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Digenic inheritance of non-syndromic deafness caused by mutations at the gap junction proteins Cx26 and Cx31

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Abstract

Mutations in the genes coding for connexin 26 (Cx26) and connexin 31 (Cx31) cause non-syndromic deafness. Here, we provide evidence that mutations at these two connexin genes can interact to cause hearing loss in digenic heterozygotes in humans. We have screened 108 *GJB2* heterozygous Chinese patients for mutations in *GJB3* by sequencing. We have excluded the possibility that mutations in exon 1 of *GJB2* and the deletion of *GJB6* are the second mutant allele in these Chinese heterozygous probands. Two different *GJB3* mutations (N166S and A194T) occurring in compound heterozygosity with the 235delC and 299delAT of *GJB2* were identified in three unrelated families (235delC/N166S, 235delC/A194T and 299delAT/A194T). Neither of these mutations in *Cx31* was detected in DNA from 200 unrelated Chinese controls. Direct physical interaction of Cx26 with Cx31 is supported by data showing that Cx26 and Cx31 have overlapping expression patterns in the cochlea. In addition, by coimmunoprecipitation of mouse cochlear membrane proteins, we identified the presence of heteromeric Cx26/Cx31 connexons. Furthermore, by cotransfection of mCherry-tagged Cx26 and GFP-tagged Cx31 in human embryonic kidney-293 cells, we demonstrated that the two connexins were able to co-assemble *in vitro* in the same junction plaque. Together, our data indicate that a genetic interaction between these two connexin genes can lead to hearing loss.

INTRODUCTION

Hearing loss is one of the most common inherited disorders and is a highly heterogeneous sensory disorder. Until now, over 100 loci and 46 different genes in which mutations cause monogenic nonsyndromic sensorineural hearing loss, have been reported (http:webhost.ua.ac.be/hhh/). Despite this heterogeneity, in many populations, up to 50% of autosomal recessive non-syndromic sensorineural hearing loss (AR-NSNHL) is associated with mutations in the locus DFNB1 (MIM 220290) on chromosome 13q12, which contains the two connexin (Cx) genes (*GJB2* and *GJB6*). Cxs are membrane-spanning proteins that coassemble into intercellular gap junction channels. Gap junction channels mediate electrical and biochemical communication between adjacent cells and play vital roles as mediators of intercellular molecular signaling. Cx-linked deafness highlights the key role of gap junctions

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in the physiological processes of hearing. Colocalization of Cxs with the gap junction system in the inner ear suggests a role in cochlear electrolyte homeostasis. During auditory transduction, they are proposed to maintain membrane potentials by regulating the flow of potassium ions between the sensory epithelia of the inner ear (Simon and Goodenough 1998; White and Paul 1999). To date, mutations in the genes encoding three of these Cxs (GJB2 for Cx26 (MIM 121011), GJB6 for Cx30 (MIM 604418), and GJB3 for Cx31 (MIM 603324) are known to result in hearing impairment (Kelsell et al. 1997; Grifa et al. 1999; Xia et al. 1998). The defect in any two of the four alleles from *GJB2* and *GJB6* could result in hearing impairment. Thus, either monogenic or digenic inheritance can occur with these genes. Among individuals with DFNB1-associated AR-NSNHL, 98% are estimated to carry two identifiable mutations in *GJB2*, whereas 2% are reported to have mutations in both *GJB2* and *GJB6* (Genetests DFNB1, http://www.genetests.org/). Mutations in *GJB3* have originally been shown to underlie an autosomal dominant form of non-syndromic deafness (DFNA2) in Chinese patients (Xia et al. 1998). We have also reported an autosomal recessive non-syndromic form of *GJB3* mediated deafness in this population (Liu et al. 2000). In Spanish patients, several *Cx31* variants have been associated with a syndromic form of neuropathy and hearing loss (Lopez-Bigas et al. 2000; 2001). Variations in the *Cx31* gene have also been linked to nonsyndromic deafness in Brazilian patients (Alexandrino et al. 2004). Mutations in the *GJB3* gene have also been reported to cause both autosomal dominant and recessive skin diseases (Plantard et al.. 2003; Richard et al. 1997; 1998; 2000).

Nevertheless, 10% to 50% of patients with prelingual nonsyndromic deafness carry a single heterozygous recessive mutation in the *GJB2* gene. Although the finding that the del(*GJB6-D13S1830*) mutation provided an explanation for the deafness in as many as 30% to 70% of affected *GJB2* heterozygotes in some populations, it has become clear that other mutations, both within DFNB1 and elsewhere involved in epistatic interactions with *GJB2*, contribute significantly to AR-NSNHL in most populations (del Castillo et al. 2003). Given the high prevalence of patients carrying only one mutant allele in *GJB2* with apparent lack of the del (*GJB6*-D13S1830) and effect of pathogenic mutations of *GJB2* and *GJB3* in Chinese patients with autosomal recessive deafness, we initiated a study to determine whether there is functional interaction between the *GJB2* and *GJB3* genes. We provide evidence that mutations in the *Cx26* and *Cx31* genes can interact to cause hearing loss in digenic heterozygotes.

