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Novel Mutations in the Connexin 26 Gene (GJB2) That Cause Autosomal Recessive (DFNB1) Hearing Loss

P. M. Kelley, D. J. Harris, B. C. Comer, J. W. Askew, T. Fowler, S. D. Smith, and W. J. Kimberling

Center for the Study of Hereditary Hearing Loss, Boys Town National Research Hospital, Omaha

Summary

Mutations in the connexin 26 (Cx26) gene (GJB2) are associated with the type of autosomal recessive nonsyndromic neurosensory deafness known as "DFNB1." Studies indicate that DFNB1 (13q11-12) causes 20% of all childhood deafness and may have a carrier rate as high as 2.8%. This study describes the analysis of 58 multiplex families each having at least two affected children diagnosed with autosomal recessive nonsyndromic deafness. Twenty of the 58 families were observed to have mutations in both alleles of Cx26. Thirty-three of 116 chromosomes contained a 30delG allele, for a frequency of .284. This mutation was observed in 2 of 192 control chromosomes, for an estimated gene frequency of .01 ± .007. The homozygous frequency of the 30delG allele is then estimated at .0001, or 1/10,000. Given that the frequency of all childhood hearing impairment is 1/1,000 and that half of that is genetic, the specific mutation 30delG is responsible for 10% of all childhood hearing loss and for 20% of all childhood hereditary hearing loss. Six novel mutations were also observed in the affected population. The deletions detected cause frameshifts that would severely disrupt the protein structure. Three novel missense mutations, Val84Met, Val95Met, and Ser113Pro, were observed. The missense mutation 101T→C has been reported to be a dominant allele of DFNA3, a dominant nonsyndromic hearing loss. Data further supporting the finding that this mutation does not cause dominant hearing loss are presented. This allele was found in a recessive family segregating independently from the hearing-loss phenotype and in 3 of 192 control chromosomes. These results indicate that 101T→C is not sufficient to cause hearing loss.

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Address for correspondence and reprints: Dr. Philip M. Kelley, Center for the Study of Hereditary Hearing Loss, Boys Town National Research Hospital, 555 North 30th Street, Omaha, NE 68131. E-mail: kelley@boystown.org

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Introduction

Mutations in the connexin 26 (Cx26) gene (GJB2), which is located on chromosome 13q11-12, are associated with the autosomal recessive nonsyndromic neurosensory deafness known as "DFNB1" (MIM 220290) (Chaib et al. 1994; Carrasquillo et al. 1997; Denoyelle et al. 1997; Kelsell et al. 1997; Van Camp et al. 1997; Zelante et al. 1997). These studies indicate that DFNB1 causes 20% of all childhood deafness and may have a carrier rate as high as 2.8%.

Connexins are a class of membrane proteins that form hexameric connexons that form gap-junction channels with identical or similar connexons in adjacent cells (Paul 1995; Bruzzone et al. 1996). Gap junctions constitute a major system of intercellular communication important in the exchange of electrolytes, second messengers, and metabolites (Dermietzel and Spray 1993; White et al. 1995; White and Bruzzone 1996). Connexins may play important roles in development, by coordinating the clonal development of groups of cells. Katz (1995) has proposed that connexin expression may direct the assembly of complex functional architecture in the developing brain by laying the foundation of cortical circuits that mature by synapse formation. In mammals, there are ~13 different connexin genes, which are divided into two classes, alpha and beta, on the basis of their primary structure (Kumar and Guila 1992, 1996). Some forms of connexin are expressed in a wide variety of tissue, whereas others are restricted to specific cell types. Turnover of connexins appears to be rapid, suggesting that the levels of most forms are dependent on transcription or mRNA turnover. Cx26 is downregulated in tumor tissue and thus is considered a class II tumor-suppressor gene. Cx26 is strongly upregulated in synchronized cells, during the S and G2 phases (Lee et al. 1992).

Immunostaining of rat cochlea, with antisera raised against cytoplasmic epitopes of Cx26, revealed that two groups of cochlear cells use the Cx26 protein (Kikuchi et al. 1995). The first group of cells includes interdental cells of the spiral limbus, inner sulcus cells, organ of Corti supporting cells, outer sulcus cells, and cells within the root process of the spiral ligament; these are all non-

sensory epithilial cells. The second group includes fibrocytes of the spiral limbus and spiral ligament, basal and intermediate cells of the stria vascularis, and mesenchymal cells lining the scala vestibuli; these are all connective-tissue cells. This observation supports the hypothesis that Cx26 mediates the recycling of endolymphatic potassium ions transiting the hair cells during the transduction process (Schulte and Steel 1994; Spicer and Schulte 1996).

