Am. J. Hum. Genet. 77:945–957, 2005

GJB2 Mutations and Degree of Hearing Loss: A Multicenter Study

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Hearing impairment (HI) affects 1 in 650 newborns, which makes it the most common congenital sensory impairment. Despite extraordinary genetic heterogeneity, mutations in one gene, *GJB2*, which encodes the connexin 26 protein and is involved in inner ear homeostasis, are found in up to 50% of patients with autosomal recessive nonsyndromic hearing loss. Because of the high frequency of *GJB2* mutations, mutation analysis of this gene is widely available as a diagnostic test. In this study, we assessed the association between genotype and degree of hearing loss in persons with HI and biallelic *GJB2* mutations. We performed cross-sectional analyses of *GJB2* genotype and audiometric data from 1,531 persons, from 16 different countries, with autosomal recessive, mild-to-profound nonsyndromic HI. The median age of all participants was 8 years; 90% of persons were within the age range of 0–26 years. Of the 83 different mutations identified, 47 were classified as nontruncating, and 36 as truncating. A total of 153 different genotypes were found, of which 56 were homozygous truncating (T/T), 30 were homozygous nontruncating (NT/NT), and 67 were compound heterozygous truncating/nontruncating (T/NT). The degree of HI associated with biallelic truncating mutations was significantly more severe than the HI associated with biallelic nontruncating mutations ($P < .0001$). The HI of 48 different genotypes was less severe than that of 35delG homozygotes. Several common mutations (M34T, V37I, and L90P) were associated with mild-to-moderate HI (median 25–40 dB). Two genotypes—35delG/R143W (median 105 dB) and 35delG/dela(*GJB6-D13S1830*) (median 108 dB)—had significantly more-severe HI than that of 35delG homozygotes.

Introduction

Hearing impairment (HI) affects 1 in 650 newborns (Mehl and Thomson 2002), which makes it the most frequent congenital sensory impairment. In most of these cases, the inheritance pattern is autosomal recessive (80%), although autosomal dominant (17%), X-linked (2%–3%), and mitochondrial (<1%) inheritance also occur. In 30% of cases, additional physical findings lead to the diagnosis of 1 of >400 syndromes in which hearing loss can be a clinical component. In the remaining 70% of cases, nonsyndromic HI is diagnosed (Morton 1991).

Nonsyndromic HI is extraordinarily heterogeneous—~100 localizations have been reported across the genome as sites of genes causally related to nonsyndromic HI, and 37 different genes encoding proteins with a wide variety of functions have been identified. Despite this degree of heterogeneity, variants of one gene, *GJB2* (MIM 121011), account for up to 50% of cases of autosomal recessive nonsyndromic HI in many world populations, which makes *GJB2* the gene most frequently associated with this condition (Kenneson et al.

Received June 22, 2005; accepted for publication September 8, 2005; electronically published October 19, 2005.

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0002-9297/2005/7706-0007$15.00

2002). A few specific mutations in *GJB2* also have been described in families with autosomal dominant HI, with and without skin manifestations, although their prevalence is low (Denoyelle et al. 1998).

GJB2 encodes the connexin 26 (CX26) protein, a member of the connexin family of highly related gap-junction proteins. Connexins oligomerize to form hexameric hemichannels called "connexons," which are present in the plasma membrane, where they can bind with connexons from adjacent cells to form functional gap junctions (Bruzzone et al. 1996). These junctional channels are permeable to ions and small metabolites with molecular masses up to 1,200 Da (Harris and Bevans 2001). They can be composed of one (homomeric) or more (heteromeric) connexins, thereby modifying selective permeability of gap junctions (Stauffer 1995).

Expression of *GJB2* has been documented in a variety of cells and tissues. In the cochlea, CX26-containing gap junctions are proposed to maintain K^+ homeostasis by ferrying K^+ away from the hair cells during auditory transduction (Kikuchi et al. 1995). Recently, it has been shown that the intercellular transduction of the second messenger inositol triphosphate (IP_3) is also essential for the perception of sound (Beltramello et al. 2005). In the epidermis, CX26-containing gap junctions play a role in coordinated keratinocyte growth and differentiation (Choudhry et al. 1997), which explains the skin abnormalities associated with autosomal dominant HI in persons who segregate a few mutations in *GJB2* that affect the first extracellular domain of CX26 and exert a dominant negative effect by impairing interconnexon docking (Maestrini et al. 1999; Heathcote et al. 2000; Alvarez et al. 2003).

Many HI-causing mutations of *GJB2* have been reported (Connexin-Deafness Home Page), some of which are very common and others of which are extremely rare. The mutation spectrum diverges substantially among populations, as reflected by specific ethnic biases for common mutations like 35delG among whites (carrier rate of 2%–4%) (Zelante et al. 1997; Estivill et al. 1998; Green et al. 1999), 235delC in the Japanese (carrier rate of 1%–2%) (Abe et al. 2000; Kudo et al. 2000), 167delT in the Ashkenazi Jewish population (carrier rate of 7.5%) (Morell et al. 1998), and V37I in Taiwan (carrier rate of 11.6%) (Hwa et al. 2003). Despite this variability, the combined frequency of all *GJB2* mutations is sufficiently high in most populations to make mutation analysis of this gene a clinically useful, and therefore widely available, genetic test.

The HI in persons with biallelic *GJB2* mutations ranges from mild to profound and is most commonly nonprogressive (Denoyelle et al. 1999; Murgia et al. 1999). Generally, phenotypic variability has been attributed to unknown modifier genes or environmental factors. Previous reports have attempted to address *GJB2* genotype-phenotype correlations, but only a few associations have been recognized, a limitation reflecting the large number of genotypes and a small number of affected patients in most series.

In the largest study reported earlier, we investigated possible genotype-phenotype correlations in a data set of 277 unrelated hearing impaired persons with biallelic *GJB2* mutations and showed that persons homozygous for the 35delG mutation have significantly more-severe HI than do 35delG/non-35delG compound heterozygotes. Persons with two non-35delG mutations have even less severe HI. Because of data set size, we were able to develop only a few specific genotype-phenotype correlations (Cryns et al. 2004). This limitation prompted us to complete this large multicenter study to describe more-detailed genotype-phenotype associations for this frequent form of hereditary HI.

