Mutations in connexin31 underlie recessive as well as dominant non-syndromic hearing loss

Xue-Zhong Liu1,2,+, Xia Juan Xia1, Li Rong Xu3, Arti Pandya1, Chuan Yu Liang3, Susan H. Blanton1,4, Steve D.M. Brown5, Karen P. Steel6 and Walter E. Nance1

1Department of Human Genetics and 2Department of Otolaryngology, Medical College of Virginia of Virginia Commonwealth University, Richmond, VA 23298-0033, USA, 3Department of Otolaryngology, West China University of Medical Sciences, Chengdu 610041, China, 4Department of Pediatrics, University of Virginia, Charlottesville, VA 22908, USA, 5MRC Mammalian Genetics Unit and UK Mouse Genome Center, Harwell, Oxon OX11 ORD, UK and 6MRC Institute of Hearing Research, University Park, Nottingham NG7 2RD, UK

Mutations in the GJB3 gene encoding connexin31 (Cx31) can cause a dominant non-syndromic form of hearing loss (DFNA2). To determine whether mutations at this locus can also cause recessive non-syndromic deafness, we screened 25 Chinese families with recessive deafness and identified in two families affected individuals who were compound heterozygotes for Cx31 mutations. The three affected individuals in the two families were born to non-consanguineous parents and had an early onset bilateral sensorineural hearing loss. In both families, differing SSCP patterns were observed in affected and unaffected individuals. Sequence analysis in both families demonstrated an in-frame 3 bp deletion (423→425delATT) in one allele, which leads to the loss of an isoleucine residue at codon 141, and a 423A→G transversion in the other allele, which creates an Ile→Val substitution at codon 141 (I141V). Neither of these two mutations was detected in DNA from 100 unrelated control subjects. The altered isoleucine residue lies within the third conserved α-helical transmembrane domain (M3), which is critical for the formation of the wall of the gap junction pore. Both the deletion of the isoleucine residue 141 and its substitution to valine in the two families could alter the structure of M3, and impair the function of the gap junction. The present data demonstrate that, like mutations in connexin26, mutations in Cx31 can lead to both recessive and dominant forms of non-syndromic deafness.

INTRODUCTION

Hearing impairment is the most common disorder of sensorineural function and is an economically and socially important cause of human morbidity. Genetic factors are known to represent a major cause of hearing loss. Approximately 80% of genetic deafness is non-syndromic (not associated with other clinical features) and, in this group, 60–75% are autosomal recessive forms (DFNB) (1). Although >22 DFNB loci have been mapped, only six genes have been identified (2), including the connexin26 (Cx26) gene (GJB2) for DFNB1, the myosin VIa gene (MYO7A) for DFNB2 (4,5), the myosin XV gene (MYO15) for DFNB3 (6), the pendrin gene (PDS) for DFNB4 (7), the tectorin gene (TECTA) for DFNB21 (8) and the otoferlin gene (OTOF) for DFNB9 (9). Mutations in the GJB2 gene have been found to cause the most common form of non-syndromic recessive deafness (DFNB1) in many populations, and are also involved with one dominantly inherited form of deafness (DFNA3) (10,11). More recently, it has been shown that another connexin gene, GJB3 [encoding connexin31 (Cx31)], underlies an additional form of dominantly inherited non-syndromic hearing loss (DFNA2) (12). Currently, none of the known DFNB loci map to the same chromosomal region as the Cx31 gene, i.e. 1p32–36. However, in the case of GJB2, different mutations have been shown to result in either dominant or recessive alleles. Therefore, we initiated a study to determine whether there are mutations in Cx31 which lead to recessive alleles. Twenty-five probands from China with non-syndromic deafness without obvious dominant inheritance patterns were screened for mutations in the GJB3 gene. We have identified two families in which compound heterozygosity for two Cx31 mutations was the cause of hearing loss in affected family members.

RESULTS AND DISCUSSION

In total, 25 Chinese families from the Sichuan province, affected with non-syndromic recessive deafness, were screened for Cx31 mutations. Three affected individuals who were born to non-consanguineous parents from two families were identified as having an early-onset bilateral sensorineural hearing loss (Fig. 1a). None had other associated abnormalities. In family DFNB.03, patients II.1 and II.2 had a severe hearing loss showing flat audiograms without apparent
progression (Fig. 1b). They both demonstrated normal vestibular function. The father and the mother (I.1 and I.2) both had good hearing with normal pure tone audiograms. In family DFNB.15, patient II.02 was first noted to have hearing loss at the age of 7 years. The hearing loss was moderate and affected all frequencies, resulting in a flat audiogram (Fig. 1b). She had normal vestibular function, and both of her parents had normal hearing with normal pure tone audiograms. There is a history of a paternal uncle who was said to have a similar early-onset hearing loss (not available for testing).