RESULTS

Mutations at the gap junction proteins Cx26 and Cx31 can interact to cause non-syndromic deafness

In total, 108 probands screened for mutations in the *Cx26* gene were found to carry a single recessive mutant allele. In those samples, no mutation was detected on the second allele either in *Cx26*-exon-1/splice sites or in *GJB6*. To investigate the role of *GJB3* variations along with *GJB2* mutations for a possible combinatory allelic disease inheritance, we have screened patients with heterozygous *GJB2* mutations for variants in *Cx31* by sequencing. Analysis of the entire coding region of the *Cx31* gene revealed the presence of two different missense mutations (N166S and A194T) occurring in compound heterozygosity along with the 235delC and 299delAT of *GJB2* in 3 simplex families (235delC/N166S, 235delC/A194T and 299delAT/A194T).

In family A, a profoundly hearing impaired proband was found to be heterozygous for a novel A to G transition at nucleotide position 497 of *GJB3*, resulting in an asparagine into serine substitution in codon 166 (N166S) and for the 235delC of *GJB2* (Fig. 1b, d). Genotyping analysis revealed that the *GJB2*/235delC was inherited from the unaffected father and the N166S of *GJB3* was inherited from the normal hearing mother (Fig. 1a). In families F and K, a heterozygous missense mutation of a G-to-A transition at nucleotide 580 of *GJB3* that causes

A194T, was found in profoundly deaf probands, who were also heterozygous for *GJB2*/*235delC* (Fig. 1g, i) and *GJB2*/299-300delAT (Fig. 1l, n), respectively. In Family F, the *GJB2*/235delC was inherited from the unaffected father and the A194T of *GJB3* was likely inherited from the normal hearing deceased mother (Fig. 1f). In Family K, genotyping analysis revealed that the father transmitted the A194T/*GJB3*, while the mother is heterozygous for the *GJB2*/299-300delAT (Fig. 1k). Carriers of a single affected allele in the two families had normal hearing. To exclude the possibility that the identified mutations were DNA polymorphisms in the studied population, samples from 200 unrelated control subjects were analyzed for both mutations by sequencing. 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 200 randomly selected, unrelated, normal hearing Chinese subjects. In addition, the pathogenicity of the N166S mutation is probable: because the asparagine is located in the second extracellular loop (E2), which is the major determinant for specificity of heterotypic interactions between hemichannels. It has also been proposed that the E2 region might be involved in targeting of the hemichannels to the plasma membrane as well as in proper cellular distribution (Verselis et al. 1994; Trexler et al. 2000). Furthermore, previous studies suggest that the specificity of heterotypic interactions between hemichannels composed of different connexins is largely dictated by the primary sequence of the second extracellular loop (White et al. 1994). Our finding is that the replacement of asparagine with serine, a smaller amino acid with a β-hydroxyl group in the N166S mutation, disrupts the primary sequence of EC2. This may induce a defect in the EC2 secondary structure that impairs Cx31 mediated coupling of cochlear cells. The multiple alignment of this domain with its homologues in rat and mouse, shows that the asparagine at position 166 is in fact a serine in these two species. Secondary structure prediction shows this asparagine to be in a short loop region in between two short beta strands. The asparagine at codon 166 is absent in the other human beta-connexins. The fact that the serine is found in rat and mouse and is not present among other human beta-connexins indicate that this mutation may be nonpathogenic. However, serines are able to be phosphorylated. We would speculate that the mutation may have caused the addition of a phosphorylation signal on the protein, and that this in turn might disrupt its usual function in humans. Altogether, these observations may give an explanation that the digenic inheritance of this *Cx31* mutation with the *Cx26* mutation can lead to hearing impairment due to impaired heterotypic interactions.

The altered alanine residue in A194T identified in Families F and K lies within the fourth transmembrane domain (M4) of the *GJB3* gene, which has been postulated to play a role in connexin trafficking (White et al. 1994). Sequence alignments of Cx31 from human, bovin, mouse, rat and chicken show that A194 is a highly conserved alanine residue. This single base-pair substitution changed a hydrophobic, non polar residue (alanine) to a neutral polar, much larger amino acid (threonine), altering the hydropathy index from 0.14 to 0.42. Because charged residues are important for proteins trafficking, the A194T may result in accumulation of the Cx31 protein in intracellular compartments such as the Golgi apparatus or in other sites such as the endoplasmic reticulum or lysosomes (Bonifacino et al. 1991; Machamer et al. 1993). The A194T substitution might cause conformational changes within the Cx31 molecule or affect the ability of Cx31 to form heteromeric hemichannels. The relationship between hemichannel assembly may be complex, considering the different paradigms for connexin oligomerization (Musil and Goodenough 1993; Kumar et al. 1995). Many of the Cx26 mutant residues lie within the EC2 and TM4 domains. Mutations affecting these regions have also been reported in *Cx32* underlying X-linked-Charcot-Marie-Tooth disease. Moreover, mutations in residues close to N166 and A194 identified in the families reported here, namely, M163L, R165W, F191L, and A197S in *Cx26* (Matos et al. 2003; Rickard et al. 2001; Feng et al. 2002; Hamelmann et al. 2001) as well as F193C, S198F and G199R in *Cx32*, have been reported previously in patients with hearing impairment (Janssen et al. 1997). Interestingly, mutations identified in patients with the skin disease erythrokeratoderma variabilis (EKV) were