Material and Methods

Patient Recruitment

Persons with congenital HI were sequentially accrued from otolaryngology departments and genetics units. All patient information was obtained between 1980 and 2003. Individuals with syndromic, unilateral, acquired, or dominant types of HI were excluded from this study. The clinical evaluation included a complete history, physical examination, and audiometry. Information on ethnicity was obtained by a combination of self-reporting and physician assessment. Most Arabs were collected by the Pediatric Molecular Genetics Unit, Ankara University School of Medicine (Ankara, Turkey), and the Department of Human Genetics and Molecular Medicine, Tel Aviv University (Tel Aviv). The latter research group also collected the majority of Ashkenazi Jews. The African and Asian participants, as well as some Arabs, were immigrants. Informed consent to allow genetic testing was obtained from all participants or from the parents of minors.

We collected audiometric data from 1,718 patients with biallelic *GJB2* mutations, including del(*GJB6-D13S1830*). Of those, 187 patients were excluded because of additional clinical features or because audiometric data were incomplete. Of the 187, 79 were persons whose data were collected using auditory-brainstem response (ABR) audiometry (five laboratories), which does not provide complete frequency-specific thresholds. To avoid bias, data from sibships with multiple affected siblings were reduced to one randomly chosen hearing impaired individual per family. In the final analysis, detailed audiometric data from 1,531 persons were included in this study. All samples were anonymized, to safeguard patient identity and to preclude the ability to link a given genotype and audiogram to a

Table 1

Frequencies of Mutations in Study Participants

Mutation	No. (%) of Alleles
Nontruncating:	
M1V	3 (.10)
T8M	1 (.03)
G12V	2 (.07)
K15T	2 (.07)
I20T	2 (.07)
V27I	10 (.33)
R32C	2 (.07)
R32H	4 (.13)
M34I	1 (.03)
M34T	***123*** (***4.01***)
I35S	1 (.03)
V37I	***75*** (***2.45***)
A40E	4 (.13)
G45E	1 (.03)
V52L	1 (.03)
V63M	2 (.07)
W77R	15 (.49)
Q80P	2 (.07)
I82M	4 (.13)
V84L	5 (.16)
L90P	***57*** (***1.86***)
L90V	1 (.03)
M93I	1 (.03)
V95M	16 (.52)
H100L	1 (.03)
H100P	1 (.03)
H100Y	3 (.10)
S113R	1 (.03)
E114G	4 (.13)
delE120	23 (.75)
R127H	2 (.07)
L132V	1 (.03)
S138N	1 (.03)
S139N	3 (.10)
I140S	1 (.03)
R143Q	1 (.03)
R143W	13 (.42)
E147K	9 (.29)
M151R	1 (.03)
V153I	5 (.16)
D159N	1 (.03)
C174R	1 (.03)
R184P	21 (.74)
R184W	4 (.13)
S199F	1 (.03)
N206S	11 (.36)
T208P	2 (.07)
Truncating:	
28delC	1 (.03)
31del14	1 (.03)
31del38	3 (.10)
35insG	4 (.13)
35delG	***2,218*** (***72.44***)
51del12insA	5 (.16)
167delT	***91*** (***2.97***)
176del16	1 (.03)
235delC	19 (.62)
269delT	1 (.03)
269insT	7 (.23)
284insdupCACGT	1 (.03)
290insA	2 (.07)
299-300delAT	4 (.13)
310del14	***52*** (***1.70***)
313insGG	1 (.03)
328delG	1 (.03)
333-334delAA	5 (.16)
355del9	1 (.03)
383ins7	2 (.07)
511-512insAACG	3 (.10)
558dup46	1 (.03)
631delGT	2 (.07)
W24X	***47*** (***1.53***)
W44X	1 (.03)
E47X	***43*** (***1.40***)
Q57X	9 (.29)
C64X	1 (.03)
Y65X	2 (.07)
W77X	6 (.20)
Q124X	2 (.07)
Y155X	1 (.03)
W172X	2 (.07)
C211X	2 (.07)
del(GJB6)-D13S1830	***51*** (***1.67***)
IVS1+1G→A	23 (.75)

(*continued*)

Table 1 (continued)

NOTE.—Alleles in bold italics had frequencies >1%.

specific individual. Institutional approval for this study was obtained from the University of Antwerp.

Audiometric Evaluation

All patients underwent otoscopic examination and audiometric testing. In most cases, hearing levels were determined by pure-tone audiometry, which was completed using a diagnostic audiometer in a soundproof room, in accordance with International Standards Organization (ISO 8253-1-3) standards. Five centers also reported behavioral testing results for 117 patients (7.6%), and one center reported the use of steady-state evoked potentials (SSEPs) for 23 patients (1.5%). SSEPs are electrophysiological measures of hearing acuity used extensively in Australia, Asia, and Canada. Because the auditory response is phase locked to changes in a continuous tonal stimulus, a higher average sound pressure level can be delivered than is possible with click stimuli; as a result, SSEPs can provide an estimate of hearing sensitivity in children who demonstrate no response to ABR testing. Pure-tone averages (PTAs) in those cases were recorded as the SSEP best response (always 100–120 dB).