We have begun an analysis of 58 multiplex families, each with at least two affected children diagnosed with autosomal recessive nonsyndromic deafness. The purpose of this study was to estimate the frequency of Cx26 mutation in the population of American families with nonsyndromic hearing loss and to identify novel mutations.

Subjects and Methods

Criteria for Inclusion in This Study

Families were included in this study if the following criteria were met (1) both parents are living and have normal hearing; (2) there exist at least two hearing-impaired siblings; (3) hearing loss is documented by audiologic testing in a clinical facility; and (4) there is no evidence of any obvious syndrome. All of the recessive nonsyndromic hearing-loss (RNSHL) families assembled were tested. A single dominant nonsyndromic hearing-loss family was tested, because of preliminary data linking it to the DFNA3 locus. Ethnicity and country of origin were self-assessed. The families included in this study are all American, primarily from Midwestern states. They are primarily Caucasian and are of northern and southern European origin. No Asian, African, or Native American families were available to us for this study.

Mutation Detection

Two methods of mutation detection were used in this study: heteroduplex analysis and sequencing. Heteroduplex analysis was done on an affected individual and the individual's parents, for each family. We focused our search for mutations on the coding region of Cx26. After the initial screen by heteroduplex analysis, we sequenced the entire coding region from at least one affected member of each family.

DNA samples were prepared for analysis by PCR amplification with the following primer pairs: Cx26A-U/Cx26A-L (TCT TTT CCA GAG CAA ACC GC/GAC ACG AAG ATC AGC TGC AG) (Kelsel et al. 1997), Cx342-U/Cx739-L (AGG CCG ACT TTG TCT GCA ACA/GTG GGC CGG GAC ACA AAG), Cx637-U/Cx1129-L (CGA AGC CGC CTT CAT GTA CG/GTG GGC CGG GAC ACA AAG). PCR products with these three amplimer pairs cover the entire coding region of Cx26 and were used for heteroduplex analysis. The primer pair CxG4352-U/Cx873-L (TCG GCC CCA GTG GTA CAG/CTG GGC AAT GCG TTA AAC TGG) amplifies the entire coding region and was used to produce a template for sequencing. Amplification conditions were as follows: reaction volume was 50 μl with a final concentration of Tris-HCl, pH 8.3, at 10 mM, KCl at 50 mM, $MgCl_2$ at 1.5 mM, dNTPs at 200 uM, AmpliTaq Gold (PE Applied Biosystems) at 1.25 units/50 μl, primers at 400 nM, and template genomic DNA at 200 ng/50 μl. Part (5 μl) of the PCR reaction volume was analyzed on a 2% agarose gel to verify the size and quantity of the PCR product.

Heteroduplex analysis was done by use of a mutation-detection enhancement (MDE) gel and a protocol provided by the manufacturer (FMC Bioproducts). Part (5–10 μl) of the PCR-reaction volume was incubated at 95°C for 3 min then was slow cooled for 30 min, to 37°C. This renatured DNA was mixed with loading buffer and was separated on a 1 × MDE gel, according to the manufacturer's directions, for 14–20 h at 800 V. Homoduplexes and heteroduplexes were visualized on a UV transilluminator, after staining in ethidium bromide (1 μg/ml) for 15 min.

Genomic DNA was PCR amplified as described above. After verification on 2% agarose gels, the PCR products were prepared for sequencing. The PCR-reaction mix was diluted with 450 μl of distilled water and was separated from unincorporated nucleotides and primers, on a Microcon-100 (Millipore). Sequencing was accomplished by use of the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) or the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 377 DNA sequencer. In general, PCR products were sequenced in both directions.

Cloning Heterozygotes

When deletions in one allele obscured a clear reading of the sequence, the PCR products were clone purified by use of the Original TA Cloning Kit (Invitrogen). PCR products were ligated, to pCR2.1, overnight at 15°C then were transformed into competent INValphaF′ *Escherichia coli*. Transformed cells were plated on Luria-Bertani (LB)/ampicillin/X-Gal plates. White colonies were picked into 25 μl of LB. Part (2.5 μl) of this bacterial suspension was used as a template in PCR reactions, under the conditions described above. PCR products from 10 colonies usually sufficed to provide sequence from both alleles.

