Published in final edited form as:

Am J Med Genet A. 2006 November 15; 140(22): 2401–2415. doi:10.1002/ajmg.a.31525.

DNA Sequence Analysis of *GJB2*, Encoding Connexin 26:

Observations From a Population of Hearing Impaired Cases and Variable Carrier Rates, Complex Genotypes, and Ethnic Stratification of Alleles Among Controls

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Abstract

Mutations in *GJB2* are associated with hereditary hearing loss. DNA sequencing of *GJB2* in a cohort of hearing impaired patients and a multi-ethnic control group is reported. Among 610 hearing impaired cases, 43 DNA sequence variations were identified in the coding region of *GJB2* including 24 mutations, 8 polymorphisms, 3 unclassified variants (G4D, R127C, M163V), 1 controversial variant (V37I), and 7 novel variants (G12C, N14D, V63A, T86M, L132V, D159, 592_600delinsCAGTGTTCATGACATTC). Sixteen non-coding sequence variations were also identified among cases including the IVS1+1A>G mutation, 2 polymorphisms, and 13 novel variants. A diagnosis of *GJB2*-associated hearing loss was confirmed for 63 cases (10.3%). Heterozygous mutations were found in 39 cases (6.4%). Eleven cases carrying novel or unclassified variants (1.8 %) and 18 cases carrying the controversial V37I variant were identified (3%). In addition, 294 control subjects from 4 ethnic groups were sequenced for *GJB2*. Thirteen sequence variations in the coding region of *GJB2* were identified among controls including 2 mutations, 6 polymorphisms, 2 unclassified variants (G4D, T123N), 1 controversial variant (V37I), and 2 novel variants (R127L, V207L). Nine sequence variations were identified among controls in the non-coding regions in and around *GJB2* exon 2. Of particular interest among controls were the variability in carrier rates and ethnic stratification of alleles, and the complex genotypes among Asians, 47% of whom carried two to four sequence variations in the coding region of *GJB2*. These data provide new information about carrier rates for *GJB2*-based hearing loss in various ethnic groups and contribute to evaluation of the pathogenicity of the controversial V37I variant.

Keywords

GJB2, connexin 26; hereditary hearing loss; hereditary deafness; genetic hearing loss; genetic deafness

INTRODUCTION

Recent studies suggest that approximately 1 in every 300–600 children born in the US has a congenital sensorineural hearing loss significant enough to affect speech and language development [Downs, 1995; Mehl and Thomson, 1998, 2002]. More than 60% of congenital hearing loss in the United States is genetic in etiology and ~60% of genetic hearing loss is non-syndromic and autosomal recessive [Keats and Berlin, 1999]. Mutations in *GJB2* are the most common cause of non-syndromic, autosomal recessive, hereditary hearing loss, and may account for 10–40% of all congenital hearing loss depending upon ethnicity [Denoyelle et al., 1997, 1999; Kelsell et al., 1997; Estivill et al., 1998; Kelley et al., 1998; Morell et al., 1998; Scott et al., 1998; Fuse et al., 1999; Green et al., 1999; Kudo et al., 2000; Rabionet et al., 2000a; Marlin et al., 2001; Tekin et al., 2001; Wiszniewski et al., 2001; Iliades et al., 2002; Kenneson et al., 2002; Liu et al., 2002; Pampanos et al., 2002; Wu et al., 2002; Bayazit et al., 2003; Hwa et al., 2003; Lopponen et al., 2003; Ohtsuka et al., 2003; Roux et al., 2004; Ballana et al., 2005].

GJB2 encodes the gap junction beta-2 protein connexin 26. Connexins are transmembrane proteins with intracellular amino- and carboxy-terminal tails and four transmembrane domains. Six connexin proteins associate to form a transmembrane hexameric gap junction hemi-channel called a connexon. Connexons embedded in the surfaces of adjacent cells associate to form a gap junction channel. Cells connected by gap junctions use the channels to transfer ions and other small molecules across cell membranes. Connexons can be homomeric, made up of a single type of connexin, or heteromeric, made up of different connexin proteins. Gap junction channels can be homotypic, made of similarly composed connexons or heterotypic, made up of differently composed connexons [Unwin, 1989; Bennett et al., 1991; Bruzzone et al., 1996; Denoyelle et al., 1997; Kelsell et al., 1997; Kelley et al., 1998; Rabionet et al., 2000a, 2002; Kenneson et al., 2002]. In the inner ear, connexin 26, in association with other connexins, is thought to play a crucial role in potassium homeostasis [Unwin, 1989; Bennett et al., 1991; Bruzzone et al., 1996; White et al., 1998; Rabionet et al., 2002; Roux et al., 2004].

Mutations in *GJB2* are associated with syndromic and non-syndromic hearing loss although non-syndromic forms of hearing loss associated with mutations in *GJB2* are far more common than syndromic forms. Syndromic forms of hearing loss associated with particular mutations in *GJB2* typically present with skin findings and include keratitis-ichthyosis-deafness (KID) syndrome, hystrix-like ichthyosis-deafness (HID) syndrome, Vohwinkel syndrome (mutilating keratoderma with hearing loss), Bart–Pumphrey syndrome, palmoplantar keratoderma with deafness, and a unique phenotype with psoriasiform skin lesions, involvement of mucous membranes and teeth, and hearing loss [Richard et al., 1998a,b, 2002, 2004; White et al., 1998; Maestrini et al., 1999; Heathcote et al., 2000; Kelsell et al., 2000; Kelsell et al., 2001; Rouan et al., 2001; Rabionet et al., 2002; van Geel et al., 2002; van Steensel et al., 2002; Brown et al., 2003]. Non-syndromic hearing loss associated with mutations in *GJB2* can be inherited in an autosomal dominant or autosomal recessive manner although recessive cases occur far more commonly than dominant cases [Denoyelle et al., 1997, 1998; Kelsell et al., 1997; Estivill et al., 1998; Kelley et al., 1998; Morell et al., 1998; Scott et al., 1998; Cohn and Kelley, 1999; Green et al., 1999; Kudo et al., 2000; Morle et al., 2000; Hamelmann et al., 2001; Kenna et al., 2001; Rouan et al., 2001; Iliades et al., 2002; Kenneson et al., 2002; Wu et al., 2002; Genetic Evaluation of Congenital Hearing Loss Expert Panel, 2004; Roux et al., 2004; Ballana et al., 2005].

DNA-based sequencing of *GJB2* is increasingly utilized in the evaluation of the infant and child with hearing loss. In 2002, the American College of Medical Genetics published guidelines for the etiologic diagnosis of congenital hearing loss that included molecular

genetic analysis of *GJB2* [Genetic Evaluation of Congenital Hearing Loss Expert Panel, 2004]. To date, more than 100 mutations, polymorphisms, and unclassified variants have been described in *GJB2* [Kenneson et al., 2002; Ballana et al., 2005]. Three non-syndromic recessive mutations, 35delG, 167delT and 235delC have been found at high frequency in Caucasian, Ashkenazi Jewish, and Asian populations, respectively [Denoyelle et al., 1997; Kelsell et al., 1997; Estivill et al., 1998; Kelley et al., 1998; Morell et al., 1998; Scott et al., 1998; Fuse et al., 1999; Green et al., 1999; Kudo et al., 2000; Rabionet et al., 2000b; Kenneson et al., 2002; Roux et al., 2004; Ballana et al., 2005]. Strong genotype/phenotype correlations have been described for some non-syndromic mutations in *GJB2* [Cohn and Kelley, 1999; Cohn et al., 1999; Lim et al., 2003; Azaiez et al., 2004; Cryns et al., 2004; Oguchi et al., 2005].

