Phenotype/Genotype Correlations in a DFNB1 Cohort With Ethnical Diversity

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Objectives/Hypothesis: The aim of this study was to 1) determine the prevalence of DFNB1 in a cohort of children with prelingual nonsyndromic sensorineural hearing loss (HL), 2) study phenotype/genotype correlations, and 3) establish guidelines for genetic counseling of DFNB1.

Study Design: Prospective cohort study.

Methods: A total of 119 unrelated children (107 sporadic and 12 familial cases) with prelingual nonsyndromic HL underwent mutational screening for DFNB1 in the noncoding and coding exons of GJB2, in addition to the del(GJB6-D13S1830) mutation of GJB6. Information regarding demographics, HL, developmental milestones, inner ear high resolution computed tomography, hearing habilitation, and associated phenotypic manifestations were collected in probands with biallelic pathogenic mutations.

Results: The prevalence of DFNB1 in cases of prelingual nonsyndromic HL was 26% (25% in sporadic and 50% in familial cases). In regards to ethnicity, 19 probands were white and 12 probands of Hispanic ancestry had a mixed racial origin (black, Native-American, white). Greater allelic heterogeneity was shown with Hispanics of mixed descent exhibiting 10 of 12 GJB2 allelic variants, whereas whites had 4 of 10 allelic variants (Fisher exact test, P = 0.033); both ethnic groups had the GJB6 deletion. The frequency of deaf carriers of the most commonly found mutation (c.35delG) was 8% and higher than that of expected for the general population (Fisher exact test, P = 0.015). The hearing phenotype was variable in terms of degree of impairment (from mild to profound), onset, symmetry and progression, and there was no correlation with any specific genotype class. DFNB1 probandshad normal gross motor development, and the frequency of computed tomography abnormalities of the inner ear was low at 8%. No other specific associated phenotypic manifestations were identified.

Conclusions: DFNB1 is the most common identifiable etiology of nonsyndromic prelingual deafness both in sporadic and familial cases in this cohort with ethnic diversity. The greater allelic variability observed in Hispanics and the high frequency of deaf probands carrying a single allelic variant of DFNB1 support extending the screening to noncoding regions of GJB2 and to the remaining DFNB1 locus. Most probands have a congenital HL that is stable, symmetrical and without associated manifestations, but the audiometric profile should not be the only criteria for offering mutational screening of DFNB1 because of the observed variability. These data can be applied to direct the clinical evaluation and effectively counsel families of children with DFNB1.

Key Words: Connexin 26, connexin 30, GJB2, GJB6, DFNB1, HL, nonsyndromic recessive HL, computed tomography, cochlear implant, genetic testing, genetic counseling, congenital HL, prevalence, motor development, Hispanics.


INTRODUCTION

Mutations in the gap-junction genes GJB2 and GJB6 at the DFNB1 locus on chromosome 13q12 are the most common cause of recessive nonsyndromic deafness in many world populations.1–8 Approximately 100 different mutations in GJB2 have been identified in patients with nonsyndromic deafness9 and a significant difference in the population distribution of the mutations has been observed. Individuals diagnosed with DFNB1 have a congenital sensorineural hearing loss (HL) of varying degrees without any other associated manifestations.10,11 The HL has been described as symmetrical and nonprogressive as well. Mutations in GJB2 do not usually result in vestibular abnormalities, visual loss, arrhythmia, or thyroid dysfunction.10,11 The habilitation of language and hearing is usually excellent in DFNB1 children when identified early and treated with amplification, cochlear implantation, and aural-verbal therapy.12,13 Despite this uniform phenotypic presentation, clinical algorithms of the evaluation of DFNB1 have been questioned after recent reports of DFNB1 children with unilateral and postnatal HL.6,14
structural, and neurocognitive disorders, and reports of a greater incidence of temporal bone imaging abnormalities than previously noted.

Genetic screening of DFNB1 has increasingly taken a major role in the evaluation of a child with sensorineural HL. The identification of recessive biallelic deafness-causing mutations in the DFNB1 locus (GJB2 and GJB6 genes) establishes the diagnosis of DFNB1. The reasons for the increasing popularity of molecular testing for DFNB1 lie on the fact that it is relatively simple, has a greater yield than traditional laboratory tests, and helps provide answers to questions about etiology and recurrence. In fact, the American College of Medical Genetics recommends the routine use of genetic testing in the evaluation of children with congenital HL. There are approximately 20 laboratories in the US that offer genetic testing for DFNB1 to patients and family members. Many of these centers provide off-site diagnostic services by mail therefore, for the clinician, it is no longer a question of the local availability of diagnostic resources. Otolaryngologists are often confronted by parents of hearing impaired children regarding issues such as etiology, prognosis, and recurrence rate. A previous survey of pediatric otolaryngologists demonstrated that 45% of those who answered the survey had a limited understanding of genetic testing for congenital HL. In this era of genomic medicine, the use of genetic testing is certainly a challenge for otolaryngologists as it is a departure from the more traditional clinical practice. In addition to being prepared to discuss the test’s limitations and benefits, the meaning of a positive and negative test, and issues such as recurrence and carrier testing, the otolaryngologist needs to become familiar with genetic terminology and procedures, all this in an environment of a rapidly evolving knowledge base and shifting clinical guidelines. In consequence, for the effective use of molecular diagnosis of DFNB1 in routine clinical practice it is important to have an understanding of the phenotype/genotype correlations, the implications of test results for genetic counseling, and the contribution of DFNB1 to HL in the target population. A panel of experts recommended that for a successful integration of genetic services (screening and counseling) into clinical practice the clinician should have a broader understanding and appreciation of individual and population variability, and this understanding is only possible by generating data that would be applicable to a broad range of ethnic groups, particularly in geographic areas of high ethnic diversity.

