

Report

Autosomal Recessive Nonsyndromic Neurosensory Deafness at *DFNB1* Not Associated with the Compound-Heterozygous *GJB2* (Connexin 26) Genotype M34T/167delT

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Previous studies of the gap-junction β -2 subunit gene *GJB2* (connexin 26) have suggested that the 101T→C (M34T) nucleotide substitution may be a mutant allele responsible for recessive deafness *DFNB1*. This hypothesis was consistent with observations of negligible intercellular coupling and gap-junction assembly of the M34T allele product expressed in *Xenopus* oocytes and HeLa cells. The results of our current study of a family cosegregating the 167delT allele of *GJB2* and severe *DFNB1* deafness demonstrate that this phenotype did not cosegregate with the compound-heterozygous genotype M34T/167delT. Since 167delT is a null allele of *GJB2*, this result indicates that the *in vivo* activity of a single M34T allele is not sufficiently reduced to cause the typical deafness phenotype associated with *DFNB1*. This observation raises the possibility that other *GJB2* missense substitutions may not be recessive mutations that cause severe deafness and emphasizes the importance of observing cosegregation with deafness in large families to confirm that these missense alleles are mutant *DFNB1* alleles.

GJB2 is one of a family of genes encoding the polypeptide components of gap junctions, which are macromolecular integral-membrane complexes that allow for the passive diffusion of water and small solutes between adjacent cells (Goodenough et al. 1996). *GJB2* encodes the gap-junction β -2 polypeptide, also known as “connexin 26” (Cx26), which is expressed within discrete regions of the cochlea (Kikuchi et al. 1995). Mutations in *GJB2* at the *DFNB1* locus (MIM 220290) on chromosome 13q12 account for up to 50% of nonsyndromic recessive deafness in some populations (Estivill et al. 1998). 35delG and 167delT are two *GJB2* alleles that cause nonsyndromic recessive deafness, and carrier rates for these mutant alleles may be as high as 4% in some ethnic populations (Estivill et al. 1998; Morell et al. 1998). Both of these frameshift mutations are predicted

to result in premature translation termination. Cosegregation of homozygosity for these alleles with recessive deafness in several different families has confirmed that the alleles are recessive mutant alleles of *GJB2* (Carrasquillo et al. 1997; Denoyelle et al. 1997; Zelante et al. 1997; Morell et al. 1998).

Numerous *GJB2* amino acid substitutions have been reported as dominant and recessive deafness alleles at the *DFNA3* (MIM 601544) and *DFNB1* loci, respectively (The Connexin-Deafness Homepage). One of these *GJB2* alleles, 101T→C (M34T), encodes a methionine-to-threonine substitution at amino acid 34 and was initially proposed to be a dominant mutation at *DFNA3*, on the basis of cosegregation with dominant profound deafness in a single small family (Kelsell et al. 1997). This conclusion appeared to be consistent with a dominant-negative effect of the M34T mutant polypeptide on the intercellular coupling activity of the wild-type *GJB2* polypeptide expressed in *Xenopus* oocytes (White et al. 1998). However, observations of normal hearing in M34T heterozygotes indicate that M34T does not function as a dominant *GJB2* allele *in vivo* (figs. 1 and 2D; Kelley et al. 1998; Scott et al. 1998).

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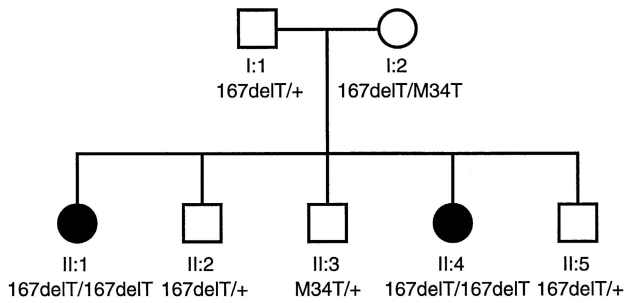


Figure 1 Pedigree and *GJB2* genotypes for the study family segregating nonsyndromic recessive deafness. Blackened symbols denote severe-to-profound sensorineural hearing loss. Audiometric data were not available for individual II:2, although his hearing was subjectively normal.