The identification of known mutations in *GJB2* after DNA sequencing analysis permits definitive etiologic diagnosis of *GJB2*-based hearing loss and improves the precision of genetic counseling and risk assessment for patients and families. The identification of novel or unclassified variants or variants of unclear pathogenicity results in equivocal DNA test results and complicates genetic counseling and risk prediction. Of particular concern are novel missense variations [ACMG Laboratory Practice Committee Working Group, 2000].

The PolyPhen (<http://coot.embl.de/PolyPhen/>) and SIFT (<http://blocks.fhcrc.org/sift/SIFT.html>) prediction tools offer an in silico mechanism by which to investigate the potential pathogenicity of novel missense variations, but the predictions made by these two sequence analysis tools can be contradictory and the accuracy of both programs averages considerably less than 100%. As a result, the predictions derived from the PolyPhen and SIFT tools must be interpreted with extreme caution [Tchernitchko et al., 2004].

Alternatively, a number of multiple sequence alignment tools are available for evaluating the evolutionary conservation of amino acids across gene families and species. Multiple sequence alignment analyses are based largely on the assumptions that evolutionarily conserved amino acids are more likely to be functionally important than nonconserved amino acids, and substitutions involving chemically similar amino acids are less likely to be damaging than substitutions involving chemically different amino acids [Miller and Kumar, 2001]. However, multiple sequence alignment analyses can only provide an illustration of the degree of evolutionary conservation of an amino acid; they cannot predict with certainty the consequences of specific missense variations [Miller and Kumar, 2001].

In vitro functional studies can be used, in some cases, to investigate the potential consequences of a sequence variation, but such analyses are time-consuming, labor intensive, and beyond the capacity of most diagnostic laboratories. In addition, the relevance of data derived from in vitro functional studies is highly dependent on the fidelity with which an in vitro system mimics the in vivo environment. Further, the results of separate in vitro studies can be contradictory, especially if investigators use different cell lines or different expression vectors, and the findings of in vitro studies are not always supported by clinical findings. As such, the results of in vitro studies must be interpreted with caution [Richard et al., 1998b; Martin et al., 1999; Thönnissen et al., 2002; Bruzzone et al., 2003; Wang et al., 2003].

Ultimately, confirmation of the pathogenicity of a DNA sequence variation requires repeated documentation that the variation segregates with a disorder in families, detection of statistically significant differences in the frequency of the variation between large populations of cases and controls, and extensive genotype–phenotype analyses. These data generally take considerable time and effort to collect, and for etiologically heterogeneous disorders such as hearing loss, these analyses can be confounded by phenocopies of the

disorder [Denoyelle et al., 1997, 1999; Kelsell et al., 1997; Estivill et al., 1998; Morell et al., 1998; Kelley et al., 1998; Scott et al., 1998; Cohn and Kelley, 1999; Cohn et al., 1999; Fuse et al., 1999; Green et al., 1999; ACMG Laboratory Practice Committee Working Group, 2000; Griffith et al., 2000; Kudo et al., 2000; Rabionet et al., 2000a,b; Wilcox et al., 2000; Abe et al., 2001; Marlin et al., 2001; Tekin et al., 2001; Wiszniewski et al., 2001; Bason et al., 2002; Iliades et al., 2002; Kenneson et al., 2002; Liu et al., 2002; Pampanos et al., 2002; Wu et al., 2002; Bayazit et al., 2003; Hwa et al., 2003; Lim et al., 2003; Lopponen et al., 2003; Ohtsuka et al., 2003; Azaiez et al., 2004; Cryns et al., 2004; Feldmann et al., 2004; Shi et al., 2004; Roux et al., 2004; Ballana et al., 2005; Oguchi et al., 2005].

In this study, 610 hearing impaired probands were evaluated for genetic deafness through DNA-based sequencing of the *GJB2* gene. A multi-ethnic control group consisting of 294 control subjects (72 African-American, 72 Asian, 74 Caucasian, and 76 Hispanic) was also evaluated to assess carrier rates, allele frequencies, and stratification of alleles across ethnic groups. These data provide new information about the carrier rates for *GJB2*-based hearing loss in various ethnic groups and contribute to evaluation of the pathogenicity of the controversial *GJB2* variant V37I.

MATERIALS AND METHODS

Subjects

Subjects with hearing loss were identified and recruited from the outpatient clinical care centers of the Bobby R. Alford Department of Otolaryngology—Head and Neck Surgery and the Department of Molecular and Human Genetics of Baylor College of Medicine as previously described [Tang et al., 2005], and from the Baylor DNA Diagnostic Laboratory of Baylor College of Medicine. Ethnicity of cases was self-described and was not always known. Severity of hearing loss in cases was not always known.

Control Subjects

Control specimens were obtained from the Baylor Polymorphism Resource (<http://www.cardiogene.org>) as previously described [Tang et al., 2005]. Control specimens were anonymized at the time of collection. Ethnicity of controls was self-reported. Nothing is known about the health or hearing status of the control subjects. Because of the anonymous nature of the control specimens, there is no opportunity to explore the hearing status of any control subject.

Institutional Review Board Review and Approval

This work was approved by the Institutional Review Board of Baylor College of Medicine.

Specimen Collection

Blood was collected by peripheral venipuncture. For controls and some cases, standard Epstein–Barr Virus-mediated transformation protocols were used to establish lymphoblastoid cell lines.

DNA Isolation

DNA was isolated according to the manufacturer's specifications as follows: from blood, using the PUREGENE® DNA Purification Kit for whole blood and bone marrow; and from cultured cells using the PUREGENE® DNA Purification Kit for cells, tissue, body fluids, and Gram-negative bacteria (Gentra Systems, Inc., Minneapolis, MN).

PCR and DNA Sequencing

PCR and sequencing of the coding region of *GJB2* was conducted as previously described [Tang et al., 2005]. PCR and sequencing of the exon 1 splice donor site of *GJB2* was conducted using the following primers: forward primer Cx26U1 5'-TGT GGG GTG CGG TTA AAA GGC GCC ACG G-3'; and reverse primer Cx26U2 5'-GCA ACC GCT CTG GGT CTC GCG GTC CCT-3'. PCR was conducted with 15 pmol of each primer, 10% DMSO, 1.25 U Taq DNA polymerase (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), 25 mM each dNTP, and 1× PCR buffer as provided by the manufacturer, in a total volume of 50 µl. PCR was conducted as follows: 94°C for 2 min; 40 cycles of 94°C for 30 sec, 64°C for 30 sec, 70°C for 1 min; and 70°C for 5 min. PCR fragments were sequenced using the forward and/or reverse primers and the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). DNA sequencing was conducted as previously described [Tang et al., 2005]. The exon 1 splice donor site was sequenced for all cases. Exon 1 was not sequenced in controls.

DNA Sequence Analysis

DNA sequence variations were identified by comparison of subject DNA sequence to *GJB2* reference sequences: Genbank Accession Numbers M86849, U43932, and/or XM_007169. Numbering of *GJB2* nucleotides starts with the A of the ATG start codon in exon 2 as position number +1.