The aims of this study are:

1. To determine the prevalence of DFNB1 and relative frequency of homozygotes, compound heterozygotes, and heterozygous carriers among children with nonsyndromic prelingual deafness.
2. To study phenotype/genotype correlations by a systematic clinical, audiological, and radiological review of probands with DFNB1 mutations; we will i) examine the association between degree of HL and genotype, ii) determine the occurrence of temporal bone abnormalities, iii) study acquisition of gross motor developmental milestones, iv) describe any associated phenotypic manifestations, and v) examine hearing habilitation.
3. To establish guidelines for genetic counseling of DFNB1 suitable to local population demographics.

**MATERIALS AND METHODS**

**Subjects Recruitment**

Subjects were accrued from referrals to an outpatient clinic in a tertiary center during the period 2002–2006. Individuals or their parents were offered voluntary entrance in this study and a total of 297 individuals were enrolled. This study met all international standards for ethical research and was approved by the governing IRB (20010738, October 7, 2002). Parental consent was obtained for all children and assent was obtained for children over the age of 7 years. Pre- and post-test genetic counseling was provided to all enrolled families.

**Subjects Clinical Evaluation**

The study population includes 119 individuals with congenital or early-onset HL. Patients were identified by one of four otologists and underwent a medical history and physical examination of the head and neck with emphasis on identifying manifestations of acquired or syndromic deafness. Individuals or their parents completed a questionnaire with specific questions regarding the onset and progression of HL, gross motor developmental milestones, previous evaluations, presence of syndromic manifestations in the proband or family members, and hearing habilitation. We obtained a family history and at least a three-generation pedigree. Probands underwent age-specific audiological evaluation. Pure-tone audiometry was obtained when possible, using a diagnostic audiometer in a sound insulated booth, in accordance with International Standards Organization (8253-1-3) standards. The threshold values in decibels (dB) for 0.5, 1, and 2 kHz were averaged for both the better and worse hearing ear [pure-tone average (PTA)]. In cases without PTA, the threshold of the wave V of the click-evoked auditory brainstem responses was used to calculate the hearing level. The degree of HL was further classified according to the binaural mean PTA (or extrapolated audiogram responses value) as mild (21–40 dB), moderate (41–70 dB), severe (71–95 dB), and profound (>95 dB). The audiometric configuration, interaural difference, progression, and age of onset were recorded according to accepted guidelines. We excluded cases of syndromic, autosomal dominant, adult-onset (after 17 years of age), and conductive-mixed HL as well as cases with failed or incomplete DNA testing. If there were two or more hearing impaired siblings of normal hearing parents in a family, the proband was classified as having autosomal recessive nonsyndromic deafness (ARNSD). In sibships with multiple affected siblings, data were obtained only from the proband or one randomly chosen hearing impaired subject per family.

**Mutation Screening**

We screened all individuals for allelic variants in the coding exon of GJB2. Allele variants of GJB2 listed as associated with nonsyndromic recessive HL were considered pathogenic. In individuals with none or only one mutated allele in GJB2, we screened for deafness-causing mutations outside the coding exon of GJB2 that have been associated with the DFNB1 locus: exon 1 and the 342-kb deletion in GJB6 [del(GJB6-D13S1830)]. The diagnosis of DFNB1 was reached in probands with two deafness-causing allele-variants in GJB2, with del(GJB6-D13S1830) in homozygosity, or with del(GJB6-D13S1830) and a GJB2 deafness-causing mutation in digenic compound heterozygosity. Persons with only one identifiable DFNB1-causing mutation were considered to have HL from causes other than DFNB1 (non-DFNB1) and be...
coincident carriers of a DFNB1 allele variant. Probands without DFNB1 allele variants were also considered to be non-DFNB1 cases. Additionally, because mitochondrial DNA (mitDNA) mutations have been associated with nonsyndromic HL, all probands were screened for the mitDNA A1555G and A7445G mutations.

**Direct sequencing of GJB2.** Genomic DNA samples were extracted from peripheral blood using standard methods. The coding exon (exon 2, 681 bp) of GJB2 was amplified by polymerase chain reaction (PCR) with primers F4 (5′-GCT TAC CCA GAC TCA GAG AAG-3′) and R1 (5′-CTA CAG GGG TTT CAA ATG GTP GC-3′) (product size 900 bp). The amplification conditions were 95°C for 5 min, then 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension for 5 min at 72°C. Both forward and reverse internal primers, F4′ (5′-CTG TCC TAG CTA TGT TCC TC-3′) and R1′ (5′-TGA GCA CGG GTC GCC TCA TC-3′), were used in cycle sequencing and the final products were visualized with an ABI 377 sequencer (ABI Applied Biosystems, Foster City, CA). To examine the possibility that the second mutant allele of GJB2 in heterozygous probands is due to either mutations at the noncoding exon 1 of GJB2, exon 1 of GJB2 was sequenced with primers described by Houseman et al. 

**Multiplex PCR amplification for the GJB6 342-kb deletion.** For each sample, PCR was used to amplify DNA segments simultaneously with each of the three sets of primers in a multiplex state. The 342-kb deletion in GJB6 [del(GJB6-D13S1830)] was screened using the method described by Wu et al. 

**Analysis for mitDNA A1555G and A7445G mutations.** We screened for the A1555G change in the 12S rRNA gene and the mutation in the 3′ end of the tRNASer(UCN) gene. Both of these regions have been successfully amplified by a multiplex PCR method in our laboratory using primer pairs described previously. The A7445G mutation was confirmed by direct sequencing.

**Inner Ear Computed Tomography**

We reviewed inner ear computed tomography (CT) images of 24 probands in this cohort. All studies were high resolution 1 mm contiguous axial and coronal images of the temporal bones. Digital or printed images were visually inspected for radiographic abnormalities of the cochlea, vestibule, semicircular canals, and endolymphatic aqueduct. We also used manual and electronic calipers to obtain measures of inner ear structures that were shown by others to be easily reproducible and clinically important. A morphological abnormality was diagnosed if one or more dimensions fell outside the normal range (mean ± 2 SD), based on the normative data provided by Purcell et al.