The binaural mean PTA for air conduction at 0.5, 1, and 2 kHz ($PTA_{0.5,1,2kHz}$) was used to compare subgroups of patients. Average thresholds in the range of 21–40

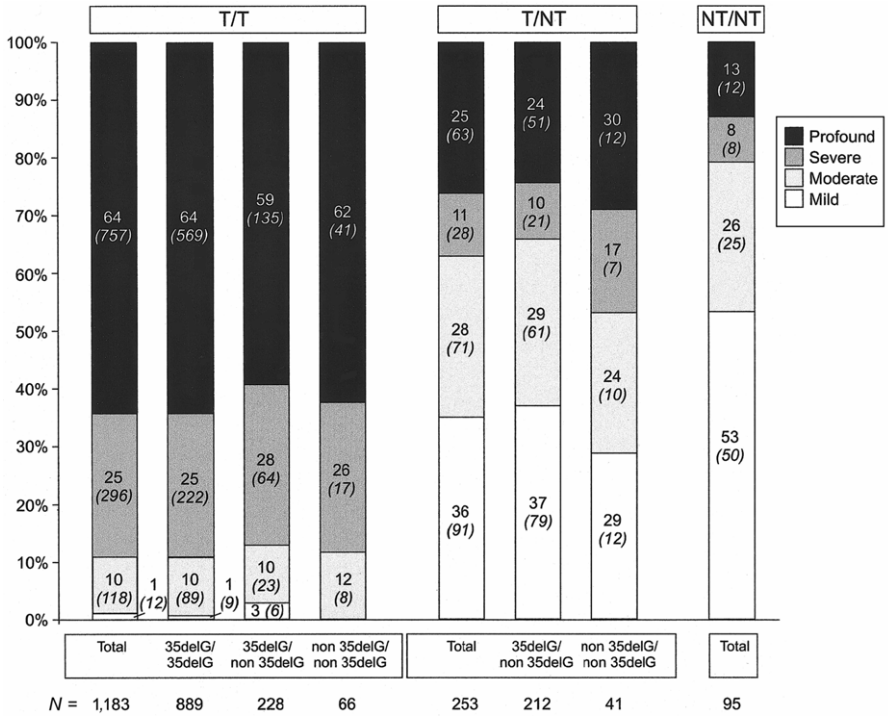


Figure 1 Relative frequencies of the degree of HI in the three classes of genotypes. The actual number of participants is given in parentheses. The three classes were biallelic truncating (T/T), compound heterozygous truncating/nontruncating (T/NT), and biallelic nontruncating (NT/NT). There were significant differences among the three classes, with χ^2 testing ($P < .0001$). An additional distinction between 35delG and non-35delG mutations in the T/T and T/NT classes was made, but statistical analysis (χ^2 test) revealed no significance among these subgroups (data not shown). The number of persons is shown under each subgroup.

dB were defined as "mild HI," in the range of 41–70 dB as "moderate HI," in the range of 71–95 dB as "severe HI," and >95 dB as "profound HI" (Smith et al. 2005).

GJB2 *Mutation Analysis*

Various methods—including DNA sequencing, SSCP, denaturing gradient gel electrophoresis, and denaturing high-performance liquid chromatography—were used for mutation analysis of *GJB2*. In some cases, the 35delG mutation was detected by an allele-specific PCR or by restriction-enzyme digestion of the PCR product (Scott et al. 1998; Storm et al. 1999). The exact nature of all *GJB2* variants was confirmed by DNA sequencing. PCR was used to detect del(*GJB6-D13S1830*), as described by del Castillo et al. (2002). We considered all allele variants of *GJB2* listed as nonsyndromic HI mutations on the Connexin-Deafness Home Page to be potentially pathologic. We also included novel allele variants not yet listed on that Web site, including M34I, I35S, L132V, S138N, I140S, M151R, and T208P. For all these mutations, at least 50 controls with normal hearing were tested, to determine whether we should exclude the mutations because they are common polymorphisms. With the exception of T208P, which was found in compound heterozygosity with W24X in two unrelated persons, these variants were found only once in compound heterozygosity with other *GJB2* mutations (table 1). Variants of debatable pathogenicity, like M34T and V37I, were also included.

Identified allele variants were classified as truncating or nontruncating mutations. The group of truncating mutations contained nonsense mutations and deletions, insertions, and duplications that introduced a shift in reading frame. The splice-site mutation (IVS1+1G→A) and one large deletion, del(*GJB6-D13S1830*), were also classified as truncating, on the basis of published data (del Castillo et al. 2002; Shahin et al. 2002). The group of nontruncating mutations contained amino acid substitutions and one inframe deletion. Although a translated protein can be made in the presence of these mutations, for some amino acid substitutions, it is possible that functional activity is lost.

Statistical Analysis

$PTA_{0.5,1,2kHz}$ was used to compare subgroups of patients. Major genotype-based pairwise comparisons were done between $PTA_{0.5,1,2kHz}$ thresholds with use of persons homozygous for the 35delG mutation as the reference group. Fisher's exact test was performed with 2 × 2 con-

tingency tables of appropriately dichotomized data at the median (50th percentile [P50]), 25th percentile (P25), or 5th percentile (P5) of the frequency distribution for $PTA_{0.5,1,2kHz}$ of the reference group. Given the multiple comparisons made, we focused on the most clearly significant test results.

The group of 35delG homozygotes was used as a reference, or "standard," group, because that group is well defined and is present in most of the previously reported studies of *GJB2* mutations as the cause of HI. Threshold data ($PTA_{0.5,1,2kHz}$) for all groups with a specific biallelic combination of mutations were screened for the presence of progression by performing analysis of the linear regression of threshold on age (at audiometry, in years). If there was no significant correlation coefficient ($P < .025$) combined with a positive slope, it was concluded that there was no significant progression.

Results

Study Sample

The study sample consisted of 638 (41.7%) males and 607 (39.6%) females; sex information was not available for 286 patients (18.7%). The median age of all participants was 8 years; 90% of persons were within the age range of 0–26 years (total age range, 0–70 years). The majority of participants were white (90%), with other subgroups fractionally represented—that is, Arabs (4.8%), Ashkenazi Jews (2.4%), Asians (1.8%), Africans (0.5%), and Roma Gypsies from the Czech Republic (0.5%). Because the different ethnic subgroups were too small for detection of a statistical difference between groups, we used the sample set as a whole for statistical evaluation. Linear regression analysis of thresholds on age in the entire study sample and in subsamples defined by genotypes did not show significant progression in any samples; therefore, age was not used as a variable for the analyses. This finding is in concordance with many other studies (Denoyelle et al. 1999; Orzan et al. 1999; Loffler et al. 2001), although progression of hearing loss cannot be definitively excluded, given the cross-sectional nature of the regression analysis. A slight degree of asymmetry was found in this study sample; however, the difference in $PTA_{0.5,1,2kHz}$ between the two ears was <15 dB in 90% of the persons.