Electropherograms were evaluated by visual inspection and pairwise alignment to reference sequences using the BCM Search Launcher BLAST2 Pairwise Sequence Alignment Tool from the Human Genome Sequencing Center of Baylor College of Medicine (<http://searchlauncher.bcm.tmc.edu/seq-search/alignment.html>), and/or by interpretation using the most up to date version of Mutation Surveyor software (Softgenetics, Inc., State College, PA) that was available at the time of sequence analysis.

Amino Acid Sequence Analysis

Connexin 26 protein domains are determined according to Kelley et al. [1998]. Amino acid sequence variations for deletion, deletion/insertion, and frameshift mutations were determined by translation of the altered nucleotide sequence in the ExPASy Translate Tool (<http://ca.expasy.org/tools/dna.html>).

Novel missense variations in connexin 26, accession number P29033, were evaluated for potential pathogenicity using the PolyPhen (<http://coot.embl.de/PolyPhen/>) and SIFT (<http://blocks.fhcrc.org/sift/SIFT.html>) prediction tools and by multiple sequence alignment. Connexin 26 amino acid sequences from human (P29033), mouse (Q00977), lowland gorilla (Q8MHW5), gibbon (Q7JGL3), rhesus monkey (Q8MIT8), sheep (P46691), orangutan (Q8MIT9), and Norway rat (P21994), and amino acid sequences from human connexins 30 (O95452), 30.3 (Q9NTQ9), 31 (O75712), 31.1 (O95377), 32 (P08034), and 43 (P17302) were obtained from the Proteome Browser Gateway of the UCSC Genome Bioinformatics web site (<http://genome.ucsc.edu/cgi-bin/pbGateway?command=start>). Amino acid sequence accession numbers are indicated in the parentheses. Amino acid sequences were compared using the BCM Search Launcher ClustalW 1.8 DNA/Protein multiple sequence alignment tool from the Human Genome Sequencing Center of Baylor College of Medicine (<http://searchlauncher.bcm.tmc.edu/>).

Sequence Variation Nomenclature

Sequence variations in *GJB2* are described according to the nomenclature recommendations of den Dunnen and Antonarakis [2001].

Data Analysis

Alleles and genotypes were recorded in spreadsheets using Microsoft Excel® 2003 software. Spreadsheets were categorized according to subject. DNA sequence variations, genotypes, and ethnicity of subjects, if known, were tabulated. Cases and controls were tabulated separately.

Statistical Analysis

Fisher's exact test of 2×2 contingency tables was used to calculate the two-tailed *P*-values associated with differences in allele frequencies of certain DNA sequence variations in case and control groups. Fisher's exact test was performed using the Graph-Pad QuickCalcs Online Calculator for Scientists (<http://www.graphpad.com/quickcalcs/index.cfm>).

RESULTS

DNA and Amino Acid Sequence Variations in *GJB2* Among Hearing Loss Cases

In the coding region of *GJB2*, 43 DNA sequence variations were identified among 1,220 alleles tested from 610 hearing impaired cases (Table I). Variations included 24 pathogenic mutations, 8 polymorphisms, 3 unclassified variants (G4D, R127C, M163V), and 7 novel variants (G12C, N14D, V63A, T86M, L132V, D159, 592_600delinsCAGTGTTTCATGACATTC). The V37I variant was also identified among cases. The pathogenicity of the V37I variation in *GJB2* is controversial as this variant has been previously reported in association with hearing loss and as a polymorphism [Kelley et al., 1998; Cohn and Kelley, 1999; Rabionet et al., 2000b; Wilcox et al., 2000; Abe et al., 2001; Marlin et al., 2001; Bason et al., 2002; Kenneson et al., 2002; Wu et al., 2002; Hwa et al., 2003; Ohtsuka et al., 2003; Cryns et al., 2004; Feldmann et al., 2004; Roux et al., 2004; Shi et al., 2004; Ballana et al., 2005].

In addition, 16 non-coding DNA sequence variations were detected among cases (Table I). These included 2 polymorphisms, 13 novel variants, and 1 pathogenic mutation (IVS1+1G>A) [Denoyelle et al., 1999; Green et al., 1999; Ballana et al., 2005]. A previously reported 765C>T polymorphism was also detected in a heterozygous state in 19 of 51 cases scored for this allele and in a homozygous state in 5 of 51 cases scored for this allele [Roux et al., 2004; Ballana et al., 2005]. Of the 102 total case alleles scored for the 765C>T polymorphism, 29 alleles (28%) were positive for the polymorphism (data not shown).

A definitive etiologic diagnosis of *GJB2*-based deafness was derived for 63 cases, 10.3% of those tested (Table II). In 39 cases, 6.4% of the total number of cases analyzed, only a single recessive pathogenic mutation was identified. In 11 cases (1.8%), the interpretation of DNA test results was complicated by the identification of an unclassified or novel sequence variation in the coding region of *GJB2*. Eighteen individuals carrying the controversial V37I variant were identified (3%). These included 4 heterozygotes, 10 homozygotes, and 4 compound heterozygotes with a known pathogenic mutation (Table II).

In addition to the hearing impaired cases reported here, five additional hearing loss cases have been previously described [Brown et al., 2003; Tang et al., 2005]. In one case, a novel, heterozygous, de novo 424T>C transition in *GJB2* that is predicted to result in the substitution of the phenylalanine at position 142 for a leucine (F142L) was detected in a child with psoriasiform skin lesions, involvement of the mucous membranes and teeth, and sensorineural hearing loss [Brown et al., 2003], and four hearing impaired cases heterozygous for the *SLC26A5* IVS2-2A>G transition were negative for pathogenic

mutations in *GJB2*, although one was heterozygous for the *GJB2* V27I polymorphism [Tang et al., 2005].

DNA and Amino Acid Sequence Variations in *GJB2* Among Controls

A total of 294 control subjects, 588 control chromosomes, were sequenced for the coding region of *GJB2*: 72 control subjects were African-American (37 males, 35 females), 76 were Hispanic (39 males, 37 females), 72 were Asian (31 males, 41 females), and 74 were Caucasian (37 males, 37 females). Among controls, 13 DNA sequence variations were identified in the coding region of *GJB2*. Three alleles previously reported to be associated with hearing loss were found (Table III): the 35delG mutation was found in a heterozygous state in 3 Caucasian control subjects ($q = 0.02$); the 235delC mutation was found in a heterozygous state in 1 Asian control subject ($q = 0.007$); and the controversial V37I variation was found in 11 of 144 Asian alleles tested ($q = 0.076$) [Denoyelle et al., 1997; Kelsell et al., 1997; Estivill et al., 1998; Morell et al., 1998; Kelley et al., 1998; Scott et al., 1998; Cohn and Kelley, 1999; Cohn et al., 1999; Fuse et al., 1999; Green et al., 1999; Gasparini et al., 2000; Kudo et al., 2000; Rabionet et al., 2000b; Wilcox et al., 2000; Bason et al., 2002; Kenneson et al., 2002; Hwa et al., 2003; Ohtsuka et al., 2003; Cryns et al., 2004; Roux et al., 2004; Shi et al., 2004; Ballana et al., 2005].

As shown in Table III, six polymorphisms were also found in the coding region of *GJB2* among controls. These include V27I, M34T, F83L, E114G, V153I, and I203T [Kelley et al., 1998; Scott et al., 1998; Fuse et al., 1999; Griffith et al., 2000; Kenneson et al., 2002; Kudo et al., 2000; Feldmann et al., 2004; Roux et al., 2004; Ballana et al., 2005]. Two unclassified variants, G4D and T123N, were also identified among controls [Park et al., 2000; Dahl et al., 2001; Hwa et al., 2003; Ohtsuka et al., 2003; Roux et al., 2004; Shi et al., 2004; Ballana et al., 2005], and two novel variants, R127L and V207L [Ballana et al., 2005], were found.