Table 1

Summary of GJB2 Mutations/Polymorphisms Detected in 58 RNSHL Families

Codon Location	Nucleotide Change	No. of Chromosomes from RNSHL Families	No. of Chromosomes from Control Group	Mutation Type	Reference(s)[a]
10	30delG	33	2	Deletion/frameshift	1, 2, 3
10	31–68del(38)	…	…	Deletion/frameshift	2
W24X	74G→A	…	…	Nonsense	4
V27I	79G→A	…	1	Polymorphism	
M34T	101T→C	2	3	Missense	4, 5
V37I	109G→A	…	1	Polymorphism	
E47X	139G→T	…	…	Nonsense	2
55	167delT	9	…	Deletion/frameshift	3
W77R	229T→C	…	…	Missense	1
W77X	231G→A	…	…	Nonsense	4
V84L	250G→C	1	…	Missense	
V95M	283G→A	1	…	Missense	
105	314–327del(14)	2	…	Deletion/frameshift	
111	333–334del(AA)	1	…	Deletion/frameshift	
S113R	339T→G	1	…	Missense	
211	631–632del(GT)	1	…	Deletion/frameshift	

[a] 1 = Carrasquillo et al. 1997; 2 = Denoyelle et al. 1997; 3 = Zelante et al. 1997; 4 = Kelsell et al. 1997; and 5 = Scott et al. 1998.

Results

Three mutations found in this study have been described elsewhere. The 30delG mutation (Carrasquillo et al. 1997; Denoyelle et al. 1997; Zelante et al. 1997) was the most common mutation found in the current study (table 1). Fourteen families were homozygous for this mutation, four families were compound heterozygous, and one family was heterozygous but with no other mutation detectable. This deletion results in a frameshift converting codon 12 from GGT glycine to GTG valine, followed by a stop codon at position 13. A 167delT, also previously described by Zelante et al. (1997), was the second-most-common mutation found. This mutation was homozygous in three families and heterozygous in three families, one of which was a compound heterozygote. It is interesting to note that all of these families reported Jewish origin. This deletion causes a frameshift at codon 56, resulting in a polypeptide that has the first 55 amino acids intact but that ends prematurely with a novel 25–amino-acid polypeptide C terminus. The third mutation, found in two families, was 101T→C and has been described elsewhere as a dominant allele of DFNA3 (Kelsell et al. 1997). In one family, we found this mutation segregating as a putative recessive allele from the father, who had normal hearing (fig. 1, family 1617). Both parents of the affected individual had normal hearing. Linkage data do not exclude a mutation on chromosome 13 as causative for hearing loss in family 1617. In this family, a second mutation, missense mutation 283G→A, Val95Met (fig. 2), was derived from the mother (fig. 1, family 1617). Codon 95 codes for an invariant valine in all beta-group connexins, although both a methionine and an alanine are found, in addition to a valine, in alpha-group connexins. A second family (fig. 1, family 1619) had the 101T→C mutation, but the mutation did not segregate with the hearing-loss phenotype. This allele was evident in the paternal grandmother and the father but was not accompanied by the hearing-loss phenotype.

Missense Mutations

In addition to the missense mutation (Val95Met) described above, two other novel missense mutations were detected. The second missense mutation was Val84Met, 250G→C (fig. 2). The valine at this position is invariant in all known alpha and beta connexin genes. This mutation was inherited from the maternal grandfather, through the mother (fig. 3, family 1612). The third missense mutation was Ser113Arg, 339T→G (fig. 2). Although this mutation does not change a conserved residue, it causes a radical change from a small polar group to a bulky charged group.