**Additional Clinical Manifestations**

Data regarding gross motor developmental milestones were obtained from questionnaires and from interviewing parents. A child was considered to have a delay in gross motor development if parents reported a failure to reach age-specific milestones by the corresponding age, based on accepted normative. The questionnaires, clinical data sheet, and medical records of DFNB1 proband were reviewed to identify additional phenotypic manifestations. Hearing habilitation strategy was also reviewed.

**Statistical Analysis**

We employed descriptive statistics to calculate the relative prevalence of DFNB1 and allelic variants in our cohort. Comparison of binaural mean PTA among genotypic groups was done by ANOVA. Other phenotype/genotype correlations were analyzed by nonparametric tests, and odds ratios (OR) and confidence intervals (CI) were calculated. The level of significance alpha was 0.05. Calculations were performed with the JMP IN statistical software package (SAS Institute, Belmont, CA).

**RESULTS**

**Study Population**

The subjects of this study are 119 unrelated children with nonsyndromic prelingual deafness identified among 297 enrolled hearing impaired individuals. All probands were 17 years old or younger at the time of molecular diagnosis. DFNB1 and mitDNA A1555G and A7445G mutation screening was performed in all of them. Thirty-one probands carried biallelic deafness-causing mutations in either GJB2 or GJB6 and they were diagnosed as having DFNB1. The prevalence of DFNB1 in this cohort is thus 26%. There were 12 women (39%) and 19 men (61%). In the remaining 88 children, two noncomplementary GJB2 or GJB6 mutations were not identified. A total of 21 children carried a single allele variant of exon 2 of the GJB2 gene: 10 of these children carried a single pathogenic allele variant therefore were considered to be DFNB1 carriers; the remaining 11 children only had a nonpathogenic polymorphism (V27I). Sixty-seven children carried two wild-type alleles. MitDNA A1555G and A7445G mutations were not identified in 113 children, and the mitDNA mutation screening failed in the remaining six children.

Among 104 children with information regarding familial HL, there were 92 sporadic cases and 12 cases of ARNSD. The prevalence of DFNB1 among sporadic cases was 25% (23 of 92 probands), and the prevalence of DFNB1 among ARNSD cases was 50% (6 of 12 probands). Although there was no significant difference in the distribution of ARNSD between DFNB1 and non-DFNB1 groups (Fisher exact test, P = .14), children with ARNSD were three times more likely to have a diagnosis of DFNB1 than those without affected siblings (OR = 3.0, 95% CI 0.880–10.223).

In regards to ethnicity among 31 DFNB1 probands, 19 were white and 12 were of mixed descent (a combination of at least two of the following races: black, Native-American, and white). The US Census Bureau considers race and Hispanic origin to be two separate and distinct concepts. Hispanic or Latino is defined as “a person of Cuban, Mexican, Puerto Rican, South or Central American, or other Spanish culture or origin regardless of race” (http://www.census.gov/prod/2001pubs/c2kbr01-1.pdf). We asked probands’ parents to report the race or races they considered themselves to be (black, white, Asian, Native-American). In a separate question, probands’ parents identified whether they considered themselves to be of Hispanic or other ancestry. In our cohort, we classified probands based on their self-reported race (black, Native-American, white), and if they reported more than one race, the denomination “mixed descent” was used. Accordingly, all of the 12 probands of mixed descent claimed also to be of Hispanic ancestry, and therefore this group will be referred as mixed-Hispanics hereafter. Because Hispanic ancestry is more a cultural identity than a race, probands reporting white race and Hispanic ancestry were counted in the white racial group. Among 19 whites, the ancestry...
was Hispanic in nine, American in five, Italian in two, German in one, Ashkenazim in one, and Irish in one. The relative frequency of whites and mixed-Hispanics in the DFNB1 group was representative of the population of 119 children with prelingual nonsyndromic HL, where there were 60 whites, 43 mixed-Hispanics, 2 Asians, and 2 blacks (ethnicity information was missing in 12 non-DFNB1 children). Among 60 whites, there were 18 children of Hispanic ancestry. In turn, the racial profile of 55 Hispanic children was white in 18 and at mixed in 37.

**Mutation Spectrum**

Among probands with DFNB1, a total of 10 \(\text{GJB2} \) mutations were identified, in addition to del(\(\text{GJB6-D13S1830}\)). Two additional \(\text{GJB2}\) mutations were identified among DFNB1 carriers. The \(\text{GJB2}\) mutations consisted of three types of frameshift mutations, six types of missense mutation (four missense mutations in probands with biallelic mutations and two missense mutations in carriers), two types of nonsense mutations, and one splice-site mutation. The most common mutation was c.35delG, accounting for 75.8% of mutant alleles in DFNB1 probands, 70% in carriers, and 75% (54 of 72) of all the mutant DFNB1 alleles (Tables I and II). This mutation was homozygous in 21 probands, and heterozygous in seven children with no other \(\text{GJB2}\) and \(\text{GJB6}\) mutations detected. The c.35delG mutations also occurred as a compound heterozygote in five probands in conjunction with other recessive mutations including c.1–3170G \(\rightarrow\) A (one child), c.229T \(\rightarrow\) C (p.W77R) (one child), c.644delT (one child), and del(\(\text{GJB6-D13S1830}\)) (two children) (Table III).

Mutations were categorized as either protein truncating (nonsense mutations and deletions or insertions leading to frameshifts) or nontruncating (missense mutations). In total, 10 different genotypes were found, of which five were homozygous truncating (T/T), one was homozygous nontruncating (NT/NT), and four were heterozygous truncating/nontruncating (T/NT). By far, the most common genotype was c.35delG/c.35delG which was present in 21 of 31 (67.7%) probands; the second most common genotype was c.35delG/del(\(\text{GJB6-D13S1830}\)) which was found in two children (6.5%); each of the remaining genotypes occurred once (3.2%) (Table III). The relative frequency of del(\(\text{GJB6-D13S1830}\)) in DFNB1 probands was 3 in 31, or 9.7%.