located within all the protein domains of the *Cx31* gene except for the EC2 and TM4 domains, which are main domains for deafness mutations. This correlation between location of mutations and phenotypes, together with the identification of pathological mutations associated with hearing loss in the same region of the EC2 and TM4 domains in these three connexin genes (*Cx26*, *Cx31*, and *Cx32*) suggested that the EC2 and TM4 domains are important to the function of the Cx31 protein in the inner ear and plays a vital role in forming connexons in the cells of the inner ear.

In the present study, we have shown that the missense N166S and A194T mutations in *GJB3* acts in a recessive manner in three unrelated Chinese patients. We have previously reported a recessive inheritance of an in-frame deletion and a missense mutation in *Cx31* in a screening of 25 Chinese families affected with non-syndromic deafness (Liu et al. 2000). A missense mutation and a nonsense mutation of *GJB3* have originally been shown to be associated with an autosomal dominant form of non-syndromic hearing loss in two Chinese DFNA2 families (Xia et al. 1998), indicating that *Cx31* alterations are a cause of deafness in non-syndromic hearing loss patients in this population. In contrast, the studies described so far have shown that variations in *Cx31* have no or a low genetic relevance in European populations and Caucasian in general. DFNA2 was first mapped in two large families with autosomal dominant non-syndromic deafness originating from Indonesia and the United States (Coucke et al. 1994). Three additional large DFNA2 families from Belgium and the Netherlands were subsequently reported (Van Camp et al. 1997). Extensive sequence analysis of the coding region and the 5′ UTR of *GJB3* in all 5 original DFNA2 families (Coucke et al. 1994) revealed no mutations (Van Hauwe et al. 1999). Several different *Cx31* alterations were found at similar frequencies in patients with deafness, patients with peripheral neuropathies and control subjects in the Spanish population (Lopez et al. 2000). Nonetheless, the dominant D66del mutation in *Cx31* linked to neuropathy and deafness, was identified in one small family within 260 unrelated deaf patients in the same population with unilateral or bilateral high frequency NSHL (Lopez-Bigas et al. 2001). The failure in finding deafness causing mutations in *Cx31* in screening of sporadic cases with NSHL performed in California and in Austria further confirms population relevance of *Cx31* mutations in Chinese population (Mhatre et al. 2003; Frei et al. 2004).

Direct physical interaction between Cx26 and Cx31 in the mouse cochlea

Co-immunolabeling of cochlear sections with antibodies against Cx26 and Cx31 shows that Cx26 was expressed in fibrocytes of the lateral wall and spiral limbus (Fig. 2a). Cx26 immunoreactivity was also found in the supporting cells and inner sulcus cells where immunolabeling of Cx31 was also positive (Fig. 2b). Superimposed image (Fig. 2d) shows that the expression of Cx26 and Cx31 overlapped in the cochlea. To examine whether the two Cxs directly interact with each other by co-assembling into the same gap junction, we performed co-immunoprecipitation experiments of gap junctions from mouse cochlear tissues. Western blots confirmed the presence of Cx31 protein in the cochlea (Fig. 3a; left panel). The predominantly single band supported that the antibody specifically recognized the Cx31 protein and there was no cross reactions with Cx26. A faint band at the location of Cx30 indicated a weak cross reaction of Cx31 antibody with the Cx30 protein. Protein complexes immunoprecipitated by Cx31 were detected by Western blots using antibodies against both Cx26 and Cx31, and both bands were detected (Fig. 3a; right panel) indicating that the two molecules physically interact with each other.

Cx26 and Cx30 are the two major Cx isoforms found in the cochlea that are known to co-assemble in cochlear gap junctions (Ahmad et al. 2003). To further examine the specificity of the interaction between Cx26 and Cx31, we performed co-immunoprecipitation using both wild type (WT) and $Cx30^{-/-}$ mice. Protein complexes with or without immunoprecipitation

by an antibody against Cx30 were examined by Western blotting analysis. Bands corresponding to Cx26 and Cx30 (Fig. 3b, lane 1) or Cx26 and Cx31 (lane 5) were detected if WT mice were used. In contrast, if cochlear tissue from $Cx30^{-/-}$ mice were used for immunoprecipitation by Cx30 antibody, no bands were detected (lanes 2 and 6). As controls testing for specificities of Cx26 and Cx30 antibodies, our Western blots showed no extra bands other than the two corresponding to Cx26 and Cx30 (Fig. 3b, lane 3) when WT mice were used. Only a single band corresponding to Cx26 remained when $Cx30^{-/-}$ mice were used (Fig. 3b, lane 4). Thus, our results support the findings of Abrams et al (2006) that Cx26, Cx30 and Cx31 can form heterotypic channels with each other. Co-assembly of Cx26 and Cx31 in the same gap junctions were further tested by reconstituting heteromeric gap junction *in vitro*. After cotransfection of HEK-293 cells using mCherry-tagged Cx26 and GFP-tagged Cx31 DNA constructs, we observed that all Cx26 gap junction plaques (labeled by mCherry in red, Fig. 4a) overlapped with GFP-tagged Cx31 gap junction plaques (Fig. 4b) (n>20). The *in vitro* data further supported that Cx26 and Cx31 are co-assembled in the same gap junctions.