The intercellular coupling activity of GJB2 polypeptides encoded by missense alleles of *GJB2* has also been recently reported for a mammalian (HeLa) cell-expression system (Martin et al. 1999). The M34T allele product appeared to be properly targeted to the plasma membrane, and it demonstrated detectable assembly into oligomers and hexamers, albeit at reduced efficiency. Coupling activity of the M34T polypeptide was also significantly reduced in comparison with that of wild-type GJB2 polypeptides. However, there was residual coupling activity that was slightly higher than that which was observed with the W44C missense-allele product or with a negative-control microinjection, although these differences did not reach statistical significance (Martin et al. 1999). These data raised the question of whether M34T is a recessive mutant allele of *GJB2* or whether the reduced but detectable gap-junction assembly and activity of the M34T allele product are sufficient to prevent the severe hearing loss usually associated with *DFNB1*.

We were able to test the hypothesis that M34T is a recessive deafness allele of *GJB2* in a single family segregating nonsyndromic recessive sensorineural deafness (fig. 1). This study was approved by institutional review boards (IRBs) at the National Institutes of Health (NIH) and the Louisiana State University Health Sciences Center, as well as by the Joint Baycrest Centre/University of Toronto IRB. Informed consent was obtained from all individuals participating in the study. Genomic DNA was prepared, by use of standard methods, from specimens taken from buccal swabs (Meulenbelt et al. 1995). The second exon of *GJB2*, encoding the entire open reading frame, was amplified, by PCR, from genomic DNA, with use of forward primer 167F (5'-TCT TTT CCA GAG CAA ACC GC-3') and reverse primer 930R (5'-CCT CAT CCC TCT CAT GCT GT-3'). The Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 377 automated fluorescent sequencer (PE Bio-

systems) were used to sequence both strands, with the use of the amplification primers and the additional reverse primer 452R (Kelsell et al. 1997; Morell et al. 1998).

Results of *GJB2* genotype analysis demonstrated that affected individuals II:1 and II:4 are 167delT/167delT homozygotes. Results of pure-tone audiometric analysis of individuals II:1 and II:4 revealed mild-to-profound and severe-to-profound bilateral sensorineural hearing loss, respectively, across all frequencies (fig. 2C and E). Their hearing losses were nonprogressive and were either congenital or prelingual in onset. Pure-tone thresholds for the father (individual I:1, who is a 167delT heterozygote) are slightly elevated only at 3 and 4 kHz (fig. 2A). His audiometric pattern is typical of noise-induced hearing loss, although there was no history of exposure to noise or ototoxic agents, trauma, otologic disease, or otologic surgery.

Individual I:2 is a compound heterozygote for 167delT and 101T→C (M34T). The genotype of individual I:2 was confirmed by similar analysis of a second DNA specimen taken from a buccal swab at a later date. Although the results of genotype analysis of her offspring suggested that individual I:2 is a compound heterozygote with 167delT and M34T alleles segregating in *trans*, the *trans* configuration was confirmed by sequence analysis of subcloned *GJB2* amplification products (data not shown).

Results of pure-tone audiometric analysis of individual I:2 demonstrated normal hearing from 250 Hz to 4 kHz, with thresholds of 45–60 dBHL bilaterally at 6 and 8 kHz (fig. 2B). The 90th-percentile thresholds for an otologically normal, age- and sex-matched population are 31 and 38 dBHL at 6 and 8 kHz, respectively (International Organization for Standardization 1984). Individual I:2 had no history of otologic disease, surgery, trauma, or exposure to ototoxic agents or excessive noise that would account for her thresholds at 6 and 8 kHz. During the preceding 2–3 years, she had noticed that she experienced gradually progressive difficulty in understanding conversational speech in the presence of background noise, which is consistent with elevated thresholds at high frequencies. The onset of her subjective hearing loss suggests that the elevated thresholds at high frequencies are not congenital. Her thresholds at 0.5, 1, 2, and 4 kHz are all within normal limits for her age (Morrell et al. 1996), and her mean pure-tone averages (mPTA) at these four frequencies are 15 dBHL for the right ear and 14 dBHL for the left ear. These values are >8 SD below the reported corresponding mPTA for 167delT/167delT homozygotes (100 ± 10 dBHL) and are >3 SD below the mPTA for 35delG/35delG homozygotes (87 ± 22 dBHL) (Cohn et al. 1999).