**Mutation Spectrum and Ethnicity**

Table IV shows the distribution of the 12 \(\text{GJB2}\) mutations and del(\(\text{GJB6-D13S1830}\)) in 103 white and mixed-Hispanic children (206 alleles). The percentage of mutated alleles between the groups was similar at 35% in whites and 34% in mixed-Hispanics. Whites carried 4 of 12 (33%) \(\text{GJB2}\) allele variants and mixed-Hispanics carried 10 of 12 (83%) \(\text{GJB2}\) allele variants, and this difference was statistically significant (Fisher exact test, \(P = .033\)). Only 2 of 12 \(\text{GJB2}\) mutations segregated in both groups, c.35delG and c.167delT. There was no difference in allelic distribution of c.35delG between groups with 18 of 86 (21%) of alleles in mixed-Hispanics carrying c.35delG compared with 36 of 120 (30%) alleles in whites (\(\chi^2 = .194\)).

The allele frequency of del(\(\text{GJB6-D13S1830}\)) was 3 of 120 (2.5%) in whites, and 1 of 86 (1.16%) in mixed-Hispanics (Fisher exact test, \(P = .8\)).

Among 31 DFNB1 probands, 17 of 19 (89.5%) whites and 9 of 12 (75%) mixed-Hispanics segregated c.35delG in either homozygosity or compound heterozygosity (Fisher exact test, \(P = .35\)). Mixed-Hispanics were almost three times more likely to have a mutation other than c.35delG than white probands (OR = 2.83, 95% CI 0.39–20.18).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Number</th>
<th>Percent</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.35delG</td>
<td>7</td>
<td>70</td>
<td>Frameshift</td>
</tr>
<tr>
<td>c.617A (\rightarrow) G</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>c.167delT</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>c.235C (\rightarrow) G</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE I.**

**Mutation Spectrum in 31 DFNB1 Probands and Frequency of Mutations in 62 Alleles.**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Number</th>
<th>Percent</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.35delG</td>
<td>47</td>
<td>75.8</td>
<td>Frameshift</td>
</tr>
<tr>
<td>del((\text{GJB6-D13S1830}))</td>
<td>4</td>
<td>6.6</td>
<td>Frameshift</td>
</tr>
<tr>
<td>c.167delT</td>
<td>2</td>
<td>3.2</td>
<td>Frameshift</td>
</tr>
<tr>
<td>c.229T (\rightarrow) C (p.W77R)</td>
<td>2</td>
<td>3.2</td>
<td>MISSENSE</td>
</tr>
<tr>
<td>c.173C (\rightarrow) G/p.P58R</td>
<td>1</td>
<td>1.6</td>
<td>MISSENSE</td>
</tr>
<tr>
<td>c.298C (\rightarrow) T/p.H100Y</td>
<td>1</td>
<td>1.6</td>
<td>MISSENSE</td>
</tr>
<tr>
<td>644delT</td>
<td>1</td>
<td>1.6</td>
<td>Frameshift</td>
</tr>
<tr>
<td>c.370C (\rightarrow) T/p.Q124X</td>
<td>1</td>
<td>1.6</td>
<td>MISSENSE</td>
</tr>
<tr>
<td>c.1-3170G (\rightarrow) A</td>
<td>1</td>
<td>1.6</td>
<td>SPFIC-SITE</td>
</tr>
<tr>
<td>c.109G (\rightarrow) A/p.V37I</td>
<td>1</td>
<td>1.6</td>
<td>MISSENSE</td>
</tr>
<tr>
<td>c.132G (\rightarrow) A/p.W44X</td>
<td>1</td>
<td>1.6</td>
<td>MISSENSE</td>
</tr>
</tbody>
</table>

Coding \(\text{GJB2}\) mutations are written as follows: nucleotide change/ amino acid change.

*Novel mutation that causes frameshift without truncation.

**TABLE II.**

**Mutation Spectrum in 10 Probands Carrying a Single \(\text{GJB2}\) Allele Variant.**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Number</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.35delG</td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td>c.617A (\rightarrow) G</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>c.167delT</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>c.235C (\rightarrow) G</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

**TABLE III.**

**Genotypes in 31 DFNB1 Probands.**

<table>
<thead>
<tr>
<th>Allele Pairs</th>
<th>Number</th>
<th>Percent</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.35delG/c.35delG</td>
<td>21</td>
<td>67.7</td>
<td>T/T</td>
</tr>
<tr>
<td>c.35delG/del((\text{GJB6-D13S1830}))</td>
<td>2</td>
<td>6.5</td>
<td>T/T</td>
</tr>
<tr>
<td>c.167delT/c.167delT</td>
<td>1</td>
<td>3.2</td>
<td>T/T</td>
</tr>
<tr>
<td>c.35delG/c.1-3170G (\rightarrow) A</td>
<td>1</td>
<td>3.2</td>
<td>T/NT</td>
</tr>
<tr>
<td>c.35delG/c.229T (\rightarrow) C</td>
<td>1</td>
<td>3.2</td>
<td>T/NT</td>
</tr>
<tr>
<td>c.35delG/c.644delT</td>
<td>1</td>
<td>3.2</td>
<td>T/NT</td>
</tr>
<tr>
<td>c.370C (\rightarrow) T/c.229T (\rightarrow) C</td>
<td>1</td>
<td>3.2</td>
<td>T/NT</td>
</tr>
<tr>
<td>c.132G (\rightarrow) A/c.109G (\rightarrow) A</td>
<td>1</td>
<td>3.2</td>
<td>T/NT</td>
</tr>
<tr>
<td>c.173C (\rightarrow) G/c.298C (\rightarrow) T</td>
<td>1</td>
<td>3.2</td>
<td>NT/NT</td>
</tr>
</tbody>
</table>

T/T – protein truncating mutations in homozygosity; T/NT – protein truncating and nontruncating mutations in compound heterozygosity; NT/NT – protein nontruncating mutations in homozygosity.
TABLE IV.
Frequency of Pathogenic GJB2 Alleles and del(GJB6-D13S1830) in 103 Proband (206 alleles) by Ethnicity.