Two methods of mutation detection were used in this study: single-strand conformation polymorphism (SSCP) and sequencing. The \textit{GJB3} gene contains a single, uninterrupted open reading frame of 813 nucleotides (12). We focused our search for mutations on the coding region of \textit{Cx}31. Five pairs of overlapping primers covering the entire coding region of \textit{Cx}31 were used for SSCP analysis (see Materials and Methods). In both families (DFNB.03 and DFNB.15), differing SSCP patterns for the PCR of primer 3FR were observed in affected and unaffected individuals (data not shown): the three affecteds showed the same homozygous variant, and the four unaffecteds had a heterozygous (subjects I.1 and I.2 of family DFNB.03, as well as subject I.2 of family DFNB.15) or a normal pattern (subject II.2 of family DFNB.15).

Sequence analysis of the SSCP variants demonstrated that the three affected individuals were compound heterozygotes with an in-frame 3 bp deletion (423–425delATT) in one allele, which leads to the loss of an isoleucine residue at codon 141 (141delIle), and a 423A→G transversion in the other allele, which creates an Ile→Val substitution, also at codon 141 (141IV) (Fig. 1c). In family DFNB.03, the 423–425delATT was inherited from the unaffected father and the 423A→G change was inherited from the unaffected mother (Fig. 1a). In family DFNB.15, neither of the mutations was found in one unaffected brother, the 423–425delATT was inherited from the unaffected mother and the 423A→G change was apparently inherited from the unaffected father (Fig. 1a). To exclude the possibility that the mutations are simply polymorphisms in the population that we have studied, samples from 100 unrelated control subjects (60 from China and 40 from other populations) were analyzed for both mutations by SSCP. Neither of the two mutations was detected in the control panel. Both mutations are believed to be pathological: first, because of their location and conservation (see below) and, second, because neither change has been observed in a series of normal controls.

The \textit{GJB3} gene is a member of the connexin family, which encodes the \textit{Cx}31 component of gap junctions. Six connexin molecules assemble to form a half-channel or connexon, which docks with its counterpart in an adjacent cell to make a complete intercellular channel. Members of the connexin family have highly conserved sequences and four transmembrane domains linked by one cytoplasmic and two extracellular loops, with cytoplasmic C- and N-terminal ends (Fig. 2) (13). To date, mutations in \textit{Cx}31 have been found to cause a dominant form of non-syndromic deafness (DFNA2) and autosomal dominant erythrokeratodermia variabilis (Table 1). The skin disorder was linked to missense mutations in either the N-terminal domain or the second transmembrane domain (Fig. 2) (14). Two different mutations in the second extracellular loop domain of \textit{Cx}31 have been identified in two Chinese DFNA2-affected families (Fig. 2) (12). One mutation is a nonsense mutation (R180X), causing a predicated truncation of the fourth transmembrane and C-terminal cytoplasmic domains, and other is a missense substitution, converting a conserved glutamine to lysine (R183K). In these two families, there was a degree of mild to moderate progressive hearing loss with gently sloping audiograms mainly affecting high frequencies. All affected male individuals from the two families had a late-onset hearing loss ranging from age 20 to 40 years (Table 1).

The altered isoleucine residue in our two recessive families lies within the third conserved \(\alpha\)-helical transmembrane domain (M3) of the \textit{GJB3} gene (Fig. 2), which is critical for the formation of the wall of the gap junction pore (13). This residue is conserved in mouse \textit{Cx}31 and rat \textit{Cx}31, whereas the other \(\beta\)-type connexins contain a phenylalanine at this position, a conservative amino acid substitution (15,16). In the \textit{Cx}26 gene, 20 different mutations have been identified in patients with non-syndromic deafness, including missense mutations, stop mutations and small deletions or insertions across all protein domains. Of these mutations in \textit{Cx}26, most are recessive alleles, some may be semidominant and at least one mutant allele (W44C) is associated with dominant deafness (2). One mutation in \textit{GJB2}, 35delG, is particularly common, accounting for two-thirds of all \textit{Cx}26 mutations in DFNB1 patients originating from various ethnic backgrounds. Several of these \textit{Cx}26 mutant residues lie within the M3 domain, and mutations affecting this region have also been reported in \(X\)-linked-Charcot–Marie–Tooth disease (CMTX; connexin 32 (Cx32)). Moreover, mutations in residues close to Ile141 affected in the families reported here, namely Arg143 and Glu147 in \textit{Cx}26 as well as Arg142 in \textit{Cx}32, have been reported previously in patients with hearing impairment (2,17).