Nine DNA sequence variations were identified in the non-coding regions of *GJB2* in and around exon 2 among controls: four variations were found 5' of the coding region; and five were found 3' of the coding region (Table III). Two of these variants, -15C>T and 765C>T, have been previously classified as polymorphisms [Roux et al., 2004; Ballana et al., 2005]. The -34C>T, -15C>T, 785A>T, and 792C>T variants were found in African-American and Hispanic controls but not in Asian or Caucasian controls. The -57G>T variant was found only in Asian controls, while the -6T>A, 777A>G, and 794T>A variants were found only in African-American controls. The 765C>T polymorphism was found at high frequency in all ethnic groups (Table III).

In the African-American control group, no DNA sequence variations were found in the coding region of *GJB2* among the 144 chromosomes analyzed (Table III). However, eight non-coding sequence variations were identified: -34C>T ($q = 0.28$); -15C>T ($q = 0.03$); -6T>A ($q = 0.02$); 765C>T ($q = 0.45$); 777A>G (0.007); 785A>T ($q = 0.25$); 792C>T ($q = 0.25$); and 794T>A ($q = 0.007$). Twelve African-American controls were homozygous for the 765C>T polymorphism, while 40 were heterozygous (data not shown). Four of 72 African-American controls were homozygous for the -34C>T variation, the 765C allele, the 785A>T variation, and the 792C>T variation (data not shown). The 785A>T and 792C>T variations were always observed together and one occurrence of the 794T>A variation occurred in an individual heterozygous for the -34C>T, 765C>T, 785A>T, and 792C>T variations. Only four occurrences of -34C>T were observed without 785A>T and 792C>T. Further, of the 32 subjects heterozygous for the -34C>T variation, 19 were also heterozygous for the 765C>T, 785A>T, and 792C>T variations while 9 were heterozygous for 785A>T and 792C>T, but homozygous for the 765C allele (data not shown).

In the Hispanic control group, 21 of 76 individuals analyzed (28%) were heterozygous for the V27I polymorphism and 2 (2.6%) were homozygous for V27I (Table IV). One copy of the M34T polymorphism was found in the Hispanic control group (Table III). A novel R127L variant was also found in the Hispanic control group (Table III), in a heterozygous state in an individual also heterozygous for the -34C>T and 765C>T variations (data not shown). Five non-coding sequence variations were also found in this group (Table III): -34C>T ($q = 0.03$); -15C>T ($q = 0.02$); 765C>T ($q = 0.3$); 785A>T ($q = 0.007$); and 792C>T ($q = 0.007$). The single 792C>T allele that was identified in this group was found in one of the five individuals heterozygous for the -34C>T variation. This individual was also heterozygous for the V27I, 765C>T, and 785A>T variations (data not shown). Further, 8 Hispanic controls were homozygous for the 765C>T polymorphism while 28 were heterozygous (data not shown).

Among Asian controls, the V27I polymorphism occurred at high frequency ($q = 0.32$). The V37I and E114G DNA sequence variations also occurred at high frequency in the Asian control group but were absent from all other ethnic groups. The V37I variant occurred at an allele frequency of 0.076 (7.6%) among Asians while the E114G polymorphism occurred at an allele frequency of 0.26 (Table III). In addition, the I203T polymorphism was observed in the Asian control group at a frequency of 0.014. One copy each of the unclassified variants G4D and T123N were observed and one copy each of two novel variants, R127L and V207L, were observed [Dahl et al., 2001; Park et al., 2000; Hwa et al., 2003; Ohtsuka et al., 2003; Roux et al., 2004; Shi et al., 2004; Ballana et al., 2005]. One individual carrying the 235delC mutation was identified. In the non-coding region, a DNA sequence variation 57 nucleotides upstream of the start codon in exon 2, -57G>T, was observed in a heterozygous state in 4 individuals ($q = 0.03$) and the 765C>T polymorphism was observed in a heterozygous state in 40 individuals, and in a homozygous state in 16 individuals ($q = 0.5$, data not shown).

In the Caucasian control group, three copies of the common 35delG mutation were found ($q = 0.02$). Three copies of the common M34T polymorphism were also found ($q = 0.02$). Two additional polymorphisms were found among Caucasians: F83L and V153I; both at a gene frequency of 0.007. Finally, 19 copies of the 765C>T polymorphism were found in Caucasian controls, $q = 0.13$ (Table III). Fifteen Caucasian controls were heterozygous for the 765C>T polymorphism while 2 were homozygous (data not shown).

Table IV shows the genotypes detected among controls. No control was homozygous or compound heterozygous for any mutation(s) known to be associated with hearing loss.

Evaluation of Novel Missense Variations

The seven novel missense variations identified in cases and controls in this study were analyzed using the PolyPhen (<http://coot.embl.de/PolyPhen/>) and SIFT (<http://blocks.fhcrc.org/sift/SIFT.html>) prediction tools and by multiple sequence alignment. A summary of these analyses is shown in Table V.

The PolyPhen prediction tool predicts the N14D and R127L variations to be probably damaging and the G12C and T86M variations to be possibly damaging, while the V63A, L132V, and V207L variations are predicted to be benign (data not shown). The SIFT prediction tool predicts that the G12C, V63A, T86M, and L132V variations would not be tolerated, while the N14D, R127L, and V207L variations would be tolerated (data not shown).

Comparison of the amino acid sequence of human connexin 26 with connexin 26 from mouse, lowland gorilla, gibbon, rhesus monkey, sheep, orangutan, Norway rat, and human

connexins 30, 30.3, 31, 31.1, 32, and 43 reveals evolutionary conservation of amino acids G12, N14, V63, and L132 across all aligned species and beta gap junction proteins (Table V). Amino acids V63 and L132 were also conserved in the human alpha gap junction protein connexin 43. The amino acid T86 was conserved across all aligned connexin 26 proteins and human connexins 30 and 32. The amino acid R127 was conserved across all aligned connexin 26 proteins and human connexin 30. The amino acid V207 was conserved only among connexin 26 proteins from human, lowland gorilla, gibbon, rhesus monkey, sheep, and orangutan, and human connexins 30 and 32. Interestingly, the valine (V) found at position 207 in human connexin 26 was replaced with a leucine (L) in human connexins 30.3 and 31.1 (data not shown).

DISCUSSION

DNA sequencing analysis of the *GJB2* gene in hearing loss patients resulted in an etiologic diagnosis of hearing loss in 10.3% of cases tested in this population. Equivocal results were obtained for 11.2% of cases due to the detection of unclassified, novel or controversial coding sequence variations, or the detection of only a single recessive mutation in *GJB2*. A negative result was obtained for 78.5% of cases (Table II). These data support predictions from prior studies that *GJB2* may be responsible for a significant percentage of early childhood onset hearing loss in many populations [Denoyelle et al., 1997; Estivill et al., 1998; Kelley et al., 1998; Morell et al., 1998; Green et al., 1999; Kudo et al., 2000; Kenneson et al., 2002; Roux et al., 2004].