<table>
<thead>
<tr>
<th>Mutation count (percent)</th>
<th>White</th>
<th>Mixed-Hispanic</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.35delG</td>
<td>36 (0.30)</td>
<td>18 (0.21)</td>
</tr>
<tr>
<td>c.109G &gt;A</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>c.132G &gt;A</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>c.167delT</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>c.172C &gt;G</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>c.229T &gt;C</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>c.235C &gt;G</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>c.298C &gt;T</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>c.370C &gt;T</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>c.617A &gt;G</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>c.644delT</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>c.1-3170G &gt;A</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>del(GJB6-D13S1830)</td>
<td>3 (0.025)</td>
<td>1 (0.011)</td>
</tr>
<tr>
<td>Mutant allele count (percentage)</td>
<td>42 (0.35)</td>
<td>30 (0.34)</td>
</tr>
<tr>
<td>GJB2 variant count (percentage)</td>
<td>4 (0.33)</td>
<td>10 (0.83)</td>
</tr>
<tr>
<td>Total No. children</td>
<td>60</td>
<td>43</td>
</tr>
<tr>
<td>Total allele count</td>
<td>120</td>
<td>86</td>
</tr>
</tbody>
</table>

Correlation Between Severity of Hearing Impairment and Genotype

The binaural mean PTA for this cohort was 91.4 dB (SD, 17 dB). In probands with truncating mutations in homozygosity, the mean PTA was 91.8 dB (SD, 15 dB). Probands with nontruncating mutations either in homozygosity or heterozygosity had a mean PTA of 88.8 dB (SD, 28 dB). There was no statistically significant difference in binaural PTA between these two groups (ANOVA, P = .719). The severity of hearing impairment ranged from mild to profound. Hearing impairment was profound in 20 probands, severe in seven, moderate in three, and mild in one. For statistical analysis we further classified HL into two categories: 1) "mild and moderate," and 2) "severe and profound." "Severe and profound" HL was equally distributed among probands with truncating mutations in homozygosity and probands with nontruncating mutations (Fisher exact test, P = .52). Similarly, "severe and profound" hearing impairment was equally distributed between probands with at least one allele carrying c.35delG and probands without the c.35delG variant (Fisher exact test, P = .345).

In our cohort there were three probands with asymmetric HL (interaural PTA difference greater than 10 dB in at least two frequencies). The interaural PTA difference was 20 dB in one, 16 dB in one, and 55 dB in one proband. Two probands had the c.35delG/c.35delG genotype, and one proband was a compound heterozygous c.109G > A/c.132G > A.

Onset and Progression of Hearing Impairment

The HL was identified at birth or shortly afterwards in 21 probands (congenital HL). Nineteen of these 21 children failed newborn hearing screening, and information regarding newborn screening was missing in two. Of 21 probands with congenital HL, 18 children had profound hearing impairment, two children had severe hearing impairment, and one had asymmetric hearing with mild HL in one ear and severe loss in the other ear. Congenital HL was equally distributed among the probands with truncating mutations in homozygosity and probands with nontruncating mutations (Fisher exact test, P = .8).

The HL was identified after age 1 year in nine children (range 1–4 year). The degree of hearing impairment was profound in one proband, severe in five probands, moderate in two probands and mild in one proband. The remaining proband deserves special mention. This child passed a newborn hearing screening (otoacoustic emissions), and was later diagnosed with HL at age 6 years. At the time of diagnosis, the hearing impairment was profound (binaural PTA of 100 dB). This child carried c.35delG in homozygosity.

Progression of HL could be studied in 10 probands with less than profound HL who had serial audiograms. Progressive HL (>15 dB change in binaural PTA within a 10-year period) was noted in two children: one had 15 dB change in 2 years, and one had 19 dB change in 3 years. The child with the largest PTA change had a Mondini anomaly and carried del(GJB6-D13S1830) in homozygosity. The binaural PTA did not change in eight probands after a mean follow-up of 4.4 years (range, 1–8 years). Between subgroups of children with documented progressive and stable HL, we did not find statistical differences in terms of distribution of genotypes (truncating vs. nontruncating), ethnicity, and onset of HL (Fisher exact test, P > .05).

DFNB1 Carriers

We identified 10 hearing impaired children with only one pathogenic allele variant in the cohort of 88 non-DFNB1 children, for an observed carrier rate of 11.4%. Seven of these children were c.35delG carriers, for a c.35delG carrier rate of 8%. This observed c.35delG carrier rate is greater than expected based on an estimate of 14 of 560 c.35delG carriers from a newborn population with high prevalence of GJB2 deafness (Fisher exact test, P = .015). This suggests the presence of additional DFNB1-causing mutations that were not identified in our screening of coding and noncoding regions of GJB2 and the 342-kb deletion in GJB6.

CT of the Inner Ear

CT images were available for 24 of 31 subjects. Twenty-two probands (44 ears) had normal CT images of the temporal bones, both by visual inspection and by radiographic measurements. One subject had increased width of the vestibule (4.5 mm, maximal normal width 3.96 mm) in both sides. One subject had Mondini triad in both sides (enlarged vestibular aqueduct, increased vestibule width, incomplete partition of the cochlear turns). In summary, 2 of 24 (8%) patients with DFNB1 in this cohort had a morphological abnormality of the inner ear by CT. The number of patients with morphological abnormalities of the inner ear in this cohort is too low to establish genotypic correlations. The child with Mondini dysplasia
had biallelic del(GJB6-D13S1830) mutations and fluctuating, progressing to severe HL. The child with enlarged vestibule had congenital profound HL and was homozygous for c.35delG. Twenty probands with normal CT scan of the inner ear had either severe or profound HL, and the remaining two probands had moderate HL.