The high-frequency hearing loss in individual I:2 may represent the auditory phenotype associated with one

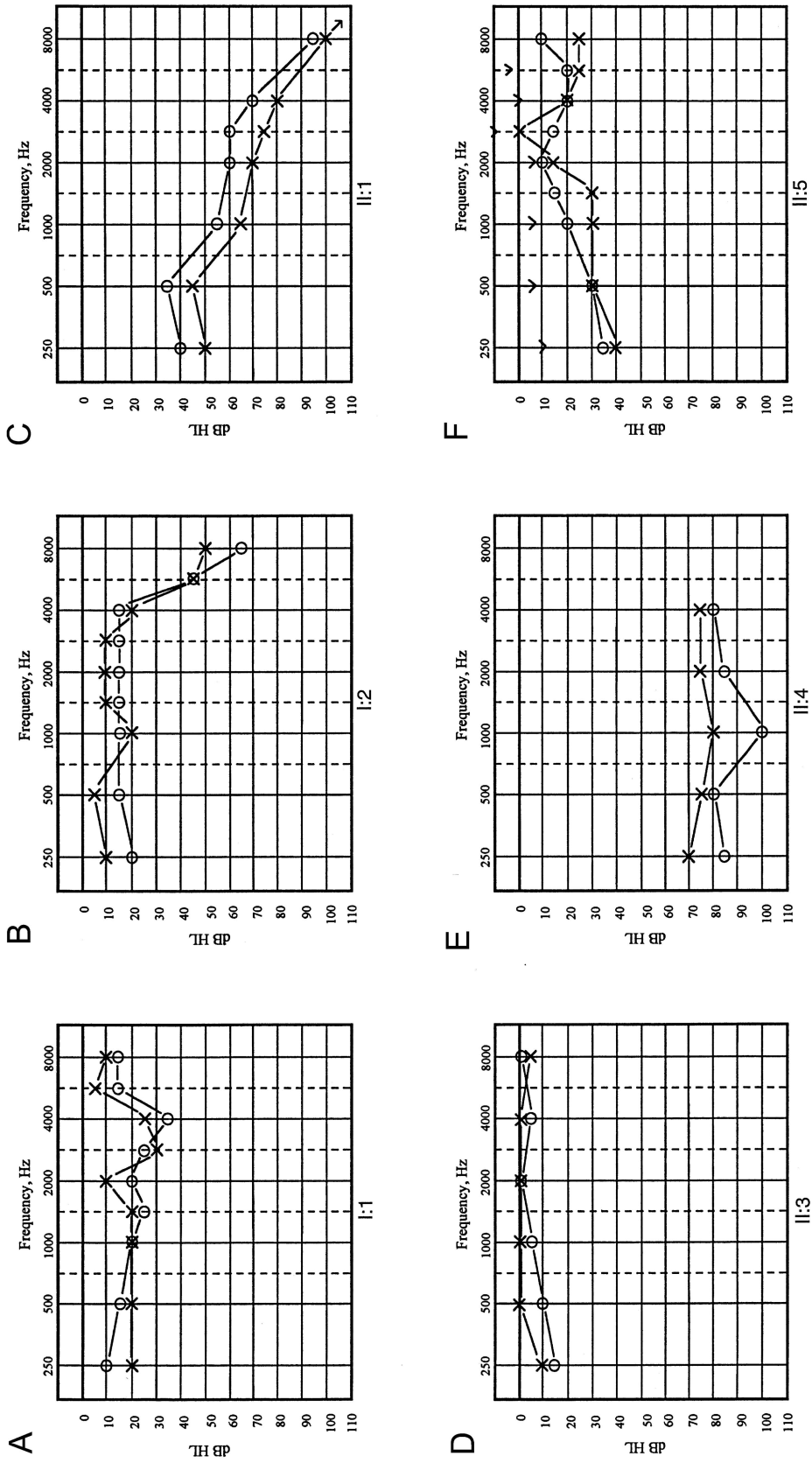


Figure 2 Pure-tone audiometric thresholds for individuals I:1, who was 52 years of age (A); I:2, who was 47 years of age (B); II:1 (C); II:3 (D); II:4 (E); and II:5 (F). Type B tympanograms (not shown) and normal midline bone conduction thresholds in individual II:5 indicate that the hearing loss is conductive, which is consistent with a known history of bilateral chronic otitis media with effusion.