DISCUSSION

Most cases of genetic deafness result from mutations at a single gene, but an increasing number of examples are being recognized in which recessive mutations at two loci are involved. In digenic inheritance, mutations in each of two unlinked genes are present in a single individual, and the combination of the two genetic hits causes a disease phenotype that is not apparent when an individual carries only one of these gene alterations. This mechanism complicates genetic evaluation and counseling, but provides an explanation for Connexin 26 heterozygotes who, for previously unknown reasons, are deaf. The del(*GJB6-D13S1830*) allele is most frequent in Spain, France, the United Kingdom, Israel, and Brazil, accounting for 5.0–9.7% of all the *DFNB1* alleles (delCastillo et al. 2003; Marlin et al. 2005) and this finding has led to a substantial decrease of the percentages of monoallelic *Cx26* mutation carriers in those countries. Interestingly, the del(*GJB6-D13S1830*) mutation is very rare in Southern Italy (one heterozygote/238 total screened cases) (delCastillo et al. 2003), but subsequent studies have shown that this large deletion is present in Northern Italy at frequencies similar to those of other European countries (Gualandi et al. 2004; del Castillo et al. 2005). However, the deletion has not been detected in Turkish, Italian, Austrian, Greek Cypriot and Chinese nonsyndromic hearing loss patients (del Castillo et al. 2003; Gunther et al. 2003; Uyguner et al. 2003; Neocleous et al. 2006; Liu et al. 2002). This may indicate that other mutations, both within DFNB1 and elsewhere may be involved in epistatic interactions with *GJB2*, in addition to the existence of mutations in the *cis* transcription regulatory elements that have not yet been considered, or it is not detected because of the technical limitations of the methods used for mutation analysis.

In the *Cx31* gene, currently more than 10 different mutations have been found in patients with deafness from Chinese, Brazilian, and Spanish populations (Xia Liu et al. 1998; Liu et al. 2001; Lopez-Bigas et al. 2001; Alexandrino al. 2004). However, the pathogenicity of most of these sequence alterations still remains questionable. The so far reported mutation spectrum in *Cx31* includes missense, deletion, and stop mutations are located in the first and second extracellular loop as well as in the third transmembrane domain of the connexin 31 protein. Of these *Cx31* mutations, two have been described to act as recessive alleles and 2 mutant alleles are associated with dominant deafness in Chinese population (Xia et al. 1998; Liu et al. 2000). Mutations in *GJB2* is one of the most common cause of non-syndromic deafness in the Chinese population (Liu et al. 2002; 2006; Liu et al. 2002; Dai et al. 2007). More than 25 different *GJB2* deafness-causing mutations have been identified in Chinese patients with non-syndromic deafness including missense, stop mutations and small deletions or insertions across all Cx26 protein domains (Dai et al. 2007; Liu et al. 2006). The high prevalence of *Cx26* mutations in the Chinese population is mainly due to the high frequency of the 235delC

deletion, which has a common ancient founder in China and Far East, as has been shown by haplotype analysis (Yan et al. 2003), at a frequency of 16.3% in Chinese cohorts with NSHL (Dai et al. 2007) and accounts for up to 80% of pathogenic *GJB2* alleles in DFNB1 patients in this population (Liu et al. 2002).

Our data have shown that Cx31 was expressed in the supporting cells and cells at the tip of the of spiral limbus that partially overlapped with the Cx26 cellular expression pattern. The overlapped localization suggested that it is feasible for the two Cxs to physically interact with each other. More evidence was provided by co-immunoprecipitation and co-localization after co-transfection. We have shown that in the cochlea Cx26 and Cx31 are able to form heterotypic channels like Cx26 and 30 (Cx26/Cx30) (Yum et al. 2007) and Cx31 and 32 (Cx31/Cx32) (Abrams et al. 2006), which provide a base of interaction of these connexins. Indeed, We have demonstrated that a digenic form of non-syndromic recessive deafness was caused by mutations in *Cx26* and *Cx31* and our data suggested that loss of any two of the four alleles from *GJB2* and *GJB3* can result in hearing impairment.