Three previously reported unclassified variants, G4D, R127C, and M163V, were identified among cases analyzed in this study (Table I) [Dahl et al., 2001; Marlin et al., 2001; Hwa et al., 2003; Roux et al., 2004; Ballana et al., 2005]. The G4D sequence variation was previously reported by Roux et al. [2004] in a heterozygous state in one control subject and by Hwa et al. [2003], in a heterozygous state in seven case subjects. In this study, the G4D variation was found in a heterozygous state in one case subject who also carried, in a heterozygous state, the -34C>T sequence variation (Table II). This subject was African-American and reported no known family history of hearing loss (data not shown). Because family members of this case were not available for analysis, it is not clear whether the G4D and -34C>T variations occur in cis or whether the G4D allele might be a de novo mutation. The G4D sequence variation was also identified in this study in a heterozygous state in one Asian control specimen (Tables III, IV). No other DNA sequence variations were observed in this control subject. Nothing is known about the hearing status or family history of controls used in this study. From these data, the pathogenicity of the G4D DNA sequence variation in *GJB2* cannot be determined definitively.

Neither the R127C nor M163V variations, which were identified among cases in this study, were found among the 588 control chromosomes analyzed (Tables I, III). In this study, the R127C variation was found in a heterozygous state in one hearing impaired case (Table II). The R127C variation has been previously reported: in one case the 35delG mutation was also found [Dahl et al., 2001]; and in another report, the context of the variant was not described [Ballana et al., 2005].

A second DNA sequence variation involving amino acid R127, R127L, was found in a heterozygous state in two controls analyzed in this study (Tables III, IV). One of the controls was Asian and was also heterozygous for the V27I, E114G, and 765C>T polymorphisms (Tables III, IV, data not shown). The other control was Hispanic and was also heterozygous for the -34C>T variation and the 765C>T polymorphism (Tables III, IV, data not shown). The R127L variant has not been previously reported [Ballana et al., 2005].

An R127H variation in *GJB2* has been previously described as a polymorphism [Estivill et al., 1998; Roux et al., 2004; Ballana et al., 2005]. It is possible that, like R127H, the R127C and R127L variants are polymorphisms; however, due to the paucity of information available about the R127C and R127L variations, determination of their pathogenicity is not possible at this time.

The M163V variation was observed in a heterozygous state in one hearing impaired case in this study (Tables I, II). This case was also heterozygous for the novel T86M variation and the R127H polymorphism. The M163V DNA sequence variation was previously reported by Marlin et al. [2001] in a heterozygous state in a hearing impaired case. Further study of the M163V DNA sequence variation is required for determination of its pathogenicity.

Seven novel variations, G12C, N14D, V63A, T86M, L132V, D159, 592_600delinsCAGTGTTCATGACATTC, were identified among cases in this study (Table I). Little is known about the individuals in whom novel sequence variations were identified, except that they carry a clinical diagnosis of hearing loss. None of the novel sequence variations identified among cases in this study was found among the 588 control chromosomes analyzed in this study (Tables I, III).

The 592_600delinsCAGTGTTCATGACATTC mutation is a complex mutation (Tables I, II). In addition to the loss of nucleotides 592–600, this mutation includes an insertion of 17 nucleotides comprised of a duplication of nucleotides 575–586 (5'-CAGTGTTCATGA-3') followed by insertion of the nucleotides 5'-CATTC-3' (data not shown). It is reasonable to predict that the novel 592_600delinsCAGTGTTCATGACATTC (V198fsX201) mutation carries a high a priori risk of being pathogenic because most of the fourth transmembrane domain of connexin 26 would be lost with this mutation [Kelley et al., 1998].

The novel 477C>T DNA sequence variation is not expected to alter the amino acid sequence of connexin 26 (Tables I, II). As a result, it is reasonable to predict that the 477C>T DNA sequence variation underlying D159 might be benign, unless a regulatory element is interrupted by this DNA sequence variation.

One of the G12C variations found in this study was identified in a heterozygous state in a hearing impaired case of Hispanic ancestry (Table II). This subject reported a family history of hearing loss consistent with an autosomal dominant pattern of inheritance (data not shown), however, because additional family members of this subject have not been available for analysis, it is impossible to determine whether the G12C variation is segregating with the hearing loss in this family. Nothing is known about the other two G12C carriers identified among cases in this study other than that they carry a clinical diagnosis of hearing loss.

Other mutations at amino acid position G12 of connexin 26 have been previously reported by others: a G12R mutation has been reported in association with KID syndrome [Richard et al., 2002]; and a G12V mutation has been reported in association with non-syndromic deafness [Rabionet et al., 2000b]. Further, mutations in the G12 amino acid position have been reported in connexin 31 (G12R and G12D) in association with erythrokeratoderma variabilis (EKV) [Richard et al., 1998a], and in connexin 32 (G12S) in association with Charcot-Marie-Tooth disease [Bergoffen et al., 1993]. Although we cannot be certain at this time that the G12C variation is pathogenic, these prior reports support the potential functional importance of the G12 amino acid position in the function of various gap junction proteins [Bergoffen et al., 1993; Richard et al., 1998a, 2002; Rabionet et al., 2000b; Richard, 2001].

For the G12C variation and the other four novel missense variations described in cases, the high degree of evolutionary conservation of these amino acids and the findings of the

PolyPhen and SIFT analyses suggest that these amino acids may play an important role in gap junction function but as is shown in Table V, the results of the PolyPhen and SIFT prediction programs do not always agree, and for amino acid T86 conservation is not complete across all aligned beta-gap junction proteins. Identification and thorough evaluation of additional individuals and families carrying the G12C, N14D, V63A, T86M, and L132V variations are required to determine whether these variations are pathogenic or polymorphic.

Two dominant mutations (D50N and R75W) were found in the coding region of *GJB2* among cases (Tables I, II). Neither of these mutations was found among the 588 control chromosomes analyzed in this study. The D50N mutation has been previously reported in association with the autosomal dominant KID syndrome [Richard et al., 2002]. The D50N mutation was found in this case group in a heterozygous state in a patient with clinical findings consistent with a diagnosis of KID syndrome (data not shown, Table II). The R75W mutation has been previously reported in association with autosomal dominant deafness and palmoplantar keratoderma [Richard et al., 1998b]. In this case group, the R75W mutation was found in a heterozygous state in a patient with hearing loss (Table II). This patient also has a hearing-impaired parent and sibling. Although neither the parent nor the sibling was counted in this study group, both were found to be heterozygous for the R75W mutation, consistent with a dominant pattern of inheritance. Little is known about the presence or absence of additional clinical features in this family, but rough skin was noted (data not shown).

Of particular interest in this study is the data derived from the control population. In 144 African-American chromosomes analyzed, DNA sequence variations adjacent to the coding region of *GJB2* occurred at a frequency of 1.3; however, it was surprising that no DNA sequence variations were found in the coding region of *GJB2* (Table III). The novel -34C>T, 785A>T and 792C>T variations are certainly polymorphisms as they occur on African-American chromosomes at a rate of 28, 25 and 25%, respectively (Table III). The -15C>T and -6T>A variations are also likely to be polymorphisms as they occur at a rate of 2.7 and 2.1%, respectively. Further investigation is required to ascertain the nature of the novel 777A>G and 794T>A variations; however, it is reasonable to expect that they too are polymorphisms. It is interesting to note that the 785A>T and 792C>T variations always occurred together and never occurred in the absence of the -34C>T variation. These data suggest that these three variations may frequently occur in cis. From these data, it can be concluded that polymorphisms in the coding region of *GJB2* are not common among African-Americans although non-coding region polymorphisms in *GJB2* are quite common among African-Americans. These data also suggest that *GJB2* is not a common cause of hearing loss in African-Americans.