GJB2 *Mutation Spectrum*

A total of 83 different mutations were identified, which were subclassified as 47 nontruncating and 36 truncating mutations (table 1). These mutations were associated with 153 different genotypes, of which 56 were homozygous truncating (T/T), 30 were homozygous nontruncating (NT/NT), and 67 were compound heterozygous truncating/nontruncating (T/NT). The nine most common mutations, including 35delG, had a frequency >1%. Of the remaining 74 mutations with a frequency >1%, 27 had frequencies >0.1%; the remainder were very rare, with frequencies <0.1%. It is possible that the two mutations del(*GJB6-D13S1830*) and IVS1+1G→A were underrepresented in this study. This might be because many patients had been ascertained before del(*GJB6-D13S1830*) was characterized (Lerer et al. 2001; del Castillo et al. 2002); also, IVS1+1G→A lies outside the *GJB2* coding region and so was not included in mutation screens by all laboratories.

The most prevalent mutations in population subgroups were 35delG (65% of mutant alleles) in Arabs, 167delT (84%) in the Jewish Ashkenazi population, and V37I (37%) in Asians. Persons of African origin did not carry a "common" mutation. Of the eight unrelated Roma Gypsies, six were homozygous for W24X, one was a compound heterozygote for W24X/35delG, and one carried the M34T/R127H combination. These findings are in accordance with the study reported by Minarik et al. (2003) and Seeman et al. (2004), which showed that W24X is a prevalent mutation in the Roma population.

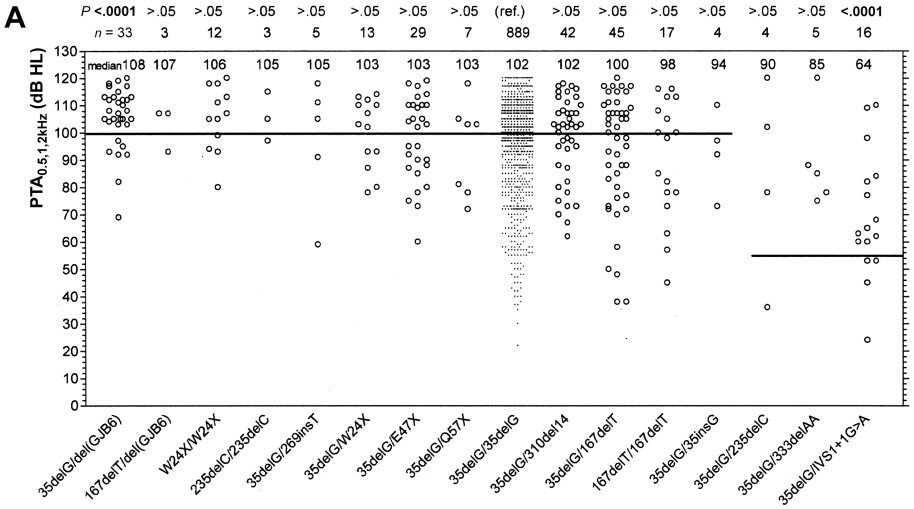
Comparison of HI between Different Genotype Classes

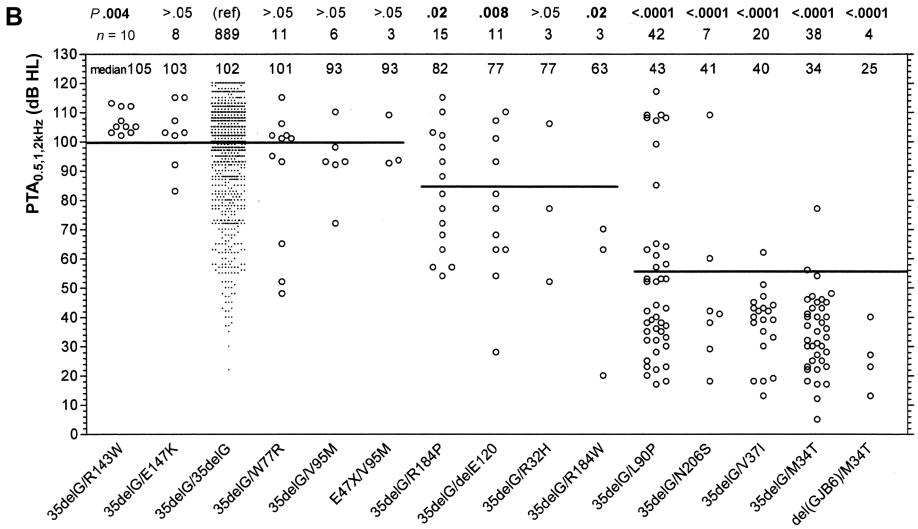
By classifying variants as truncating or nontruncating, we defined three genotype classes: biallelic truncating (T/T) (1,183 persons [77.3%]; 56 genotypes [37%]), biallelic nontruncating (NT/NT) (95 persons [6.2%]; 30 genotypes [20%]), and compound heterozygous truncating/nontruncating (T/NT) (253 persons [16.5%]; 67 genotypes [44%]). The HI across classes is nonrandomly distributed (χ^2 testing, $P < .0001$) (fig. 1), with the HI in the biallelic T/T class more severe than in the T/NT class, which is more severe than in the NT/NT class. Within the T/T and T/NT classes, we distinguished between 35delG and non-35delG mutations. This distinction had little impact on the distribution of HI, which remained similar irrespective of the nature of the mutation (35delG vs. non-35delG) (χ^2 testing: T/T class $P = .39$; T/NT class $P = .42$).