null allele and one hypomorphic allele of *GJB2*. It is also possible that the M34T/167delT genotype can cause severe deafness in some individuals; however, in individual I:2, the mutant auditory phenotype has been modified by environmental or genetic factors. The observation of less-severe degrees of hearing loss in some 35delG homozygotes suggests that such factors exist and can modify expression of mutant *GJB2* auditory phenotypes (Cohn et al. 1999; Denoyelle et al. 1999). We have preliminarily addressed the possibility of modifying genetic loci by DNA sequence analysis of two other gap-junction genes that have been implicated in hereditary deafness, *GJB3* (*Cx31*; Xia et al. 1998; Liu et al. 2000) and *GJB6* (*Cx30*; Grifa et al. 1999). The results of these analyses of individual I:2 revealed no polymorphisms or mutations in the protein-coding regions of either *GJB3* or *GJB6* (data not shown).

Current assays for gap-junction assembly and activity may not accurately reflect the *in vivo* consequences that residual *GJB2* activity has for auditory function. The 35delG and 167delT alleles encode markedly truncated *GJB2* polypeptides that would be predicted to exhibit no assembly or functional activity in gap-junction assays, and their causative role in recessive deafness *DFNB1* has been confirmed by cosegregation with the deafness phenotype in multiple families (Denoyelle et al. 1997; Zelante et al. 1997; Morell et al. 1998). In contrast, our results indicate that M34T is not a recessive mutant *GJB2* allele associated with severe deafness, although it may be associated with less-severe forms of sensorineural hearing loss, such as presbycusis (fig. 2B). The residual *GJB2* activity associated with a single M34T allele thus appears to be sufficient for normal auditory function at most frequencies. The carrier rate for M34T has been estimated to be as high as 2.3% in the general population (Green et al. 1999), and its detection in sporadic cases and small sibships with deafness is probably a coincidental result of the large numbers of deaf probands undergoing *GJB2* genotype analysis.

The W77R missense allele also encodes a *GJB2* polypeptide with residual gap-junction assembly and intercellular coupling activity similar to those of M34T (Martin et al. 1999). W77R was initially detected in a large pedigree cosegregating recessive deafness *DFNB1* with the 35delG and W77R alleles of *GJB2* (Carrasquillo et al. 1997). Affected individuals included 35delG homozygotes as well as W77R homozygotes and W77R/35delG compound heterozygotes, indicating that W77R is indeed a mutant allele of *GJB2*. It is possible that the difference in auditory phenotypes of affected individuals segregating W77R and the M34T/167delT compound heterozygote in the family investigated in the present study may be due to differences in environmental factors or modifying genetic loci. Alternatively, there may be significant *in vivo* functional differences between W77R

and M34T. In either case, the gap-junction activity levels reported for these missense alleles expressed in *Xenopus* oocytes (White et al. 1998) or HeLa cells (Martin et al. 1999) are not predictive of the auditory phenotype.

This observation shifts the burden of proof that M34T may be a recessive mutant allele at *DFNB1*. Confirmation of M34T as a mutant *DFNB1* allele will require demonstration of its cosegregation with recessive deafness in large families. In the absence of large families cosegregating M34T with *DFNB1* deafness, this hypothesis may be tested by prospective *GJB2* genotype analyses of large numbers of normal-hearing individuals, to identify additional homozygotes and compound heterozygotes for M34T. Similar analyses should also be performed for other hypomorphic missense alleles of *GJB2*. These results will have important implications for genetic counseling of individuals in families segregating these alleles, as well as for improving our understanding of the structure and function of gap junctions in the auditory system.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Connexin-Deafness Homepage, The, <http://www.iro.es/cx26deaf.html>
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for *DFNB1* [MIM 220290] and *DFNA3* [MIM 601544])

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