**Gross Motor Milestones**

All of the 31 DFNB1 children in this series acquired gross motor milestones within the normal time period. The mean age of onset of walking was 11.9 months (SD, 1; range 11–15 months).

**Additional Clinical Manifestations**

Twenty-nine of 31 DFNB1 probands had no other phenotypic manifestations which could be identified by medical history or physical examination. One proband with congenital profound HL and c.35delG in homozygosity had myopia. One proband had congenital hyperbilirubinemia, profound HL, and c.35delG in homozygosity. This proband and another one in this cohort also suffered sepsis and were treated in the neonatal intensive care unit for 7 and 21 days, respectively, including the use of intravenous gentamicin. These children did not carry mtDNA mutations. These two children failed newborn hearing screening, and their HL was presumed to be acquired before DFNB1 testing.

Hearing habilitation was evaluated in 20 DFNB1 probands. Seven probands with mild and moderate HL use hearing aids, attend regular schooling, and showed no delays in speech development. One proband with profound HL was enrolled in a total communication program since early childhood and uses hearing aids only for lip-reading and noise awareness. The remaining 12 DFNB1 children received cochlear implants and their performance was studied and reported elsewhere. At the 48-months follow-up all DFNB1 children were cochlear implant users, and 75% of them had achieved excellent open-set word recognition (compared with 53% of non-DFNB1 patients). Furthermore, they showed faster gains in tests of language skills than the non-DFNB1 implanted children.

**DISCUSSION**

In this study, the prevalence of DFNB1 among all children with prelingual nonsyndromic sensorineural HL was 26%. In sporadic cases, the prevalence was 25% and in probands with ARNSD, DFNB1 was diagnosed 50% of the times. For a condition as genetically heterogeneous as hereditary deafness, the fact that mutations in one gene cause approximately one of four cases of congenital HL in this and other world populations is remarkable, ascertainment bias not withstanding. Thus, DFNB1 is the single most common identifiable cause of childhood sensorineural HL in the geographical area under study.

Most prevalence studies of DFNB1 have been derived from populations of Northern European extraction. This study represents a comprehensive analysis of genotype and phenotype features of subjects who were drawn from a diverse ethnic population. According to the 2000 demographic census, the countywide population of 2,253,362 inhabitants had a self-reporting racial profile as follows: white in 69%, black in 20%, and other races in 11%; 51% of the population was foreign-born. Hispanic ancestry was reported by 57% of the population. Among Hispanics, 80% claimed at least two races. In the largest study so far comparing DFNB1 mutation spectrum and ethnicity in probands from North America, Pandya et al. reported 29 allelic variants in whites compared with only 10 in Hispanics. The relative frequency of c.35delG was 25% in whites and 12% in Hispanics. Contrary to their findings, this study shows greater allelic heterogeneity in mixed-Hispanics with 10 of the 12 GJB2 mutations, and similar distribution of c.35delG alleles and del(GJB6-D13S1830) as whites, whereas whites (of Hispanic and non-Hispanic ancestry) carried 4 of 12 GJB2 mutations. Both studies used the same strategy for mutation analysis but used different criteria for racial profiling; recognizing a difference between race and ancestry, in this study whites of Hispanic ancestry were counted as whites, whereas this distinction is not apparent in the study by Pandya et al., where probands of Hispanic ancestry were classified as a distinct racial group. Moreover, African-Americans were notably absent in our DFNB1 cohort except for one mixed-Hispanic family that claimed black, Native-American, and white racial origin. This finding is in agreement with the low prevalence of DFNB1 in African Americans previously reported.

The most common mutation among our cohort of DFNB1 children was c.35delG, which was present in homozygosity (21 probands) or compound heterozygosity (five probands), accounting for 75% of all mutant alleles in DFNB1 probands and about 25% of mutant alleles in children with nonsyndromic deafness. c.35delG has been reported in up to 70% of Northern and Southern European, American white, and Middle Eastern DFNB1 cases, with a carrier frequency ranging from 1.3% to 4%. The studies have shown a south-to-north European gradient, where a high prevalence of the mutation in the populations of Southern Europe has been explained by the founder effect. The c.35delG mutation is the deletion of a guanine (G) in a stretch of six guanines in the coding region of the connexin 26 gene, GJB2, at position 30 to 35. This leads to a frameshift, resulting in a stop codon at position 13 and in the synthesis of a truncated 12-amino acid polypeptide instead of the normal 226-amino acid polypeptide.

The c.35delG mutation occurred in conjunction with other recessive mutations including c.229T >C, c.644delT, c.1-3170G >A, and the 342-kb GJB6 deletion. c.229T >C is a missense mutation. c.229T >C is a thymidine to cytosine substitution at 229 leading to a change of tryptophan into arginine at position 77. A novel mutation reported in this study, c.644delT changes the last 11 amino acids of the connexin-26 protein without causing truncation. c.1-3170G >A is a splice-site mutation consisting in a 3170 guanine to adenosine transition. It has been observed that c.35delG/c.1-3170G >A compound heterozygotes have less severe hearing impairment compared with c.35delG homozygotes. The HL in our proband with c.35delG/c.1-3170G >A was of moderate degree. Furthermore, the prevalence of this splice-site mutation has been estimated at 4% in some populations.
The c.167delT mutation also occurred once in our study in homozygosity in one mixed-Hispanic proband, and in one deaf carrier of Ashkenazi ancestry. This deletion of a thymidine at position 167, results in a frameshift at codon 56 and subsequent premature stop, 25 amino acids downstream, at position 81. The c.167delT mutation has been demonstrated in several populations but is most prevalent in Ashkenazi Jews, accounting for up to 40% of the pathogenic alleles and with 4% carrier frequency in this population. The c.167delT mutation present in Ashkenazi Jews has also been attributed to a founder effect.8

The c.109G > A mutation was detected in compound heterozygosity with c.132G > A in one proband. c.109G > A, a guanine-to-adenine missense substitution that replaces a valine with an isoleucine (p.V37I), was originally described as a nonpathogenic polymorphism, but later was shown to be a pathogenic variant when associated with another mutated GJB2 allele.38