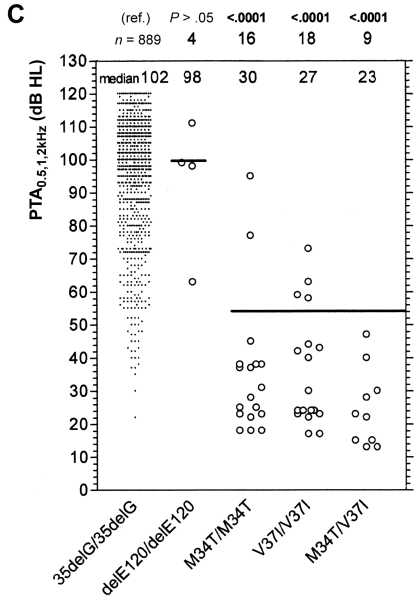
In the T/T class, 59%–64% of persons had profound HI, 25%–28% had severe HI, 10%–12% had moderate HI, and 0%–3% had mild HI. In the T/NT class, 24%–30% of persons had profound HI, and 10%–17% had severe HI, with a shift toward severe-to-profound degrees of HI in persons with two non-35delG mutations, although this difference was not significant (χ^2 testing, $P = .42$). More than half (53%) of persons in the NT/NT class had only a mild degree of HI, and only one person in five in this class had severe-to-profound HI.

Comparison of HI between Specific GJB2 *Genotypes*

To investigate hearing thresholds by genotype, we constructed scatter diagrams showing the binaural mean $PTA_{0.5,1,2kHz}$ for each person within each genotype class (fig. 2). The reference group (35delG/35delG) is included


A
P <.0001 >.05 >.05 >.05 >.05 >.05 >.05 >.05 (ref.) >.05 >.05 >.05 >.05 >.05 >.05 <.0001
n = 33 3 12 3 5 13 29 7 889 42 45 17 4 4 5 16
median 108 107 106 105 105 103 103 103 102 102 100 98 94 90 85 64
PTA0.5,1,2kHz (dB HL)
35delG/del(GJB6)
167delT/del(GJB6)
W24X/W24X
235delC/235delC
35delG/269insT
35delG/W24X
35delG/E47X
35delG/Q57X
35delG/35delG
35delG/310del14
35delG/167delT
167delT/167delT
35delG/35insG
35delG/235delC
35delC/333delAA
35delG/IVS1+1G>A


B
P .004 >.05 (ref) >.05 >.05 >.05 .02 .008 >.05 .02 <.0001 <.0001 <.0001 <.0001 <.0001
n = 10 8 889 11 6 3 15 11 3 3 42 7 20 38 4
median 105 103 102 101 93 93 82 77 77 63 43 41 40 34 25
PTA0.5,1,2kHz (dB HL)
35delG/R143W
35delG/E147K
35delG/35delG
35delG/W77R
35delG/V95M
E47X/V95M
35delG/R184P
35delG/delE120
35delG/R32H
35delG/R184W
35delG/L90P
35delG/N206S
35delG/V37I
35delG/M34T
del(GJB6)/M34T


C
(ref.) P > .05 <.0001 <.0001 <.0001
n = 889 4 16 18 9
median 102 98 30 27 23
PTA0.5,1,2kHz (dB HL)
35delG/35delG
delE120/delE120
M34T/M34T
V37I/V37I
M34T/V37I

in each panel to facilitate visual comparisons, and biallelic *GJB2* genotypes are listed in descending order of $PTA_{0.5,1,2kHz}$. In figure 2, we have not included genotypes that were present in only one or two persons. Instead, these data are listed in table 2 and table 3. Table 2 contains the genotypes of study subjects whose degree of HI was significantly different from that of the reference group, and table 3, those that were not significantly different. The associated *P* value, for comparison of the $PTA_{0.5,1,2kHz}$ with the reference group. is included in table 2 only. These results must be interpreted with some caution because of small sample sizes.

Within the T/T genotype class shown in figure 2*A*, only two genotypes differed significantly from the reference group, persons segregating 35delG/del(*GJB6-D13S1830*) had significantly more HI (median $PTA_{0.5,1,2kHz}$ = 108 dB; $P < .0001$), whereas persons segregating 35delG/IVS1+1G→A had significantly less HI (median $PTA_{0.5,1,2kHz}$ = 64 dB; $P < .0001$). Among persons with T/NT genotypes (fig. 2*B*), one genotype, 35delG/R143W, showed significantly more HI than did the reference group, and eight genotypes had significantly less HI. The three genotypes with the least HI were 35delG/V37I (median $PTA_{0.5,1,2kHz}$ = 40 dB; $P < .0001$), 35delG/M34T (median $PTA_{0.5,1,2kHz}$ = 34 dB; $P < .0001$), and del(*GJB6-D13S1830*)/M34T (median $PTA_{0.5,1,2kHz}$ = 25 dB; $P < .0001$). In the T/NT genotype class, the threshold distribution in persons with 35delG/L90P suggests a bimodal distribution (fig. 2*B*). Seven 35delG/L90P compound heterozygotes had $PTA_{0.5,1,2kHz} > 95$ dB, and 34 had $PTA_{0.5,1,2kHz} < 65$ dB, with the $PTA_{0.5,1,2kHz}$ of only one person falling between the two values (65–95 dB). Among the NT/NT genotype class, participants with three genotypes showed HI significantly different from that of the reference group: M34T/M34T (median $PTA_{0.5,1,2kHz}$ = 30 dB; $P < .0001$), V37I/V37I (median $PTA_{0.5,1,2kHz}$ = 27 dB; $P < .0001$), and M34T/V37I (median $PTA_{0.5,1,2kHz}$ = 23 dB; $P < .001$).

Figure 3 shows the P50, P10, and P90 of hearing thresholds in audiogram format for genotypes with HI significantly different from that of the reference group. We did not plot P10 and P90 values when $n < 10$. Genotypes represented by a small number of persons or a large variation in threshold ($n < 5$ and SD > 25 dB) also were excluded. It is noteworthy that the audiogram slope of nearly all genotypes is fairly similar to that of the 35delG homozygote reference group (i.e., mildly downsloping), although the rather flat configuration seen with the 35delG/IVS1+1G→A genotype is an exception.