Several mutated connexins have been reported to mistarget gap junctions and/or fail to oligomerize correctly into hemichannels, or to alter specific permeability properties of gap junctions (Martin et al. 1999; Marziano et al. 2003; Bruzzone et al. 2003; Mese et al. 2004). In the present study, we show that double heterozygous patients for *Cx26* and *Cx31* mutations are deaf. The simultaneous loss of one *GJB2* and one *GJB6* allele may have reduced the dosage of both genes' products. This finding suggests that the two connexins may not be functionally equivalent and cannot thus compensate each other. The inability of other connexins to functionally compensate for the absence of another is a common feature of connexin-based disorders (Simon and Goodenough 1998), and has been interpreted as an indication that a specific complement of connexin channels is required to meet the specific physiological needs of a particular tissue. It is well known now that all connexins are not made equal: in fact they are differences in size and ionic selectivity and have distinct gating mechanisms. It has also been shown that channels composed of different connexins have different conductances (Veenstra 1996), and permeabilities to ions (Beblo and Veenstra 1997) and fluorescent dyes (Elfgang et al. 1995; Cao et al. 1998). We demonstrate that Cx26/Cx31, like the Cx26/Cx30 proteins, are co-expressed and can physically interact in the mouse cochlea. The cochlear cells would then be expected to exhibit a variety of single-channel events consisting of homomeric/homotypic Cx26 or Cx31 channels or homomeric/heterotypic Cx26/Cx31 channels, depending on combinations of connexins forming connexons, hemichannels, and full channels. However, it is not clear exactly what proportion of cochlear gap junctions exist in homomeric and heteromeric forms, which makes analysis of different Cx26/Cx31 molecular configurations difficult. We have examinated the functional status of homomeric gap junction channels formed by the N166S and A194T mutant proteins by monitoring the calcium diffusion and by using dye transfer studies in transfected gap junctions-deficient HEK293 cells. Compared with WT-Cx31, neither of the two *Cx31* variants showed obvious changes in ionic permeability (Data not shown). However, we cannot exclude the possibility that the pathogenesis of hearing loss caused by the N166S and A194T mutations in *GJB3* is due to their deleterious effect on biochemical coupling. Thus, the patho-genetic nature of the N166S and A194T remains to be elucidated. Nevertheless, an important implication of our results is that it would be useful to screen patients from other populations to establish the likely frequency of *GJB2*/*GJB3* mutations among those in whom deafness could be attributed to *GJB2* mutation in a heterozygous condition.

MATERIAL AND METHODS

Subjects and DNA samples

Probands/families were ascertained through the nationwide epidemiologilcal survey in Beijing, China (Dai et al. 2007). Informed consent was obtained from all of the participants. The study protocol was approved by the Ethics Committee of the Chinese PLA General Hospital and from institutional review board (IRB) at University of Miami. In total, 108 Chinese families affected with non-syndromic deafness without obvious dominant inheritance patterns were included in this study. All the probands showed a congenital, bilateral, severe to profound, sensorineural hearing impairment with normal hearing parents. 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 for evidence of syndromic forms of deafness. The hearing of all affected and unaffected individuals in the present series was examined using pure tone audiometry. Air conduction thresholds were measured at 250 Hz, 500 Hz, 1 kHz, 2 kHz, 4 kHz, 6 kHz 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. In addition, 200 Chinese control individuals with normal hearing were also analyzed. DNA was extracted from peripheral blood leukocytes using a commercially available kit.

Mutational analysis

After exclusion of *GJB2* including exon 1, *GJB6*, *SLC26A4*, and the A1555G mutation in the 12SrRNA gene (MTRNR1) as potential causes of hearing loss in the 108 probands with only one GJB2 mutant allele, the full *GJB3* coding region was analyzed. The coding exon (exon 2) and flanking intronic regions of *GJB2* gene were PCR amplified with forward primer 5′-TTGGTGTTTGCTCAGGAAGA-3′ and reverse primer 5′GGCCTACAGGGGTTTCAAAT 3′. PCR amplification of Cx31 was performed using the forward 5′-TACGATGGTTTTTCCTCTAATTCT-3′ and reverse 5′-TTGCATAACTTAGTGAACTCAGAG-3′ primer sets based on the *Cx31* sequence (BC012918). The PCR products were purified with the QIAquick spin column purification kit (Qiagen, Valencia, CA). Purified amplicons were cycle sequenced by using the Big Dye Terminator Cycle Sequencing Kit (PE Biosystems) and run on an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA,) according to manufacturer recommendations (PE Biosystems). DNA sequence variations were identified by alignment of the subject's DNA sequence to the wild type sequences AY280971 (*GJB2*) and BC012918 (*GJB3*) at the NCBI interface (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). Numbering of the genes begins with the nucleotide A of the ATG start codon in exon 2 as cDNA position number 1. The sequences were analyzed using Genetool Lite software and the genes Genebank sequences.

Immunolabeling of Cx31 in cochlear cryosections

Cochleas were obtained from adult mice (strain CD-1) and cochlear cryosections were prepared as previously described (Sun et al., 2005). Cochlear sections were blocked first with bovine serum albumin (2%) plus the serum (20%) of the host animal to generate the secondary antibodies prior to incubating with primary antibodies (1:200 dilution in PBS) overnight at 4 °C. Anti-Cx31 (Rabbit IgG, Zymed Laboratories, Southern San Francisco, CA) was used to label the cryosections. After washing in PBS three times the sections were incubated with secondary antibodies (1:500 dilution in PBS) for about 2 h at room temperature. Labeled sections were mounted in an anti-fade medium (Molecular Probes, Eugene, OR) and examined with a confocal microscope (Zeiss LSM, Carl Zeiss USA, Shrewsbury, PA).