Small Deletions

In addition to the two common deletions reported above, three new small deletions have been detected. A 14-bp deletion, 314–327del(14), was found in two families (fig. 4). This deletion causes a frameshift following codon 105, resulting in four additional novel amino acids and premature termination at codon 110. This mutation was inherited from the mother, in one of the families (fig. 3, family 1599). A second new small-deletion

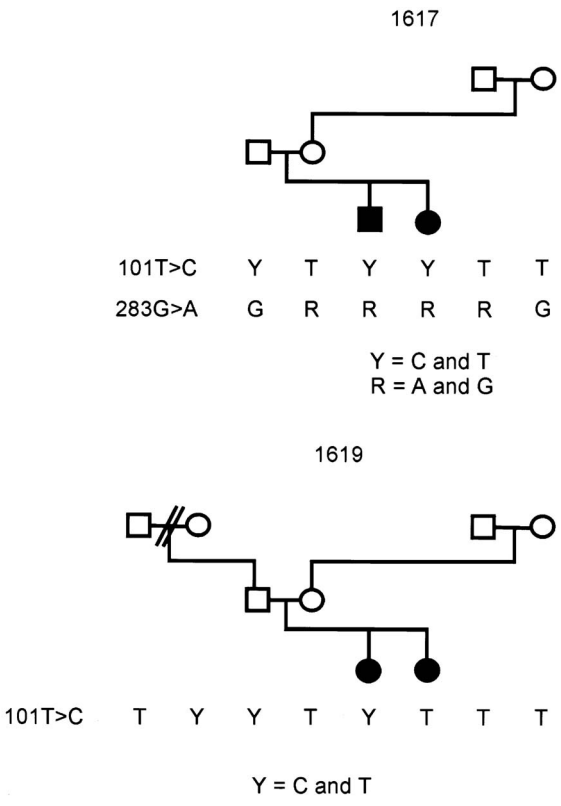

Figure 1 Pedigree and sequence summary for families with 101T→C. Below the pedigrees are shown the sequences of the individual patients, at position 101 or 283 (position 1 is the first base in the first codon of GJB2), derived from PCR amplimer CxG4352-U/Cx873-L by use of Cx26A-U as the sequencing primer, for family 1617, and the base at position 101, for family 1619. The family number is given above each pedigree.

mutation, 333-334del(AA), was observed at codon 111 (fig. 4) in a single family. This deletion causes a frameshift resulting in chain termination after an additional novel amino acid. This deletion was inherited from the paternal grandfather, through the father (fig. 3, family 1621). A third, 2-bp deletion, 631-632del(GT), was observed at codon 211 in a single family (fig. 4) and results in a frameshift and terminates the polypeptide product four codons downstream. This mutation was inherited from the mother (fig. 3, family 1692).

Mutations Found in Unaffected Controls

DNA from 96 unrelated individuals known not to have noticeable hearing loss were analyzed for their Cx26 genotype. PCR products were analyzed by heteroduplex analysis, to detect mutations or polymorphisms. For primers Cx26A-U/Cx26A-L, eight heterozygous changes were detected, and DNA from individuals with these changes was sequenced. Three individuals had the previously described dominant missense mutation 101T→C. Two individuals had the deletion frameshift mutation 30delG, described above. Two novel changes were detected, both resulting in conversion of a valine codon to an isoleucine, at codon 27 (79G→A) and at codon 37 (109G→A) (also see table 1). No mutations in the Cx26 coding region were found in the one dominant family that linked to chromosome 13.

Discussion

The structure and function of human connexins have been extensively studied at the biochemical and physiological levels. Studies of mutant forms of connexin have preceded their connection to human disease. Site-directed mutagenesis and domain replacement forms of human Cx26 have aided in the definition of important and essential elements of connexin primary structure (Rubin et al. 1992; Ek et al. 1994). The topology of Cx26 has been verified by use of specific antibodies and partial proteolysis (Zhang and Nicholson 1994) (fig. 5). Cx26, like other connexins, has a common sequence of structural motifs. The N-terminal domain (fig. 5), together with the transmembrane domain M1 border, forms a charge complex that acts as a voltage sensor (Verselis et al. 1994). The extracellular domains E1 and E2 determine the heterotypic compatibility, whereas the cytoplasmic linking (CL) domain and the C-terminal domain are involved in pH gating of the channel. Connexins are unique among integral multimeric membrane proteins in that they do not oligomerize within the endoplasmic reticulum (Laird 1996). This assembly appears to occur in the trans-Golgi network (TGN) and may be dependent on an undefined TGN chaperone.