The distribution of the degree of HI for the most prev-

Table 2

Genotypes of Study Subjects Whose Degree of HI Was Significantly Different from That of the Reference Group (35delG/35delG)

Mutation	$PTA_{0.5,1,2kHz}$	P[a]
Truncating/truncating (T/T):		
35delG/631delGT	22	.0022
35delG/W77X	32	.0034
35delG/W172X	45	.0191
310del14/W24X	55	.0416
310del14/Q57X	49	.0247
W24X/IVS1+1G→A	53	.0337
Truncating/nontruncating (T/NT):		
35delG/I20T	52	.0337
35delG/H100Y	47	.0213
35delG/M101T	32	.0034
35delG/S139N	29	.0022
35delG/A40E	***50***	***.0045***
35delG/G160S	16	.0011
31del38/L90P	53	.0337
167delT/V37I	50	.0281
167delT/L90P	45	.0191
235delC/V37I	29	.0022
310del14/V37I	30	.0034
310del14/L90P	***25***	***.0045***
Y65X/L90V	30	.0034
IVS1+1G→A/L90P	48	.0247
IVS1+1G→A/R184P	40	.0112
Nontruncating/nontruncating (NT/NT):		
M34T/V95M	5	.0011
M34T/R143W	41	.0112
M34T/V153I	28	.0022
V37I/R143W	23	.0022
V37I/G160S	20	.0011
V37I/N206S	24	.0022
V63M/D159N	38	.0079
L90P/S139N	22	.0022
L90P/V153I	38	.0079
V95M/L90P	***72***	***.0165***
V153I/T8M	1	.0011
T123N/T123N	***56***	***.0028***

NOTE.—All genotypes were present in only one participant except for the genotypes 35delG/I20T, 310del14/L90P, V95M/L90P, and T123N/T123N (shown in bold italics), which were present in two participants.

[a] *P* values were calculated by comparing the median $PTA_{0.5,1,2kHz}$ of each genotype with the median $PTA_{0.5,1,2kHz}$ of the reference group, by use of Fisher's exact test.

Figure 2 Scatter diagrams of the $PTA_{0.5,1,2kHz}$ of groups with specific genotypes. The genotypes were divided into three classes, truncating/truncating (*A*), truncating/nontruncating (*B*), and nontruncating/nontruncating (*C*). The genotypes are shown (*left to right*) in descending order of group median $PTA_{0.5,1,2kHz}$. The dividing lines used to dichotomize the threshold data in any group, as well as the reference group ("ref"), are shown as horizontal lines at 100 dB (~P50 of the reference group), 85 dB (~P25), and 55 dB (~P5). The *P* value indicated above the panel frame relates to the Fisher's exact test applying this dichotomy to both the reference group and the test group. HL = hearing loss; "del(GJB6)" represents the del(*GJB6-D13S1830* mutation.

Table 3

Infrequent Study *GJB2* Genotypes, with HI Not Significantly Different from That of the Reference Group (35delG/35delG)

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

alent genotypes ($n \geq 10$) is given in table 4. Of 18 genotypes, 10 (shown in bold italics) had an HI that was significantly different from that of the homozygous 35delG reference group.

Discussion

In this study of persons segregating *GJB2*-related deafness, we found truncating mutations of *GJB2* to be associated with a greater degree of HI than were nontruncating mutations. Several of the common genotypes in this study group were associated with mild-to-moderate HI, which suggests that complete *GJB2* mutation screening, including IVS1+1G→A and del(*GJB6-D13S1830*), should be offered to all children with nonsyndromic HI, regardless of severity.

The pathogenicity of missense mutations depends on many factors, including the position of the mutation in the protein and the nature of the substitution. For example, a change in an amino acid that is positioned in a functional domain or that is conserved in related genes or species is likely to be pathogenic. However, given the complex structure and function of gap junctions, it is extremely difficult to predict pathogenicity of some missense mutations. This limitation reflects our incomplete understanding of the molecular basis for gap-junction function, and it is for this reason that data from animal models and recombinant expression systems, although valuable for the investigation of mutations, should be extrapolated to humans with caution.

Gap-junction channels are permeable not only to ions but also to small metabolites with relative molecular masses up to ~1,200 Da (Harris and Bevans 2001), with differences in ionic selectivity and gating mechanisms among gap junctions that reflect the existence of >20 different connexin isoforms in humans. Of the handful of *GJB2* mutations that have been tested in recombinant expression systems, most show a loss of function due to altered sorting (G12V, S19T, 35delG, and L90P), inability to induce formation of homotypic gap-junction channels (V37I, W77R, S113R, delE120, M163V, R184P, and 235delC), or interference with translation (R184P). Most *GJB2* mutations, however, have not been studied, and their impact on gap-junction function remains speculative.

Of the reported functional studies, some results are in apparent contradiction with our data. In particular, the predicted gating properties of IVS1+1G→A, V37I, and L90P are discrepant with the degree of HI we observed. Expression studies have demonstrated complete loss of channel activity for V37I and L90P (Bruzzone et al. 2003; Skerrett et al. 2004), although we found these mutations to be associated with mild-to-moderate HI, and functional studies of 35delG and IVS1+1G→A do not yield detectable CX26 protein and mRNA, respectively (D'Andrea et al. 2002; Shahin et al. 2002), which is inconsistent with our observation that 35delG/IVS1+1G→A compound heterozygotes had significantly less-severe HI ($P < .0001$) compared with 35delG homozygotes. For the M34T allele variant, data are even more contradictory. Some functional studies have demonstrated a complete loss of channel activity for this mutation, whereas other studies have shown that this variant affects neither the permeability of dyes nor the formation of stable connexons (Oshima et al. 2003; Skerrett et al. 2004).

The difficulty in data interpretation is illustrated by functional studies of V84L, which have shown no appreciable effect on channel properties, although the mutation has been proven to be clearly associated with HI in humans (D'Andrea et al. 2002; Bruzzone et al. 2003). A recent study by Beltramello and colleagues (2005), which reviews the limitations of our knowledge of gap-junction function, reports that the V84L mutant causes HI due to an impaired permeability to IP_3. The spreading of an IP_3-mediated Ca^{2+} signal is essential for the propagation of Ca^{2+} waves in cochlear supporting cells. This discovery implicating IP_3 as an essential component for the perception of sound is very promising for future studies of *GJB2* mutations, especially when functional studies and clinical data are discordant (i.e., for M34T and V37I).