No known mutations in *GJB2* were found in among Hispanic controls although four known polymorphisms were identified and four novel variants were identified (Table III). Overall, DNA sequence variations in and around the coding region of *GJB2* occurred at a frequency of 0.5 among Hispanics. The V27I polymorphism was found at high frequency in this group ($q = 0.16$). Identification of the M34T polymorphism among Hispanic controls suggests that this variation is not limited to Caucasian populations. Further, as noted above, a novel R127L variant was identified in a heterozygous state in an individual also heterozygous for the -34C>T and 765C>T variations (data not shown). These data suggest that *GJB2* is not a common cause of deafness in the Hispanic community.

Among Caucasian controls, variability in *GJB2* DNA sequence was far less frequent than that observed in other groups in terms of the overall number of DNA sequence variations identified, with DNA sequence variations in and around the coding region of *GJB2*

occurring at a frequency of 0.2, but deafness-causing mutations occurred at a frequency of 0.02, a higher rate than in any other ethnic group. The 35delG mutation and the M34T polymorphism were both observed in this control group at a frequency of $q=0.02$ (Tables III, IV). These frequencies are similar to what has been reported by others [Denoyelle et al., 1997; Kelsell et al., 1997; Estivill et al., 1998; Kelley et al., 1998; Morell et al., 1998; Green et al., 1999; Gasparini et al., 2000; Kenneson et al., 2002; Roux et al., 2004; Ballana et al., 2005].

Among Asian controls, DNA sequence variations in and around the coding region of *GJB2* were observed at a frequency of 1.2 (Table III). Only one known pathogenic mutation, 235delC, was identified in a heterozygous state in a single individual among Asian controls. Two unclassified variants (G4D, T123N), three novel variants (–57G>T, R127L, V207L), four polymorphisms (V27I, E114G, I203T, 765C>T), and one controversial variant (V37I) were also found (Table III).

For the two novel missense variations described in controls, predictions of pathogenicity are not possible. The R127 and V207 positions are not completely conserved across aligned beta-gap junction proteins and the PolyPhen and SIFT analyses suggest that certain variations at these amino acid positions might be tolerated but as is shown in Table V, the results of the PolyPhen and SIFT prediction programs do not always agree. Identification and thorough evaluation of additional individuals and families carrying the R127L and V207L variations are needed to determine whether these variations are polymorphic or pathogenic.

The complexity of genotypes observed in the Asian control group was of particular interest (Table IV). Forty-seven percent of Asian controls carried two to four DNA sequence variations in the coding region of *GJB2* including: 1 V27I homozygote; 1 V37I homozygote; 1 V27I/V37I compound heterozygote; 1 V27I/T123N compound heterozygote; 1 V37I/I203T compound heterozygote; 18 V27I/E114G compound heterozygotes; 7 V27I/V27I +E114G/E114G double homozygotes; 3 V27I/V37I/E114G triple heterozygotes; and 1 V27I/E114G/R127L triple heterozygote (Table IV). Further, one Asian control was heterozygous for the unclassified variant G4D and one was heterozygous for a novel variant, V207L. A heterozygous V27I polymorphism was identified in conjunction with a heterozygous E114G polymorphism in 22 individuals. V27I occurred without E114G in only nine individuals, while E114G occurred without V27I in only one individual. Seven individuals were homozygous for both V27I and E114G. These data suggest that V27I and E114G may frequently occur in cis (Table IV).

Among controls, 3 copies of the 35delG allele, 4 copies of the M34T allele, and 11 copies of the V37I allele were identified in 588 alleles tested (Table III). Among cases, 120 copies of the 35delG allele, 19 copies of the M34T allele, and 28 copies of the V37I allele were identified in 1,220 alleles tested (Table I). As shown in Table VI, the difference in the allele frequency of the 35delG allele between cases and controls is statistically significant ($P<0.0001$). In contrast, the difference in the allele frequency of the M34T allele between cases and controls is not statistically significant ($P=0.1770$). This finding is similar to those of prior studies that suggest the M34T variant is a polymorphism [Griffith et al., 2000; Feldmann et al., 2004; Roux et al., 2004]. Further, the difference in the allele frequency of the V37I allele between cases and controls is not statistically significant ($P=0.4895$). The V37I variant has been previously reported as a pathogenic mutation and as a polymorphism [Kelley et al., 1998; Cohn and Kelley, 1999; Rabionet et al., 2000b; Wilcox et al., 2000; Abe et al., 2001; Bason et al., 2002; Kenneson et al., 2002; Hwa et al., 2003; Ohtsuka et al., 2003; Cryns et al., 2004; Roux et al., 2004; Shi et al., 2004; Ballana et al., 2005]. In this study, all 11 copies of the V37I variant found among controls occurred in Asian controls

(Table III). As shown in Table IV, 1 individual among the 72 Asian controls analyzed was found to be homozygous for V37I. These data suggest that the V37I variant occurs among Asians at an allele frequency of approximately 0.076 (7.6%).

In summary, these data support and extend prior studies of the association between *GJB2* and hereditary hearing loss. The stratification of alleles among various ethnic groups informs evaluation of the potential pathogenicity of DNA sequence variations in *GJB2* and interpretation of DNA test results in diagnostic settings, and contributes to risk assessment and genetic counseling of hearing impaired patients and families. Of particular interest is the complexity of genotypes found among Asian controls, demonstrating the importance of ethnicity information for the interpretation of DNA-based test results and risk assessment in hearing impaired patients and families. These data also contribute new information about the controversial V37I allele, adding to discussions of whether this allele has any pathogenic effect.

Acknowledgments

Grant sponsor: Allbritton-Alford Fund; Grant sponsor: Alkek Foundation; Grant sponsor: Brown Foundation.

The authors thank Laura Molinari, Susan D. Fernbach, John W. Belmont, and the Baylor Polymorphism Resource (<http://www.cardiogene.org>) for the availability of the control specimens used in this work. The authors also thank the current and prior members of The Hearing Center and The Audiology Center of Texas Children's Hospital including Jody Hamond, Ross Tonnini, Claudia Emery, Janet Maxian, and Heidi Heilstedt. The authors also thank Allason James, JoAnna Leshner, Tony Cirigliano, and Kelly Hoon for technical assistance, and Dr. Lee-Jun Wong for critical evaluation of this manuscript. This work was supported by the Allbritton-Alford Fund, the Alkek Foundation, and the Brown Foundation (to H.Y.T. and R.L.A.).