The most common Cx26 mutation found in the 58 RNSHL families analyzed was a G deletion converting a GGGGGG to a GGGGG, 30delG. This results in a change from a glycine residue at codon 12 to a valine residue and a stop codon at position 13. A possible origin of this mutation is due to slipped mispairing during DNA synthesis (Cooper and Krawczak 1993). The site of the mutation, TGGGGGG, is very similar to a consensus deletion–hot spot sequence, TG(A/G)(A/G)(G/T)(A/C). The frequency of this mutation in a control (unaffected) population was 2/192 chromosomes, or approximately 1%, which is consistent with the high frequency we found in the affected population. This allele has also been reported as the most common form found in Mediterranean families (Zelante et al. 1997). It was also found in a heterozygous state in a Muslim Israeli-Arab kindred (Carraquillo et al. 1997). Denoyelle et al. (1997) found this mutation in the homozygous state in 19 families and in the heterozygous state in 16 families. These families originated from Tunisia, France, New Zealand, and the United Kingdom. One of their families had a second deletion, 31del38, beginning in the same

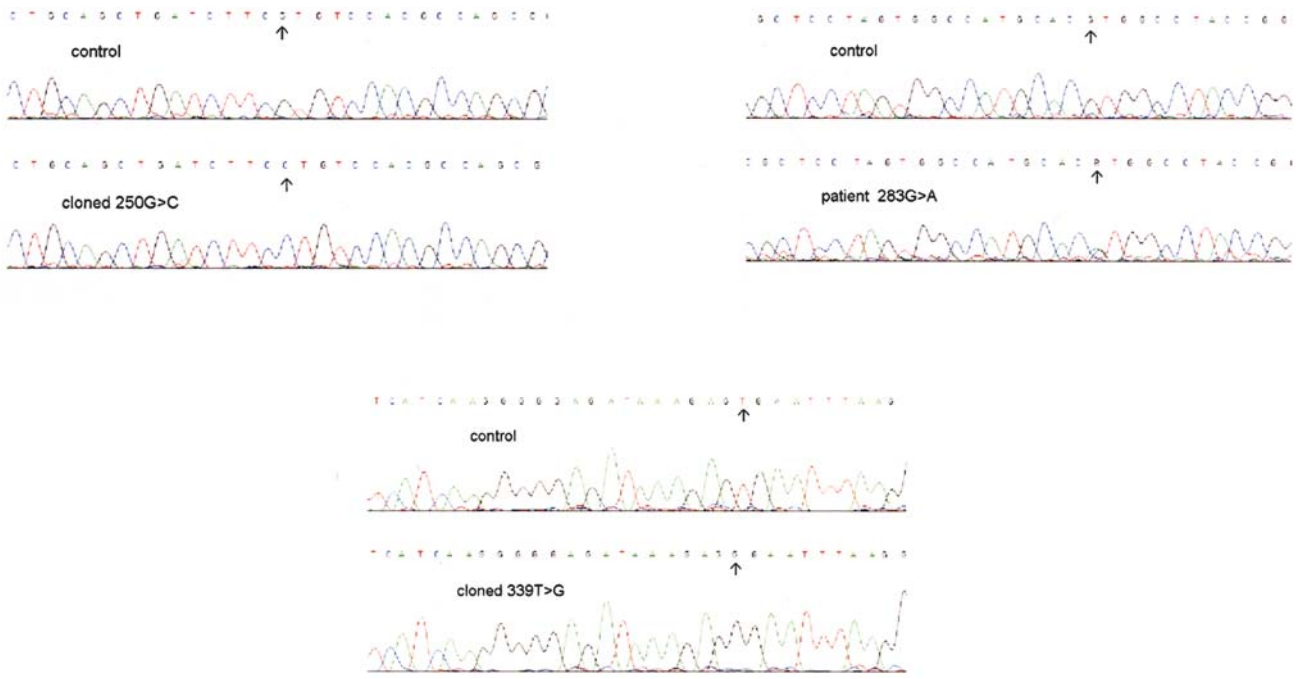

Figure 2 Sequences of novel missense mutations. Electropherograms from an ABI 377XL DNA sequencer are shown. An arrow indicates the location of the base change.

