearing loss from a multiplex family with hearing parents. Thus, it is not clear if this change is actually responsible for the hearing loss in the family. The proline at position 70 is also highly conserved in the human beta connexins, and the P70S and P70A substitutions were both found in association with a 35delG mutation in separate probands with profound congenital SNHL, making a strong case as to their pathogenic effects. The P70S substitution occurred in a multiplex sibship with hearing parents, whereas the P70A change was observed in a simplex sibship whose father was reported to have late

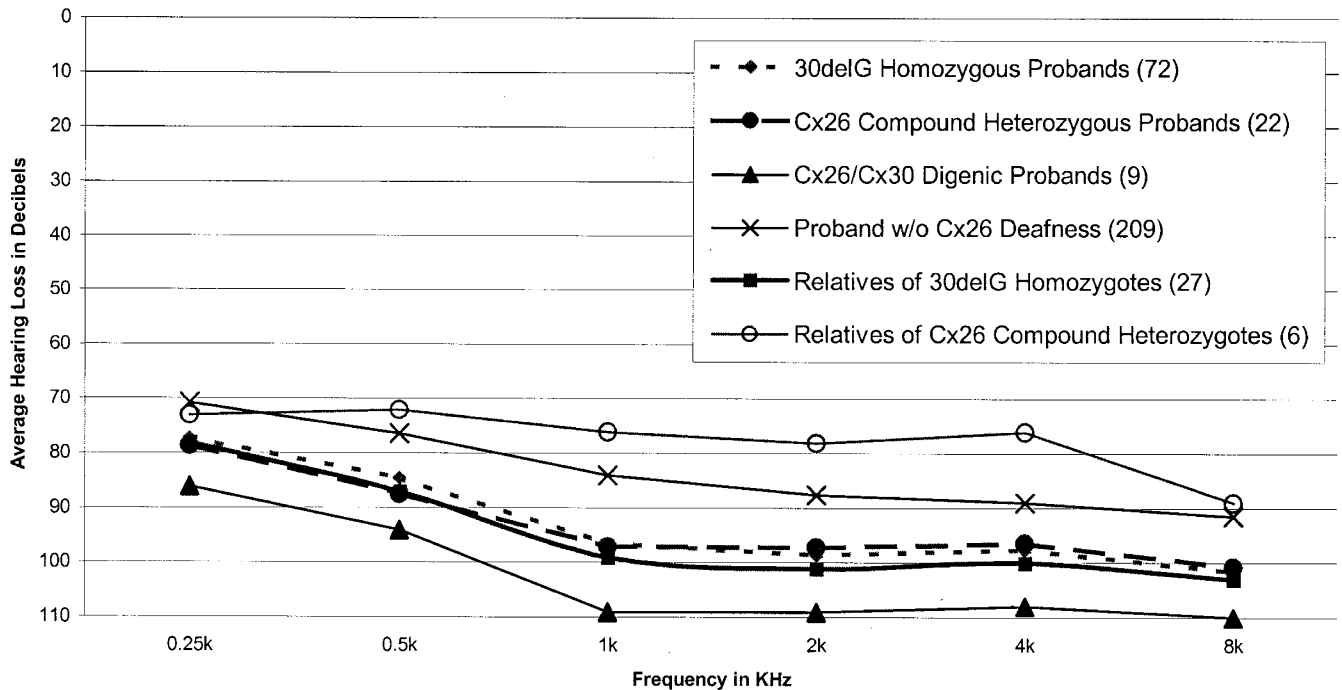


Fig. 2 Average hearing loss in deaf subjects by connexin mutation status.

onset hearing loss. The valine at position 84 is also highly conserved, and another pathogenic change involving this residue (V84L) has previously been reported.²⁹ However, the pathogenicity of the V84M substitution is less clear because it was found without a second accompanying GJB2 mutation in a 17-year-old simplex female with profound deafness who had hearing parents and in a 26-year-old Jewish male who was the product of a DxD mating, with hearing grandparents and all deaf siblings. Yet another substitution at this same locus, V84A, reported by Park et al.,³⁰ is also of uncertain pathogenicity. In our sample it occurred in combination with a 35delG allele in the profoundly deaf 20-year-old daughter of deaf parents. Marriages among the deaf bring together rare genes for deafness, and it is not uncommon to observe two or more forms of genetic deafness in a single nuclear family. This non-random association of unlinked genes that results from assortative mating is referred to as “gametic phase disequilibrium” because it can mimic linkage disequilibrium or ethnic stratification by producing individuals who transmit pairs of rare genes in their gametes with greater than expected frequency even though the loci are actually unlinked.³¹ Thus, for probands who are products of a DxD mating, even if a single GJB2 variant is not the cause of the proband’s deafness, it could still be a pathologic allele whose presence is explained by gametic phase disequilibrium.