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TABLE I

Variations in *GJB2* Identified by DNA Sequencing of Hearing Loss Cases

Amino acid variation	Nucleotide variation	Gene/mRNA/protein domain ^d	Characterization of variant	Number of occurrences total n =1,220
	-3464C>G	5' of exon 1	Novel	2
	-3364T>G	5' of exon 1	Novel	1
	-3360T>C	5' of exon 1	Novel	1
	-3275G>C	5' UTR, exon 1	Novel	1
	-3265G>A	5' UTR, exon 1	Novel	1
	IVS1+1G>A (-3170G>A)	IVS1 splice donor site	Pathogenic	3
	IVS1+12G>A (-3158G>A)	IVS1	Novel	1
	-60C>T	IVS1	Novel	1
	-57G>T	IVS1	Novel	1
	-40T>A	IVS1	Novel	1
	-34C>T	IVS1	Novel	46
	-28T>C	IVS1	Novel	1
	-15C>T	5' UTR, exon 2	Polymorphism	11
	-6T>A	5' UTR, exon 2	Novel	2
G4D	11G>A	IC1	Unclassified	1
G12fsX13	35delG	IC1	Pathogenic	120
G12C	34G>T	IC1	Novel	3
N14D	40A>G	IC1	Novel	1
V27I	79G>A	TM1	Polymorphism	64
M34T	101C>T	TM1	Polymorphism	19
V37I	109G>A	TM1	^d	28
W44X	131G>A; 132G>A	EC1	Pathogenic	3 ^b
E47X	139G>T	EC1	Pathogenic	2
L56fsX81	167delT	EC1	Pathogenic	6
D50N	148G>A	EC1	Pathogenic (dominant, syndromic, KID syndrome)	1
Q57X	169C>T	EC1	Pathogenic	2
V63A	188T>C	EC1	Novel	1
R75W	223C>T	EC1	Pathogenic (dominant, syndromic, deafness with PPK)	1
W77X	231G>A	TM2	Pathogenic	1
L79fsX81	235delC	TM2	Pathogenic	1
Q80X	238C>T	TM2	Pathogenic	1
F83L	249C>G	TM2	Polymorphism	1
V84L	250G>C	TM2	Pathogenic	1
T86M	257C>T	TM2	Novel	1
L90P	269T>C	TM2	Pathogenic	8
V95M	283G>A	IC2	Pathogenic	2

Amino acid variation	Nucleotide variation	Gene/mRNA/protein domain ^d	Characterization of variant	Number of occurrences total n =1,220
H100Y	298C>T	IC2	Pathogenic	1
E114G	341A>G	IC2	Polymorphism	2
K105fsX109	313_326delAAGTTCATCAAGGG	IC2	Pathogenic	2
E120del	358_360delGAG	IC2	Pathogenic	1
K122I	365A>T	IC2	Pathogenic	2
R127C	379C>T	IC2	Unclassified	1
R127H	380G>A	IC2	Polymorphism	7
L132V	394C>G	TM3	Novel	1
S139N	416G>A	TM3	Pathogenic	2
R143W	427C>T	TM3	Pathogenic	1
V153I	457G>A	EC2	Polymorphism	2
D159	477C>T	EC2	Novel	1
G160S	478G>A	EC2	Polymorphism	1
M163V	487A>G	EC2	Unclassified	1
V178A	533T>C	EC2	Pathogenic	1
R184W	550C>T	EC2	Pathogenic	2
R184P	551G>C	EC2	Pathogenic	2
V198fsX201	592_600delinsCAGTGTTTCATGACATTC	TM4	Novel	1
I203T	608T>C	TM4	Polymorphism	2
R216fsX232	647_650delGATA ^c	IC3	Pathogenic	2
	682C>T	3'UTR	Polymorphism	1
	684C>A	3'UTR	Novel	1

n, number of alleles tested.

^aIVS, intervening sequence/intron; UTR, untranslated region; IC, intracellular; TM, transmembrane; EC, extracellular; KID, keratitis-ichthyosis-deafness; PPK, palmoplantar keratoderma.

^bTwo of the W44X mutations were 131G>A while one was 132G>A.

^cThe 647_650delGATA mutation has not been previously described as such but a mutation designated as 645-648delTAGA has been reported [Prasad et al., 2000; Ballana et al., 2005]. Due to the DNA sequence in this region of *GJB2*, the 647_650delGATA and 645-648delTAGA DNA sequence variations would be indistinguishable by DNA sequencing and might both be the same mutation.

^dThe V37I variant has been previously reported by others as a pathogenic mutation and as a polymorphism [Kelley et al., 1998; Cohn and Kelley, 1999; Rabionet et al., 2000b; Wilcox et al., 2000; Abe et al., 2001; Bason et al., 2002; Kenneson et al., 2002; Hwa et al., 2003; Ohtsuka et al., 2003; Cryns et al., 2004; Roux et al., 2004; Shi et al., 2004; Ballana et al., 2005].

TABLE II

GJB2 Genotypes of Hearing Loss Cases

Coding region genotype	Non-coding region genotype	Number of occurrences total n =610
[S139N] + [wt]	[-3464C>G; -34C>T] + [-3464C>G; -34C>T]	1
	-3360T>C/wt + -34C>T/wt ^a	1
	[-3275G>C] + [wt]	1
	-3158G>A/wt + -34C>T/wt ^a	1
	[IVS1+1G>A] + [wt]	2
	[IVS1+1G>A] + [wt]	1
	[-60C>T] + [wt]	1
	[-40T>A] + [wt]	1
	[-34C>T] + [wt]	20
	-34C>T/wt + -15C>T/wt ^a	4
	[-34C>T] + [-34C>T]	5
	-34C>T/wt + 682C>T/wt ^a	1
	[-28T>C] + [wt]	1
	[-15C>T] + [wt]	6
	[-6T>A] + [wt]	2
[G4D] + [wt]	[-34C>T] + [wt]	1
[35delG] + [wt]		16
[35delG] + [wt]	[-15C>T] + [wt]	1
[35delG] + [35delG]		36
35delG/wt + M34T/wt ^a		7
35delG/wt + V37I/wt ^a		1
[35delG] + [167delT]		4
[35delG] + [V27I; E114G] ^b		1
[35delG] + [W44X] + V27I/wt ^a		1
[35delG] + [wt]	[-3265G>A] + [wt]	1
[35delG] + [W44X]	[-34C>T] + [wt]	1
[35delG] + [E47X]		2
[35delG] + [W77X]		1
[35delG] + [V84L]		1
[35delG] + [L90P]		3
[35delG] + [H100Y]		1
[35delG] + [K105fsX109]		2
[35delG] + [S139N]		1
[35delG] + [R184W]		2
[35delG] + [R184P]		1
[35delG] + [R216fsX232]		1
[G12C] + [wt]		3

Coding region genotype	Non-coding region genotype	Number of occurrences total n =610
[N14D] + [wt]		1
[V27I] + [wt]		43
[V27I] + [wt]	[-34C>T] + [wt]	1
[V27I] + [V27I]		5
[V27I; M34T] + [V27I]		2
V27I/wt + E114G/wt ^a		1
V27I/wt + R127H/wt ^a		1
V27I/wt + L132V/wt ^a		1
[V27I; W44X] + [Q80X]		1
[M34T] + [wt]		7
[M34T] + [wt]	[-34C>T] + [wt]	1
[M34T] + [M34T]		1
[V37I] + [wt]		4
[V37I] + [V37I]		9
[V37I] + [V37I]	[-57G>T] + [wt]	1
V37I/wt + L90P/wt ^a		2
V37I/wt + R216fsX232/wt ^a		1
[D50N] + [wt]		1
[Q57X] + [wt]		2
V63A/wt + 235delC/wt ^a		1
[R75W] + [wt]	[-34C>T] + [wt]	1
[167delT] + [167delT]		1
[F83L] + [wt]		1
T86M/wt + R127H/wt + M163V/wt ^a		1
[L90P] + [wt]		1
[L90P] + [L90P]		1
[V95M] + [wt]		1
V95M/wt + R127H/wt ^a		1
[E120del] + [wt]		1
[K122I] + [wt]		2
[R127C] + [wt]		1
[R127H] + [wt]		4
[R143W] + [wt]	-3364T>G/wt + -34C>T/wt ^a	1
[V153I] + [wt]		2
[D159] + [wt]		1
[G160S] + [wt]		1
[V178A] + [wt]	[-34C>T] + [wt]	1
[R184P] + [wt]		1
[592_600delinsCAGTGTTCAT- GACATTC] + [wt]		1
[I203T] + [wt]		2

Coding region genotype	Non-coding region genotype	Number of occurrences total n =610
	[684C>A] + [wt]	1

n, number of individuals tested; wt, wild type. For all hearing impaired cases carrying homozygous or compound heterozygous recessive mutations, opposition of the mutations has been presumed, unless otherwise known.