Preparations and immunoprecipitation of gap junctions

The purified gap junctions from mouse cochlear tissues were prepared as previously described. Primary antibody against Cxs26, 30 or 31 (Zymed Laboratories, Southern San Francisco, CA) was added to the supernatant at 1:250 dilution and incubated overnight. Antibodies bound to connexons were precipitated by protein A-linked Sepharose beads using protocol as described earlier (Diez et al. 1999). Individual connexin protein or immunoprecipited protein complexes were separated by SDS PAGE and detected by immunoblotting using specific antibodies (1:1000 dilution in blocking solution) at 4 °C for 12–15 h using protocol previously described (Diez et al. 1999). Immunolabeled proteins were visualized by using enhanced chemiluminescence (Super-Signal, Pierce, Rockford, IL) exposed to X-ray films (Hyper Film, Amersham Biosciences, Piscataway, NJ).

Construction of the pCx31-eGFP-N1 clone

The human *Cx31* cDNA was epitope tagged at the carboxyl terminus by subcloning into the green fluorescent protein vector, pEGFP-N1 (Clontech Laboratories, Inc., S. San Francisco, CA). The primers (5′-GTCAGATCCGCTAGCATGGACTGGAAGACA -3′ and 5′-AAGCTTGAGCTCGAGGATGGGGGTCAGGTT -3′) were designed such that the PCR product contained NheI and XhoI restriction enzyme sites for subsequent subcloning into the peGFP-N1 vector.

Expression analysis of wild-type and mutant human Cx31 in Human Embryonic Kidney (HEK) 293 cells

GJ-deficient HEK293 cells (American Type Culture Collection, Manassas, VA) grown to 80%. HEK 293 cells were grown at 37°C in an incubator (5% CO_2, under a moist atmosphere), in MEM medium supplemented with penicillin and streptomycin (0.5% v/v final concentration) and foetal bovine serum (10% v/v final concentration; all reagents for cell culture were from CellGro, Mediatech, Herndon, VA, USA). One day prior to transfection, cells were dissociated by trypsin-EDTA for 2 min, seeded on glass coverslip in the culture medium and allow to grow up to 60–80% confluence. Transfection with pCx31-eGFP-N1 was performed using the FuGene6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN, USA) according to the protocol provided by the manufacturer. After 24h of incubation, culture medium was replaced. Functional assays were carried out at room temperature, 36 to 72h after transfection.

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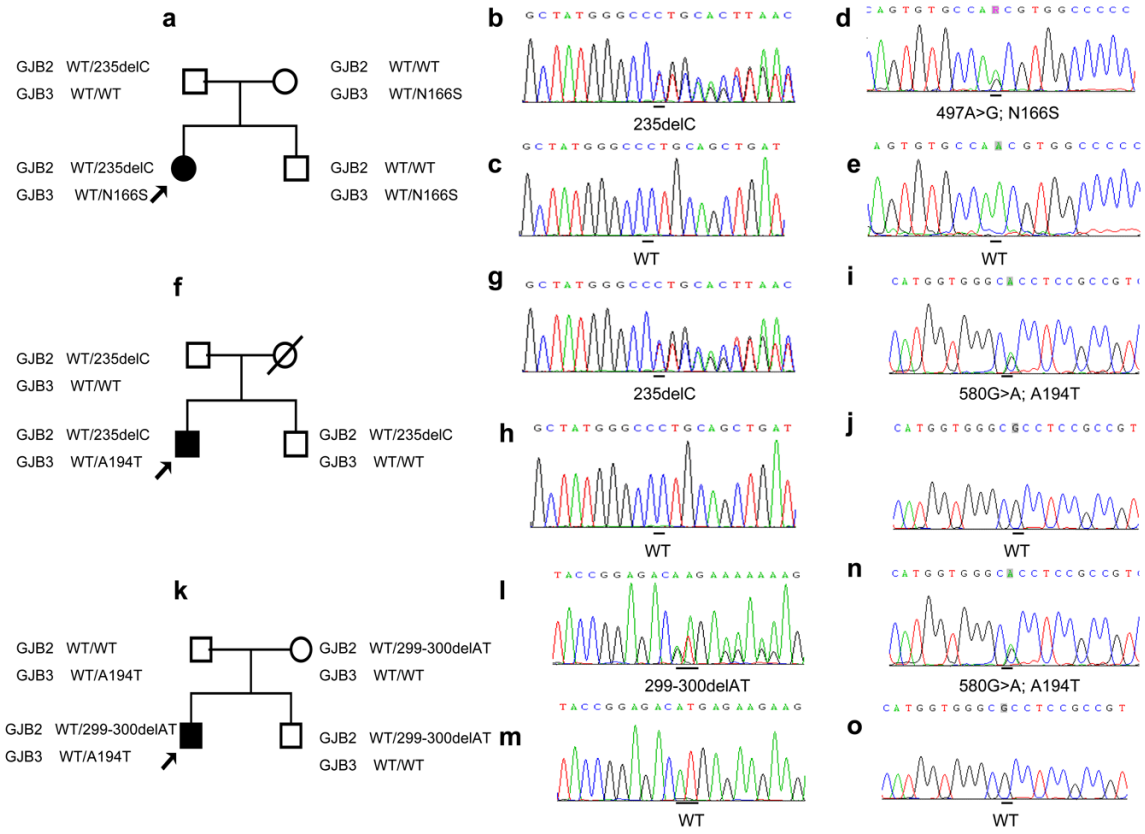