The 342-kb deletion [del(GJB6-D13S1830)] in GJB6 was detected in homozygosity in one proband, and in digenic compound heterozygosity with c.35delG of GJB2 in two probands. In one family, the deaf proband was found to be heterozygous for c.35delG and heterozygous for del(GJB6-D13S1830). Genotyping revealed that the mother was heterozygous for c.35delG, the father was heterozygous for del(GJB6-D13S1830), and the normal hearing brother was homozygous for the wild-type allele of GJB2 and heterozygous for del(GJB6-D13S1830). This 342-kb deletion is most prevalent in Spain, France, the UK, Israel, and Brazil (5.9%–9.7% of all DFNB1 alleles); it is less frequent in the US, Belgium, and Australia (1.3%–4.5% of all DFNB1 alleles), and is very rare in Southern Italy.4 In this cohort, del(GJB6-D13S1830) was the second most common mutation causing DFNB1, with a relative allele frequency of 6.6% among DFNB1 cases, but its frequency among all children with nonsyndromic deafness was low (2.5% in whites and 1% in mixed-Hispanics) (Table IV). When considering probands with one GJB2 pathogenic variant but no mutations in either the coding and noncoding regions of GJB2, 3 of 13 (23%) carried del(GJB6-D13S1830) in the other allele.

No mitDNA mutations were identified in this cohort of children with nonsyndromic HL. It has been reported that the A1555G and A7445G mtDNA mutations are associated with nonsyndromic HL in children, but these mutations were not found among 20 white probands with nonsyndromic deafness identified through a newborn hearing screening program.39

**Association of Genotype Classes and Hearing Loss**

Our data did not show a correlation between level of hearing impairment and genotype classes (truncating mutations in homozygosity vs. nontruncating mutations in homo- or heterozygosity), and level of hearing impairment and distribution of the c.35delG allele variant. Severe and profound HL was equally distributed among GJB2 genotype classes, but the three children with del(GJB6-D13S1830) either in homo- or heterozygosity with GJB2 had profound HL and required cochlear implantation.

The hearing impairment in this cohort varied from mild to profound. Variability of hearing impairment in DFNB1 even between persons with the same mutations has been reported.6,10,11 More recently, phenotype/genotype correlations have been demonstrated in large series.5,35 Probands with biallelic frameshift or nonsense mutations had more severe hearing impairment than those with biallelic missense mutations.5,35 Differences in the audiological phenotype throughout the mutation spectrum were also identified, with del(GJB6-D13S1830) and c.35delG associated with more severe HL, and c.101T > C and c.109G > A with mild to moderate HL.5

In our cohort there were cases of asymmetric, progressive, and noncongenital HL. HL has been found asymmetric in about 25% of subjects in some series,10,11 and unilateral in rare cases.6 Typically, the HL in DFNB1 is stable and congenital.10,11 However, not all cases of DFNB1 are detected by newborn hearing screening, as one of our cases and some cases of others illustrate.14,40 This variability in expression of HL should be taken into account, and mutation analysis for DFNB1 should be considered in all children with nonsyndromic deafness regardless of an atypical audiological presentation.

**Additional Phenotypic Findings**

In our series of children with DFNB1, we found a prevalence of inner ear dysplasia of 8% (2 of 24 individuals) when using both visual inspection and systematic measurements. This prevalence of 8% is lower than that reported for childhood deafness in general, and likely indicates a coincidental occurrence rather than disruption of labyrinth development by GJB2 mutations. Of note, the only proband in this cohort with a major inner ear anomaly (Mondini anomaly) was homozygous for the large deletion in GJB6, carried c.35delG in heterozygosity, and exhibited progressive HL. Our data are in agreement with others41 and do not support the routine use of CT in GJB2 deafness. Because a child with diagnosis of GJB2 deafness has a low probability (<10%) of having an inner ear anomaly, CT could probably be avoided or delayed until it becomes necessary (i.e., cochlear implant preoperative evaluation or evidence of progressive HL).

Because it is presumed that mutations in connexin-26 and -30 genes cause HL by altering potassium homeostasis and thus altering the endocochlear potential, a similar mechanism could affect the function of the vestibular receptors. Children with bilateral vestibular loss have delayed gross motor development; they stand and walk later than healthy children. However, with one exception,42 vestibular dysfunction has not been reported in series of patients with DFNB1.10,11 Our DFNB1 children did not have delays in their gross motor milestones. Todt et al.42 reported absent vestibular evoked myogenic potentials (VEMP) in two adults with DFNB1, one patient.

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**Notes:**

- **DFNB1** is the gene for deafness, non-syndromic, autosomal recessive 1.
- **GJB2** is the gene for connexin-26.
- **GJB6** is the gene for connexin-30.
- **MTDND** is the gene for mitochondrial DNA.
- **HL** is the abbreviation for hearing loss.
- **CT** is the abbreviation for computed tomography.
was homozygous for c.35delG whereas the other patient was compound heterozygous c.35delG/c.313-326del. These two cases, as well as three of five DFNB1 carriers had absent VEMP and normal caloric testing. Absent VEMP indicates loss of saccular function. Moreover, most of the patients in their series had normal subjective visual vertical test, suggesting intact utricular function. Possible explanations for the observed difference in cochlear, saccular, utricular, and semicircular canal function include differential expression of connexin proteins within the inner ear, and collapse of the saccule because of its proximity with the ductus reuniens and the cochlea. The sacculus and the cochlea share a common embryological origin (pars inferioris), and cochleo-saccular degeneration was found in the only temporal bone study of DFNB1.43 Furthermore, postural control has been evaluated in a cohort of DFNB1 children who had undergone cochlear implantation; the testing paradigm evaluated the vestibulospinal reflex under conditions of sensory modification to which the otolith organs contribute. All DFNB1 children scored similarly to control children in measures of postural control.44 These results underscore findings that isolated saccular dysfunction is unlikely to significantly affect postural control and gross motor development.