GJB6 is unique because of its chromosomal localization within 50 kb of *GJB2*. A frequent mutation included in this study, del(*GJB6-D13S1830*), leaves the *GJB2* coding region intact but deletes a large region close to *GJB2* and truncates *GJB6*. This deletion is frequently found in compound heterozygosity with a *GJB2* mutation, and the associated HI is assumed to be caused either by the deletion of a putative *GJB2* regulatory element or by digenic inheritance (del Castillo et al. 2002). Pure digenic inheritance, however, seems unlikely, since compound heterozygosity with a *GJB2* mutation has not been found for other *GJB6* mutations. We found del(*GJB6-D13S1830*) to be one of two mutations associated with HI that is significantly worse than that of the homozygous 35delG reference group (the other is 35delG/R143W). The 33 35delG/del(*GJB6-D13S1830*) compound heterozygotes we ascertained showed the highest median $PTA_{0.5,1,2kHz}$ (108 dB) in this study. The regulatory-element hypothesis could explain this finding, since deletion of this element would both

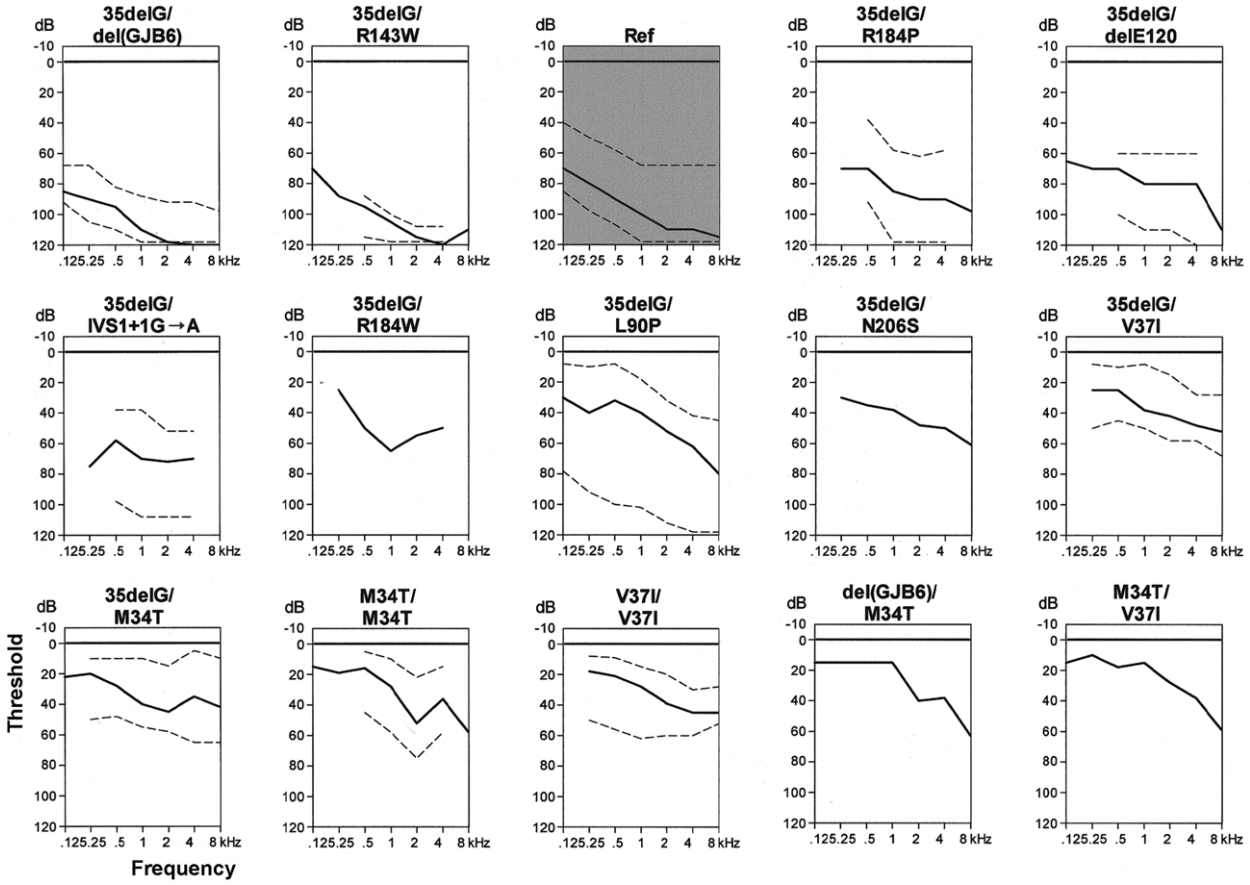


Figure 3 Audiogram format for genotypes with an HI that was significantly different from that of the reference group of 35delG homozygotes. Only genotypes represented by a minimum of five persons and with an SD <25 dB are included. Median (P50) threshold (*solid line*) and P10 and P90 thresholds (*dashed lines*) are shown only for $n > 10$. The reference group ("Ref") is included and is shaded in gray. "del(GJB6)" represents the del(*GJB6-D13S1830* mutation.

abolish *GJB2* expression and inactivate one *GJB6* allele. If *GJB6* partially substitutes for *GJB2* inner ear function, as has been suggested (Ahmad et al. 2003), this substitution would then be less efficient, thus leading to more-severe HI.

The 35delG/L90P genotype was associated with a bimodal distribution of the binaural mean $PTA_{0.5,1,2kHz}$ (fig. 2*B*). Although, as a whole, those with this genotype had significantly less HI than did the reference group, a small group of 35delG/L90P compound heterozygotes had severe-to-profound HI. The generally mild character of the L90P mutation was corroborated by six additional compound heterozygous L90P combinations that all had significantly less HI than did the reference group—that is, combinations involving S139N, V153I, 31del38, 167delT, IVS1+1G→A, and 310del14. Only two genotypes involving L90P (i.e., L90P/R143Q and L90P/delE120) failed to show a significant difference from the 35delG/35delG genotype.