^aPhase unknown.

^bThis individual has a hearing impaired sibling with genotype [35delG] + [V37I] that was not counted.

TABLE III

Variations in *GJB2* Identified by DNA Sequencing of Control Subjects

Amino acid variation	Nucleotide variation	Gene/mRNA/protein domain ^a	Characterization of variant	African-Americans n = 144	Hispanics n = 152	Asians n = 144	Caucasians n = 148	Number of occurrences total n = 588
	-57G>T	IVS1	Novel			4		4
	-34C>T	IVS1	Novel	40	5			45
	-15C>T	5'UTR	Polymorphism	4	3			7
	-6T>A	5'UTR	Novel	3				3
G4D	11G>A	IC1	Unclassified			1		1
G12fsX13	35delG	IC1	Pathogenic				3	3
V27I	79G>A	TM1	Polymorphism		25	46		71
M34T	101C>T	TM1	Polymorphism		1		3	4
V37I	109G>A	TM1	<i>b</i>			11		11
L79fsX81	235delC	TM2	Pathogenic			1		1
F83L	249C>G	TM2	Polymorphism				1	1
E114G	341A>G	IC2	Polymorphism			37		37
T123N	368C>A	IC2	Unclassified			1		1
R127L	380G>T	IC2	Novel		1	1		2
V153I	457G>A	EC2	Polymorphism				1	1
I203T	608T>C	TM4	Polymorphism			2		2
V207L	619G>C	TM4	Novel			1		1
	765C>T	3' UTR	Polymorphism	64	44	72	19	199
	777A>G	3' UTR	Novel	1				1
	785A>T	3' UTR	Novel	36	1			37
	792C>T	3' UTR	Novel	36	1			37
	794T>A	3' UTR	Novel	1				1

n, number of alleles tested. Exon 1 was not sequenced in controls.

In the African-American control group, the portion of the gene including and 3' to position 765 was read for 142 chromosomes only. In the Hispanic control group, the portion of the gene including and 3' to position 765 was read for 146 chromosomes only. In the Asian control group, the portion of the gene including position 765 was read for 142 chromosomes only, the portion of the gene including and 3' to position 785 was read for 132 chromosomes only, the portion of the gene including position 792 was read for 130 chromosomes only, and the portion of the gene beyond position 792 was read for 128 chromosomes only.

^aIVS, intervening sequence/intron; UTR, untranslated region; IC, intracellular; TM, transmembrane; EC, extracellular.

^bThe V37I variant has been previously reported by others as a pathogenic mutation and as a polymorphism [Abe et al., 2001; Ballana et al., 2005; Bason et al., 2002; Cohn and Kelley, 1999; Cryns et al., 2004; Hwa et al., 2003; Kelley et al., 1998; Kenneson et al., 2002; Ohisuka et al., 2003; Rabtonet et al., 2000b; Roux et al., 2004; Shi et al., 2004; Wilcox et al., 2000].

TABLE IV

GJB2 Genotypes of Control Subjects

Genotype ^a	African-Americans n =72	Hispanics n =76	Asians n =72	Caucasians n =74	Number of occurrences total n =294
[G4D] + [wt]			1		1
[35delG] + [wt]				3	3
[V27I] + [wt]		21	6		27
V27I/wt + V37I/wt ^b			1		1
V27I/wt + E114G/wt ^b			18		18
V27I/wt + E114G/wt + R127L/wt ^b			1		1
V27I/wt + V37I/wt + E114G/wt ^b			3		3
V27I/wt + T123N/wt ^b			1		1
[V27I] + [V27I]		2	1		3
[V27I; E114G] + [V27I; E114G]			7		7
[M34T] + [wt]		1		3	4
[V37I] + [wt]				4	4
V37I/wt + I203T/wt ^b			1		1
[V37I] + [V37I]			1		1
[235delC] + [wt]			1		1
[F83L] + [wt]				1	1
[E114G] + [wt]			1		1
[R127L] + [wt]		1			1
[V153I] + [wt]				1	1
[I203T] + [wt]				1	1
[V207L] + [wt]				1	1

n, number of individuals tested; wt, wild type.

^aDNA sequence variations in non-coding regions not included.

^bPhase unknown.

TABLE V

Comparison of Results From PolyPhen, SIFT, and Multiple Sequence Alignment Analysis of Novel Missense Variations in Connexin 26

Amino acid change	PolyPhen	SIFT	Conservation of human connexin 26 amino acid in
G12C	Possibly damaging	Not tolerated	Connexin 26 from mouse, lowland gorilla, gibbon, rhesus monkey, sheep, orangutan, Norway rat, human connexins 30, 30.3, 31, 31.1, 32
N14D	Probably damaging	Tolerated	Connexin 26 from mouse, lowland gorilla, gibbon, rhesus monkey, sheep, orangutan, Norway rat, human connexins 30, 30.3, 31, 31.1, 32
V63A	Benign	Not tolerated	Connexin 26 from mouse, lowland gorilla, gibbon, rhesus monkey, sheep, orangutan, Norway rat, human connexins 30, 30.3, 31, 31.1, 32, 43
T86M	Possibly damaging	Not tolerated	Connexin 26 from mouse, lowland gorilla, gibbon, rhesus monkey, sheep, orangutan, Norway rat, human connexins 30, 32
R127L	Probably damaging	Tolerated	Connexin 26 from mouse, lowland gorilla, gibbon, rhesus monkey, sheep, orangutan, Norway rat, human connexin 30
L132V	Benign	Not tolerated	Connexin 26 from mouse, lowland gorilla, gibbon, rhesus monkey, sheep, orangutan, Norway rat, human connexins 30, 30.3, 31, 31.1, 32, 43
V207L	Benign	Tolerated	Connexin 26 from lowland gorilla, gibbon, rhesus monkey, sheep, orangutan, human connexins 30, 32

PolyPhen Prediction Tool: <http://coot.embl.de/PolyPhen/>; SIFT Prediction Tool: <http://blocks.fhcrc.org/sift/SIFT.html>; Multiple Sequence Alignment Tool: <http://searchlauncher.bcm.tmc.edu/>. For all novel missense variations, the amino acid sequence of human Connexin 26 was aligned with Connexin 26 from mouse, lowland gorilla, gibbon, rhesus monkey, sheep, orangutan, and Norway rat, and human Connexins 30, 30.3, 31, 31.1, 32, and 43.

TABLE VI

Comparison of Allele Frequencies Between Cases and Controls: 35delG, M34T, and V37I

Allele	Frequency in cases	Frequency in controls	Two-tailed <i>P</i> value
35delG	0.098	0.005	<i>P</i> <0.0001
M34T	0.016	0.007	<i>P</i> =0.1770
V37I	0.023	0.019	<i>P</i> =0.4895