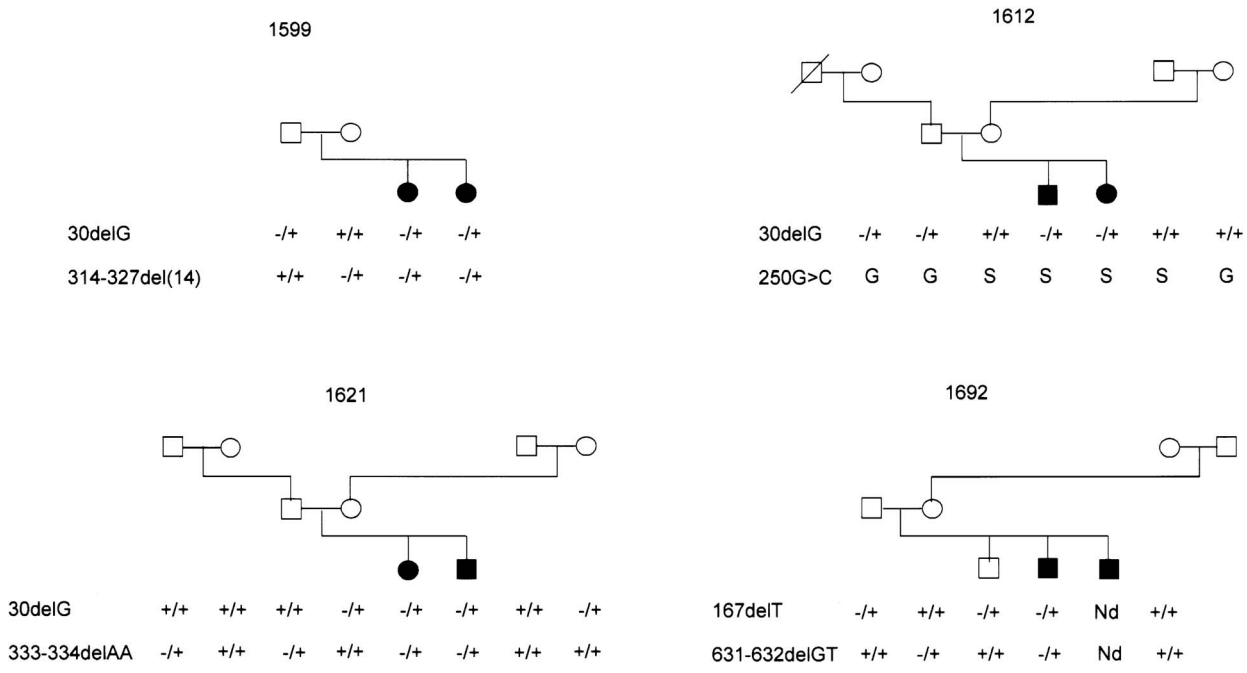

Figure 3 Pedigrees of RNSHL families and individual sequence summaries. Below the pedigrees are shown the sequences of the individual patients, at the positions indicated, derived from PCR amplimer CxG4352-U/Cx873-L by use of Cx26A-U or Cx432-U as the sequencing primer. A hyphen (-) indicates the deletion allele; and a plus sign (+) indicates the normal sequence. The family number is given above each pedigree. No DNA was available from the second affected son in family 1692, and, therefore, the sequence is marked "nd," for "not done."