All except one child in our cohort exhibited nonsyndromic deafness without associated clinical findings. The finding of myopia in one child is felt to be coincidental and not secondary to GJB2 mutations. Clearly, ascertainment bias has to be considered, as it is possible that children with syndromic deafness were excluded from DFNB1 screening. GJB2 mutations are primarily associated with nonsyndromic deafness, with many DFNB1 series reporting no abnormalities in tests of equilibrium, vision, intelligence, cardiac conductivity, and thyroid function.10,11 A few reports of dominant dermatologic syndromes (keratitis-ichthyosis-deafness and palmoplantar keratoderma) have been found in association with GJB2 deafness, but these reports have been on small families. More recently, Kenna et al.15 reported on 163 children with DFNB1, 29 (18%) of those presenting with structural or neurocognitive abnormalities. Two children in their series were thought to have deafness because of systemic gentamicin treatment before DFNB1 testing, similarly as in two of our probands. Although these authors were unable to show any specific genotype-phenotype correlations, and conceded that these clinical findings are likely coincidental and not resulting directly from GJB2 mutations, they justifiably pointed out that DFNB1 screening should be considered beyond the spectrum of nonsyndromic deafness.

**Genetic Counseling**

Genetic counseling is the process of providing individuals and families with information on the nature, inheritance, and implications of genetic disorders to help them make informed medical and personal decisions. The purpose of genetic counseling is to ensure that the parents and probands understand the findings and limitations inherent in any genetic test. There are three important aspects of counseling: 1) to provide accurate factual information about the disorder, 2) to explain the implications of a test results, and 3) to describe available therapies and supportive resources. Factual information is based on phenotype/genotype correlation studies. Most individuals in this and other series have a bilateral, stable HL that ranges from mild to profound, without other associated clinical manifestations, and with a family history compatible with autosomal recessive inheritance.5,6,10,11 There is good prognosis for hearing and language habilitation. Ethnic bias for certain allele variants has been demonstrated. Detailed description of genotype/phenotype correlations have been possible after pooling many probands into large case-series: despite some variability, biallelic missense mutations are usually associated with a milder hearing impairment than biallelic frameshift or nonsense mutations.5,35 Although there have been an explosion of reports on clinical and genetic aspects of DFNB1, most studies include individuals to whom mutational screening was offered because their phenotypic profile was akin with that expected for DFNB1, and therefore an ascertainment bias is possibly present. Some important gaps in our knowledge of DFNB1 have only become evident after studies combining universal newborn hearing and mutational screening. Most studies have shown that DFNB1 can be congenital, but this and other series14,40 have reported on neonates with documented normal hearing tests at birth who were later diagnosed with severe or profound GJB2 deafness. Given the observed variability of DFNB1 in terms of the audiological profile (mild to profound, congenital vs. postnatal, bilateral vs. unilateral, stable vs. progressive HL), providing prognostic information regarding hearing impairment may be difficult in some cases. Another confounding issue is the large proportion of deaf individuals carrying only one pathogenic allele variant of GJB2 reported by this and other series,35 underscoring the need to provide families with a comprehensive screening of noncoding regions of GJB2 and the remaining DFNB1 locus. The reported c.35delG carrier rate among deaf individuals is greater than that expected in the general population, making it difficult to determine whether these are non-DFNB1 deaf individuals and coincidental carriers of DFNB1, or whether the allelic variant contributes to the HL singly or in combination with another yet unidentified pathogenic variant. The combination of DFNB1 screening and audiometry in a two-tiered screening of entire populations has great potential to answer questions about carrier rates and natural history,39 but it is still undetermined if such screening strategy will be beneficial and cost-effective. Although this type of population screening certainly minimizes ascertainment bias, the spectrum of allele variants to be tested may be large and currently cost-prohibitive, particularly in geographical areas with ethnic diversity. In fact, 25% of mixed-Hispanics with DFNB1 in this study would not have been detected by a screening program that only uses the most common mutation (c.35delG). Other controversial issues are determining the pathogenesis of some allelic variants, the contribution of known pathogenic variants in syndromic deafness, and the disclosure of results to carriers. Careful research must be done to answer these and other questions.
Based on data from this study, if two mutated alleles are found after genetic testing it will be possible to diagnose DFNB1 in one of four children with sporadic nonsyndromic deafness, and in one of two children with ARNSD. We then know the cause of the child’s deafness with certainty, can accurately predict the chance of recurrence in subsequent children (25%), and can avoid unnecessary tests. Additional testing can be indicated for the rare child with other manifestations; many of the extra-auditory manifestations that have been reported coincidently with DFNB1 are identifiable after a thorough and focused history and physical examination. In children with atypical audiology profiles (unilateral or progressive HL) or anticipating a cochlear implant, CT of the temporal bones should be obtained.

Alternatively, DFNB1 screening may be negative. A negative screening test does not mean that the deafness is not genetic. This distinction is subtle but important, and must be conveyed to parents before testing. In patients with a negative family history and a negative test for DFNB1, the probability that the deafness is genetic can be given based on the number of hearing siblings and the ethnic group.

CONCLUSIONS
1. The prevalence of DFNB1 in our cohort was 26% making it the most common identifiable etiology of nonsyndromic prelingual deafness. In families with ARNSD, the prevalence is 50%. The greater allelic heterogeneity shown in mixed-Hispanics and the high frequency of deaf probands carrying a single allelic variant of DFNB1 support extending the screening to noncoding regions of GJB2 and to the remaining DFNB1 locus.
2. The hearing phenotype was variable in terms of degree of impairment (from mild to profound), onset, symmetry, and progression, and there was no correlation with any specific genotype class. The hearing phenotype should not be the only criteria for offering mutational screening of DFNB1.
3. DFNB1 probands had no specific phenotypic manifestations cosegregating with HL. Gross motor development was normal. The frequency of CT abnormalities of the inner ear was low at 8%. Hearing habilitation was excellent.

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