Figure 1. Evidence for digenic inheritance of deafness involving *GJB2* and *GJB3*. Pedigree and segregation of the mutations in *GJB2* and *GJB3*

The deaf proband is indicated by an arrow. *GJB2*/*GJB3* genotypes are given below the respective pedigrees symbol (a, f and k). Direct sequence analysis showing the 235delC mutation (b and g) and wild type (WT) allele (c and h) of *GJB2*. Direct sequence analysis showing the 299–300delAT mutation (l) and wild type (WT) allele (m) of *GJB2*. Direct sequence analysis showing the 497A>G (N166S) mutation (d) and WT allele (e) of *GJB3*. Direct sequence analysis showing the 580G>A (A194T) mutation (i and n) and WT allele (j and o) of *GJB3*.

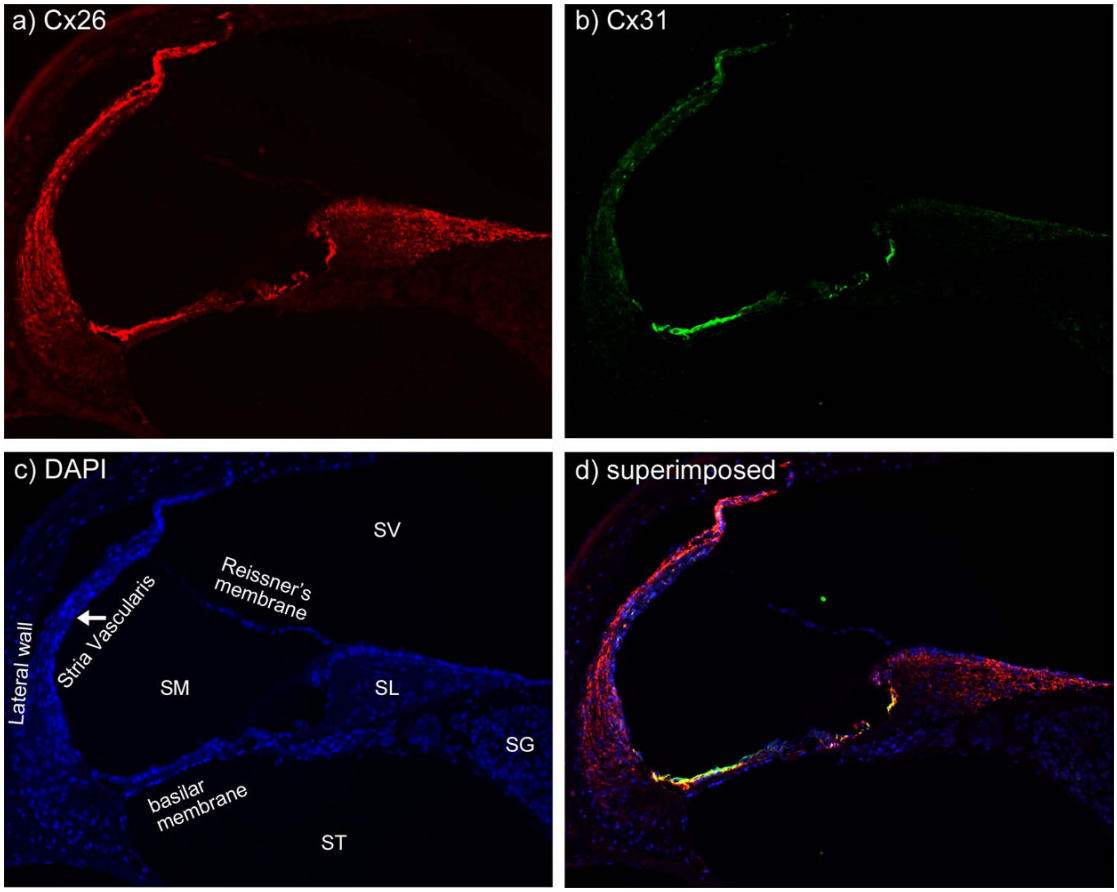

Figure 2. Expression of Cx31 and Cx26 in the mouse cochlea examined by coimmunostaining

Cochlear cryosections were cut at a thickness of 8 μm and labeled with an antibody against Cx26 (a) and Cx31 (b). DAPI staining gives the outline of cochlear structure (c). The superimposed image is given in (d). Abbreviations: SV: scala vestibule; SM: scala media; ST: scala tympani; SL: spiral limbus; SG: spiral ganglia.