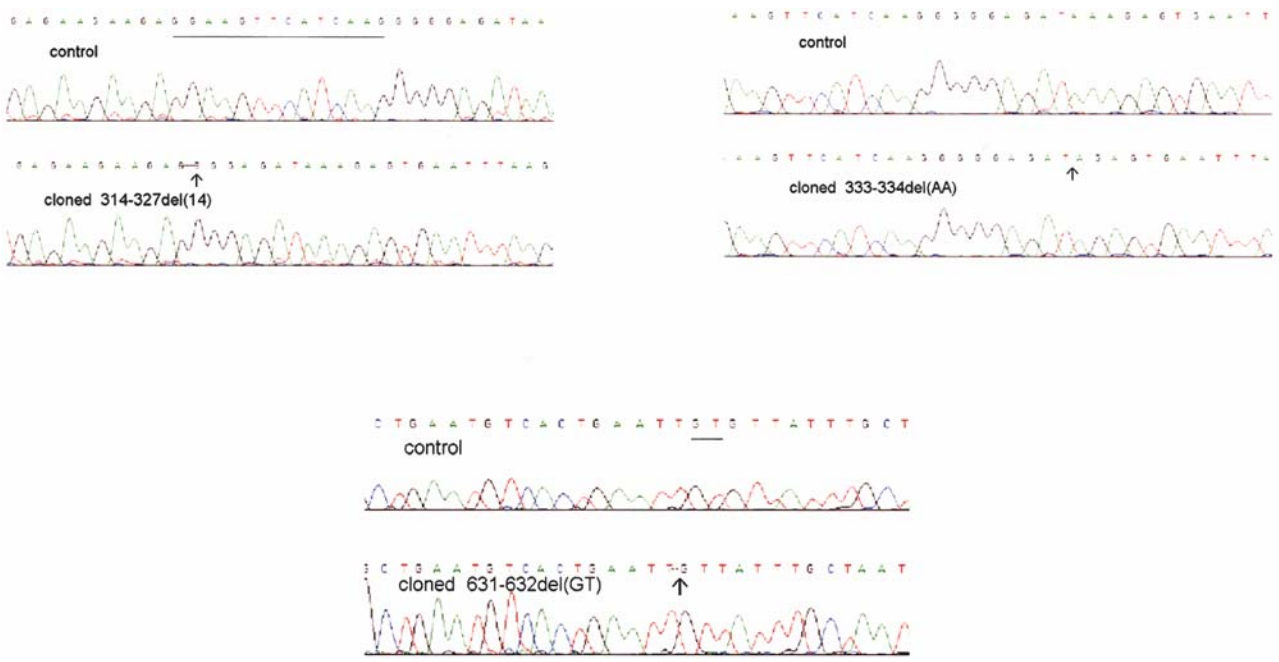

Figure 4 Sequences of novel small-deletion mutations. Electropherograms from an ABI 377XL DNA sequencer are shown. An arrow indicates the location of the deletion.

GGGGGG sequence. These findings further support the hypothesis that this site is a mutational hot spot. The 30delG mutation would appear to be the most common cause of RNSHL in northern and southern European populations. The estimated gene frequency is .01 ± .007, based on our control population of 96 unaffected families. The homozygous frequency of the 30delG mutation then was estimated at .0001, or 1/10,000. Given that the frequency of all childhood hearing impairment is 1/1,000 and that half of that is genetic, the specific mutation 30delG is responsible for 10% of all childhood hearing loss and for 20% of all childhood hereditary hearing loss. This places Cx26 in the same category of common recessive disorders as cystic fibrosis, sickle-cell anemia, and Tay-Sachs disease. If the hypothesis regarding the hypermutable nature of the GGGGGG motif is correct, the same high frequency of 30delG should occur in all ethnic groups worldwide.

The missense mutation 101T→C has been reported to be a dominant allele of DFNA3 (Kelsell et al. 1997). Our data indicate that this mutation may not have a dominant effect. In one of our RNSHL families the 101T→C allele segregated independently from the hearing-loss phenotype. Furthermore, we have found this mutation in 3 of 192 control chromosomes, for a frequency of ~1.5%. Our data indicate that the 101T→C mutation is not sufficient to cause hearing loss. Scott et al. (1998) also detected this mutation in the heterozygous state in their control population. The frequency of the 101T→C mutation in both control populations suggests that the incidence of homozygous affected individuals to be similar to the incidence of affected individuals having the 30delG mutation (~1/10,000). This suggests that the 101T→C mutation has no effect on hearing loss. However, no individual homozygous for 101T→C has been detected yet. Such an individual would clarify the role of 101T→C in causing nonsyndromic hearing-loss type DFNB1. Our observations, however, do not rule out the possibility of an interaction of 101T→C with a second gene, at the same or another locus, to exert a dominant effect. In vitro and in vivo studies indicate that specific heterotypic channels can form that have communication properties distinct from homotypic channels. Connexin 32 (Cx32) mutations are causative for X-linked dominant Charcot-Marie-Tooth neuropathy (CMTX). CMTX also causes deafness in some families. In vitro studies indicate a dominant negative effect on Cx26 activity by some Cx32 mutants (Bruzzone et al. 1994). A mutation in a second connexin gene, such as the CMTX gene, might act with a specific allele of GJB2 to cause a digenic form of deafness that could exhibit a dominant pattern of inheritance.