The most common variant among Caucasians was the 35delG allele, which accounted for a fourth of all of the GJB2 alleles in our deaf probands. Interestingly, the 35delG allele and the V271 polymorphic variant were noted with almost equal frequencies in the Hispanic probands. In contrast, the M34T variant was seen only in the Caucasian probands. We

observed 4 changes whose pathogenic nature remains unclear (V271 + E114G, V84A, M34T, and K224Q). The clinical significance of the M34T substitution remains uncertain.^{32,33} Nine of our ten probands were heterozygotes and the other was a homozygote for the M34T mutation, showing no significant departure from Hardy-Weinberg expectations ($\chi^2 = 3.54$, 2df, $P \geq 0.25$). However the father of one 35delG homozygous proband had a moderately severe lifelong hearing loss and was a M34T/35delG compound heterozygote. Functional studies for the M34T substitution using the *Xenopus laevis* oocyte and the HeLa cell systems showed decreased mean conductance compared to wild type, and less efficient trafficking and connexon formation in these studies respectively.^{34,35} Thus the possibility that M34T may represent a hypomorph that can sometimes contribute to hearing loss as a recessive trait remains a possibility.

Several studies have tried to determine the carrier frequency of pathologic Cx26 mutations in the population, yielding estimates which range from 1% to 2.5% in the US for the most common 35delG allele. In deaf probands in our study, 11.8% had a single pathologic GJB2 mutation, 3 of whom carried the dominant mutations W44C or R184Q. Screening for the 342-kb deletion in GJB6 in all probands identified a digenic cause for deafness in 14 (15.9%) of the heterozygotes, which is much lower than the 67% reported by del Castillo¹¹ in Spanish subjects. In our sample, the GJB6 deletions were virtually confined to Caucasian probands. Whether there are populations that lack the GJB6 deletion or other genes that can interact with GJB2 alleles to cause deafness needs to be determined (Arnos et al., unpublished data, 2003). The overall frequency of the GJB6 deletion in all 737 probands tested was 2.57% and included one

homozygote and 19 heterozygotes, 14 of whom also carried a pathogenic mutation in the GJB2 gene.

Several smaller studies have assessed the auditory findings in individuals with GJB2 deafness and attempted to establish genotype-phenotype correlations.³⁶ Most subjects with GJB2 deafness exhibit a severe to profound sensorineural hearing loss with little evidence for more severe losses in 35delG homozygotes than among compound heterozygotes. Our results were similar when we compared probands, showing that a large proportion of those with two pathogenic GJB2 alleles had severe to profound SNHL. As others have reported, we did encounter some subjects with lesser degrees of hearing loss as assessed by the pure tone averages, but among the probands there was no apparent difference in severity between 72 35delG homozygotes and 22 compound heterozygotes (Fig. 1). When we compared the average hearing thresholds at six frequencies in probands with their deaf family members carrying the same mutations, we found no significant differences between 14 deaf relatives and the 35delG homozygous probands. However, the six relatives of compound heterozygotes were consistently less severely affected than probands with the same mutation (Fig. 2). Although the sample size is very small, these findings raise the possibility that the phenotypes of compound heterozygotes may in fact be more variable, but that there is an ascertainment bias associated with proband status. As in the case of Waardenburg syndrome, the analysis of affected family members may provide a more reliable picture of the full range of phenotype than studies based entirely on probands. Furthermore, although the pure tone average in the better ear is a very valuable measure of the clinical significance of a hearing impairment and the functional ability of the patient, it is clearly less useful for genetic studies than an average from both ears. For this reason, it may not be the most appropriate measure to study genotype-phenotype correlations.

Finally, the observation of a more severe hearing loss in our small sample ($n = 9$) with digenic deafness suggests a role of the GJB6 gene product on the phenotype rather than merely interfering with the function of the adjacent normal GJB2 allele in double heterozygotes with mutations in trans. GJB2 and GJB6 sub units are known to be capable of forming heteromeric connexons.³⁷ This redundancy may account for the residual hearing in 35delG homozygotes, which may be impaired in individuals with mutations at both loci.

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