Mutations in the M3 domain of \textit{Cx}32 have been shown to cause loss of intracellular transport in CMTX: connexons are formed but transport is deficient because of the incorrect alignment of two connexons or the inability to form a functional gap junction between two connexons (18). Both the deletion of Ile141 and its substitution to valine in the two families reported here would cause a change in the structure of M3, which might impair or block channel formation and result in impaired function of the gap junction. Interestingly, a deletion of Leu143/144 of \textit{Cx}32, one codon upstream of the \textit{Cx}31 deletion reported here, has been implicated in CMTX (19). The identification of several pathological mutations associated with hearing loss in the same region of the M3 domain in these three connexin genes (\textit{Cx}26, \textit{Cx}31 and \textit{Cx}32) suggests that the M3 domain is important to the function of the \textit{Cx}31 protein and plays a vital role in forming connexons in the cells of the inner ear.

The heterozygous carriers in our two families with recessive deafness had normal hearing, suggesting that half of the usual amount of normal \textit{Cx}31 is sufficient to preserve adequate cochlear function. In contrast, the heterozygous carriers of the previously reported \textit{GJB3} mutations in dominantly inherited deafness (DFNA2) showed late-onset, progressive hearing loss (Table 1). One possible explanation for this difference is that the dominant mutations might lead to a dominant-negative effect, for example by resulting in the presence of an abnormal \textit{Cx}31 molecule which affects the activity of any connexin
Figure 1. Mutation analysis of two families. (a) Pedigrees of families DFNB.03 and DFNB.15. (b) Audiograms of patients from families DFNB.03 (II.1 and II.2) and DFNB.15 (II.1) as well as a typical audiogram of patients carrying a dominant Cx31 mutation (12). (c) Sequencing gels showing two mutations of Cx31 in two DFNB families. (1) Normal sequence (antisense strand); (2) sequence of clones from patient II.1 in family DFNB.03 showing a 3 bp deletion (antisense strand); and (3) showing A→G substitution (sense strand) as for patient II.2 in family DFNB.03 and patient II.1 in family DFNB.15 (data not shown). The mother (I.2) of family DFNB.03 is heterozygous for the A→G substitution, and the father (I.1) of family DFNB.03 is heterozygous for the 3 bp deletion, as was the mother (I.2) of family DFNB.15 (data not shown). The 3 bp deletion was confirmed by direct sequencing of PCR products from five family members.
multimers (connexons) containing the mutant protein, whereas the recessive mutations might instead result in the absence of a protein product of the mutated gene, which in turn may have a less deleterious effect on gap junction formation. Alternatively, the recessive mutations could result in a Cx31 molecule with only reduced activity, allowing heterozygotes to have normal cochlear function, and the dominant mutations could be null alleles, producing no protein and leading to hearing loss by haploinsufficiency. Further work on the nature of the connexins produced by the mutant alleles will be needed to resolve the molecular basis of the pattern of inheritance.

The present data demonstrate that Cx31, like Cx26, can be responsible for both recessive and dominant forms of nonsyndromic deafness. The identification of the same mutations in two separate families suggests that both mutations may be a relatively common cause of recessive hearing loss in the Chinese population. This increases the number of cloned genes involved in both DFNA and DFNB forms to four: the three other genes are GJB2 (Cx26) for DFNB1 and DFNA3 (3,10), MYO7A for DFNB2 and DFNA11 (4,5,20), and TECTA for DFNB21 and DFNA8/12 (8,21). Nevertheless, an important implication of our results is that it would be useful to screen samples from other populations to establish the likely frequency of GJB3 mutations among those with recessive nonsyndromic deafness.

**MATERIALS AND METHODS**

**Clinical evaluation of patients**

Families were ascertained through the Clinic for the Deaf (Chengdu, China). Informed consent was obtained from all participants and from parents of patients younger than 18 years. In total, 25 Chinese families affected with nonsyndromic deafness without obvious dominant inheritance patterns were included in this study. In all families, there existed at least two hearing-impaired members with normal hearing parents. Eighty percent of the families had profound prelingual deafness, and 20% showed moderate/severe deafness. The clinical history was obtained and an examination was conducted on each individual by one of the investigators, with special emphasis on identifying potential environmental causes of hearing loss such as infections, trauma and information on exposure to known or possible ototoxic drugs or on evidence of syndromic forms of deafness (22). The hearing of all affected and unaffected individuals (individuals I.1 and II.2 of family DFNB.3; individuals I.1, I.2 and II.2 of family DFNB.15) in the present series was examined using pure tone audiometry. Air conduction thresholds were measured at 0.25, 0.5, 1, 2, 4, 6 and 8 kHz. Bone conduction thresholds were determined to identify the type of hearing loss. Oto-immittance measurements were undertaken on all individuals and all were otoscopically examined. Ice-water caloric using Frenel’s glasses were undertaken in these patients. Further details of clinical evaluations can be found in Liu and Xu (22).