Three of the new frameshift mutations discovered eliminate major functional domains of Cx26 and are presumed to severely disrupt the topological state of these polypeptides. A deletion frameshift at codon 104 in the CL domain (fig. 5) terminates four later codons, eliminating two transmembrane domains, the E2 do-

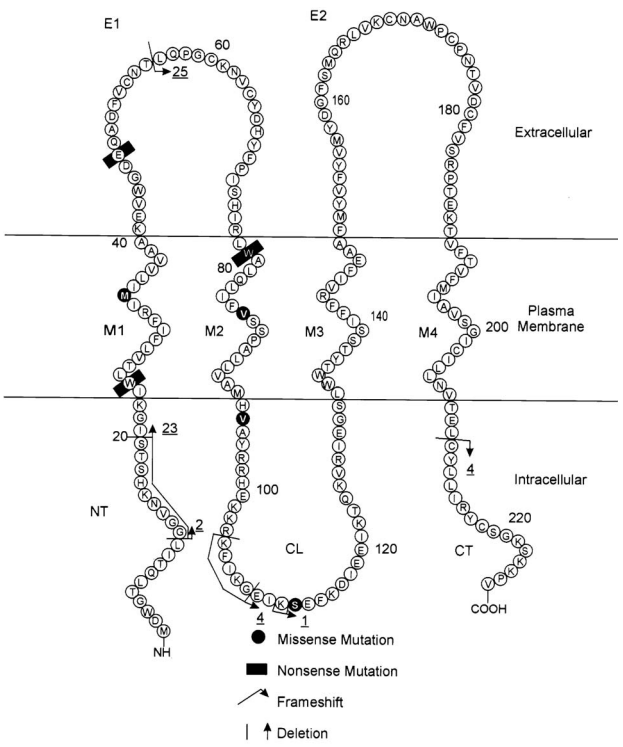

Figure 5 Schematic representation of Cx26 and its mutations. NT = N-terminal domain; M1, M2, M3, and M4 = transmembrane domains; E1 and E2 = extracellular domains; CL = cytoplasmic linking domain; and CT = C-terminal domain. Underlined numbers next to a frameshift or deletion arrow indicate the number of residues, after the frameshift, before a termination codon is found. The polypeptide chain is numbered every 20th residue. Mutation type is designated according to the key at the bottom of the figure.

main and the C-terminal domain. This 14-bp deletion is flanked by a 2-bp GA repeat that may have allowed slipped mispairing during DNA synthesis. A second deletion at codon 111 terminates two later codons, with similar consequences. A 2-bp deletion mutation at codon 210 causes a frameshift and termination four codons downstream, eliminating the C-terminus domain.

Three novel missense mutations were found. One of the mutations, Val84Met, occurs in the second transmembrane domain, M2 (fig. 5). This methionine replaces an invariant valine. The next missense mutation, Val95Met, is a similar change occurring at the interface of the CL domain and the M2 domain. The pathologic significance of this change is more difficult to explain, since a methionine has been found in this position in alpha connexins; however, only valine has been found in beta connexins such as Cx26. An additional missense mutation is found at codon 113, where a serine codon has been replaced with an arginine codon in the middle of the CL domain. Two other changes have been found only in unaffected controls and may represent polymorphic changes. Both occur in the M1 domain and involve the conversion of a valine codon to an isoleucine codon (Val27Iso and Val37Iso). Val27Iso is at an invariant valine, in all connexins. Val37Iso is at an invariant valine, in beta connexins. In alpha connexins, glycine, serine, and alanine, but not valine, codons are found at that position. Since these mutations were observed only as heterozygotes, the possible pathologic significance must await further studies.

The frequency of childhood deafness is ~.001, or 1/1,000 (Van Camp et al. 1997). Half of this hearing loss is genetic, whereas about half of this genetic hearing loss is recessive nonsyndromic. This study indicates that ~40% (49/116 chromosomes, excluding 101T→C) of childhood nonsyndromic hearing loss is caused by mutations in DFNB1. These data suggest that ~20% of all childhood hearing loss is caused by mutations in DFNB1, most by the 30delG mutation, for a frequency of .0001, or 1/10,000, of all childhood hearing loss. No consistent audiologic phenotype was observed in individuals with mutations in Cx26. Hearing loss varied from mild to profound even within the group of families homozygous for the common mutation 30delG. These results suggest that other factors may significantly modify the effects of mutations in Cx26.

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