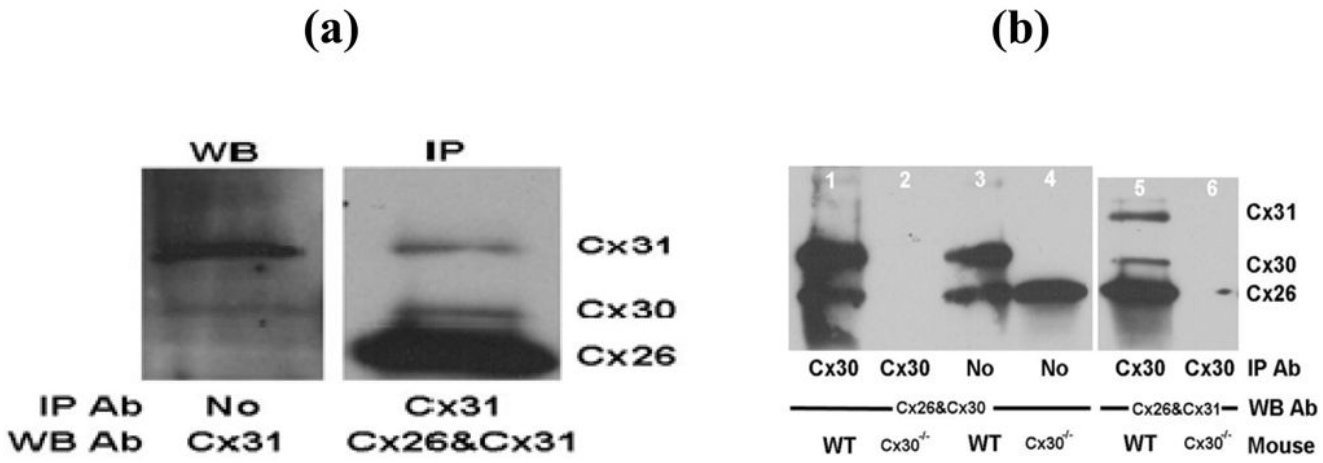

Figure 3. Direct physical interaction between Cx26 and Cx31 in the cochlea

Western blot data confirmed the presence of Cx31 protein in the cochlea and the specificity of the antibody used. No cross-reactions were observed between the Cx26 and Cx31 antibodies (a; left panel). Detection of the Cx26 and Cx31 proteins by immunoblotting of the Cx31 immunoprecipited protein complex using antibodies against both Cx26 and Cx31 supported the physical interaction between the two connexins (a; right panel). The specificity of the interaction between Cx26 and Cx31 as well as that of the antibodies used is further confirmed by co-immunoprecipitation using both wild type (WT) and $Cx30^{-/-}$ mice, showing only the respective protein bands on the immunoblot in WT mice (b, lane 3). But a single band corresponding to Cx26 remained when $Cx30^{-/-}$ mice were used (b, lane 4).

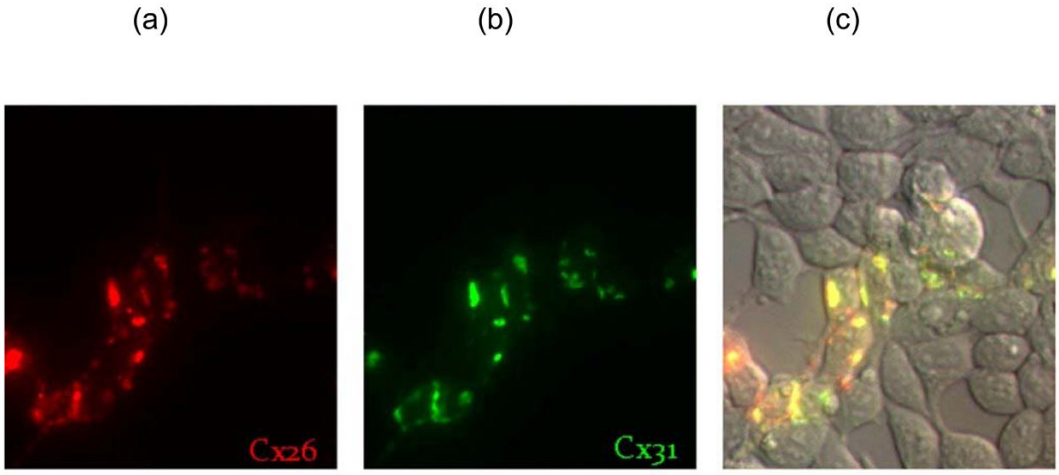

Figure 4.

Cx26 and Cx31 colocalize in the same reconstituted gap junction –plaque by cotransfection and coimmunolabeling of mCherry -tagged Cx26 and GFP-tagged Cx31 in HEK-293 cells. Cx26-labeled gap junction plaques in red (a) overlapped with GFP-tagged Cx31 gap junction plaques (b) in cell pairs (n>20) we examined. Simultaneous presence of red (mCherry) and green (GFP) fluorescence in the same cell (c) was observed in the majority of cells from random views. Using the FuGene6 transfection reagent, about 40% cells was transfected with the Cx plasmids as determined by the expression of fluorescent protein.