**SSCP and sequence analysis of Cx31**

Blood was obtained from affected individuals and available unaffected family members, and DNA was extracted. Linkage

---

### Table 1. Phenotype–genotype correlations in patients with GJB3 mutations

<table>
<thead>
<tr>
<th>Mutations (reference)</th>
<th>Family</th>
<th>Protein modification</th>
<th>Phenotype</th>
<th>Age of onset</th>
<th>Audiogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant alleles (12,14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>538C→T</td>
<td>Chinese</td>
<td>R180stop</td>
<td>Deafness</td>
<td>20–40 years</td>
<td>Mild–moderate, 20–55 dBHL, middle–high frequencies, gently sloping shape</td>
</tr>
<tr>
<td>547G→A</td>
<td>Chinese</td>
<td>E183K, E2 domain</td>
<td>Deafness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34G→C</td>
<td>Caucasian</td>
<td>G12R</td>
<td>Keratodermia variabilis</td>
<td>At birth or early childhood</td>
<td>Normal</td>
</tr>
<tr>
<td>35G→A</td>
<td>Caucasian</td>
<td>G12D, NT domain</td>
<td>Keratodermia variabilis</td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>256T→A</td>
<td>Caucasian</td>
<td>C86S, TM2 domain</td>
<td>Keratodermia variabilis</td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Recessive alleles (this study)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>423A→G</td>
<td>Chinese</td>
<td>141delI</td>
<td>Deafness</td>
<td>Pre-lingual or early childhood</td>
<td>Moderate–profound, 45–85 dBHL, all frequencies, flat shape</td>
</tr>
<tr>
<td>423–425delAT</td>
<td>Chinese</td>
<td>I141V , TM3 domain</td>
<td>Deafness</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Audiograms were categorized in affected individuals based on the classification of Liu and Xu (22).
analysis for the GJB3 locus was not performed to exclude/include families prior to mutation screening. The Cx31 gene has a single exon with a coding region of 810 bp (12,14). Five pairs of overlapping primers were designed from the complete coding and flanking untranslated region sequence of Cx31 (GenBank accession no. AF099730) using the Primer 3 program (http://www-genome.wi.mit.edu/genome-software/other/primer3.html). The amount of overlap between each of the primer pairs ranged from 21 to 61 bp. Primers were: 1F, 5'-ATT CTC TCA AGG CAC GG -3', and 1R, 5'-TGG TGT AGT CAA AGT CC-3' (210 bp PCR product); 2F, 5'-TAT ACG TGG TGG CTG CAG AG-3', and 2R, 5'-CTG CTG TGT AGT ACA GCT TG-3' (270 bp PCR product); 3F, 5'-CTC GCT GGT CAT CTT CCT-3', and 3R, 5'-CAT ATT GAA GCC ATG CCA GA-3' (223 bp PCR product); 4F, 5'-OTTC CTC TAC CTG CTG CAC AC-3', and 4R, 5'-GGC AGA TGA GGT AGC AGA GC-3' (202 bp PCR product); 5F, 5'-CCG TCT GCA TCG TAC TCA CC-3', 5R, 5'-CCT GCC TGG TCA GAT-3' (255 bp PCR product). The coding region of the Cx31 gene was amplified from genomic DNA by PCR using the above primers. The amplification conditions were 95°C for 5 min, then 30 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, with a final extension for 5 min at 72°C.

For mutation analysis, the PCR products were run on a 1 mm thick 8% non-denaturing polyacrylamide gel (acrylamide:methylene bisacrylamide 49:1) at 4°C. When a variant band was seen in one of the patients, samples from all family members were tested.

Direct sequencing of PCR products with SSCP variants from patients and carriers were performed on both strands using the T7 Sequenase v2.0 (USB, Carlsbad, CA) for single-strand sequencing. For each sample, at least eight clones were sequenced using the T7 Sequenase v2.0 sequencing kit method.

ACKNOWLEDGEMENTS

We thank the families for their contribution to this study. We thank Wanda Hunt for her technical help. This work was supported by the Jeffress Research grant J-523, Defeating Deafness, AD, Williams Trust Funds and NIHRO1 grant DC02530.

REFERENCES