The M34T variant was described first as an autosomal dominant mutation (Kelsell et al. 1997), consistent with the study by White and colleagues (1998), in which it was reported to have a dominant negative effect over wild-type CX26 in *Xenopus* oocytes. These dominant effects were later attributed to an artifact in the expression levels of mutant and wild-type RNA that was not controlled in the exogenous system (Wilcox et al. 2000). Other reports list the M34T allele as an autosomal recessive mutation in the presence of other *GJB2* mutations or in the homozygous condition (Wilcox et al. 2000; Houseman et al. 2001; Kenneson et al. 2002; Wu et al. 2002), whereas other studies have stated that this variant is not pathogenic (Griffith et al. 2000; Feldmann et al. 2004). If M34T is indeed a polymorphism, persons with the 35delG/M34T genotype are carriers of only one *GJB2* mutation (35delG), and their HI must be caused by other unidentified mutations in *GJB2* or by other genes. Because of the large phenotypic variability among genetic causes of HI, we would expect a highly variable degree of HI in these persons, with a

Table 4

Degree of HI for the Most-Prevalent Genotypes ($n \geq 10$)

Mutation and Genotype	No. of Subjects	No. (%) of Subjects, by HI Severity			
		Mild	Moderate	Severe	Profound
Truncating/Truncating (T/T):					
35delG/del(GJB6-D13S1830)	33	0 (0)	1 (3)	5 (15)	27 (82)
35delG/35delG	889	9 (1)	89 (10)	222 (25)	569 (64)
W24X/W24X	12	0 (0)	0 (0)	3 (25)	89 (75)
35delG/W24X	13	0 (0)	0 (0)	5 (38)	8 (62)
35delG/E47X	29	0 (0)	1 (3)	10 (34)	18 (63)
35delG/310del14	42	0 (0)	3 (7)	11 (26)	28 (67)
35delG/167delT	45	2 (4)	4 (9)	13 (29)	26 (58)
167delT/167delT	17	0 (0)	3 (18)	5 (29)	9 (53)
35delG/IVS1+1G→A	16	1 (6)	9 (56)	3 (19)	3 (19)
Truncating/nontruncating (T/NT):					
35delG/R143W	10	0 (0)	0 (0)	0 (0)	10 (100)
35delG/W77R	11	0 (0)	3 (27)	2 (18)	6 (55)
35delG/R184P	15	0 (0)	5 (34)	5 (33)	5 (33)
35delG/delE120	11	1 (9)	4 (36)	3 (28)	3 (27)
35delG/L90P	42	20 (48)	14 (33)	1 (2)	7 (17)
35delG/V37I	20	11 (55)	9 (45)	0 (0)	0 (0)
35delG/M34T	38	26 (68)	11 (29)	1 (3)	0 (0)
Nontruncating/nontruncating (NT/NT):					
M34T/M34T	16	13 (81)	1 (6)	2 (13)	0 (0)
V37I/V37I	18	10 (55)	7 (39)	1 (6)	0 (0)

Note.—Genotypes with HI that is significantly different from that of the reference group (35delG/35delG) are shown in bold italics.

range from mild to profound. However, all persons with a 35delG/M34T genotype had mild-to-moderate HI, with a median $PTA_{0.5,1,2kHz}$ of 34 dB. Persons homozygous for M34T had an even lower median $PTA_{0.5,1,2kHz}$ value (30 dB). M34T is reported to have a high frequency in the general white population, comparable to that of 35delG (Green et al. 1999; Roux et al. 2004). The lower frequency of M34T, compared with 35delG, in the patient sample of this study may reflect reduced penetrance or possible ascertainment bias toward more-severe HI, since persons with mild HI are less likely to see an otorhinolaryngologist for audiologic or genetic testing. Although some studies report that V37I is not pathogenic (Kelley et al. 1998; Kudo et al. 2000; Hwa et al. 2003; Wattanasirichaigoon et al. 2004), we documented an association with mild HI in 9 of 10 genotypic combinations in our study sample. This result is consistent with other studies of this allele (Abe et al. 2000; Wilcox et al. 2000; Kenna et al. 2001; Lin et al. 2001; Marlin et al. 2001).

In spite of the genotype-phenotype correlations we observed, significant phenotypic variability within genotypes remains. This variability may reflect the effect of modifier genes and/or environmental factors that lead to incomplete penetrance and variable expression (Nadeau 2001). If modifier genes are involved, their characterization will be essential for refining genotype-phenotype correlations and improving the accuracy of phenotype prediction.

Acknowledgments

This study was supported by grants from the University of Antwerp, the Vlaams Fonds voor Wetenschappelijk Onderzoek and the Interuniversity Attraction Poles program P5/19 of the Belgian Federal Science Policy (to G.V.C.), and the National Institutes of Health (National Institute on Deafness and Other Communication Disorders [NIDOCD] R01-DC02842) (to R.J.H.S.). Additional grants are from the Internal Grant Agency of the Ministry of Health of the Czech Republic (IGA MH CR NM 7417-3), the Israel ministry of science and technology (to K.B.A.), the Turkish Academy of Sciences in the framework of the young scientist award program (MT/TUBA-GEBIP/2001-2-19), the Polish State Committee for Scientific Research (3P05A15024), the Programa Ramón y Cajal of Ministerio de Ciencia y Tecnología (to I.d.C.), the Spanish Fondo de Investigaciones Sanitarias (FIS G03/203 and FIS PI020807), the G. Passe & R. Williams Foundation (to H.H.M.D), and NIDOCD grant DC005248 (to M.A.K.). This study was initiated through the European Union GENDEAF study group.

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Web Resources

The URLs for data presented herein are as follows:

Connexin-Deafness Home Page, http://davinci.crg.es/deafness/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *GJB2*)

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