Different Functional Consequences of Two Missense Mutations in the GJB2 Gene Associated with Non-syndromic Hearing Loss

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ABSTRACT: Mutations in the GJB2 gene, which encodes the gap junction (GJ) protein connexin26 (Cx26), are the most common cause of inherited non-syndromic hearing loss (NSHL). We identified two missense mutations, p.D46E (c.138T>G) and p.T86R (c.257C>G), of GJB2 in Korean HL families. The novel p.D46E mutation exhibited autosomal dominant inheritance, while the p.T86R mutation, which is exclusively found in Asians, segregated with an autosomal recessive pattern. Thus, we sought to elucidate the pathogenic nature of such different inherited patterns of HL. We studied protein localization and gap junction functions in cells transfected with wild-type or mutant Cx26 tagged with fluorescent proteins, which allowed visual confirmation of homozygous or heterozygous mutant GJs. The Cx26-D46E mutant was targeted to the plasma membrane, but this mutant protein failed to transfer Ca²⁺ or propidium iodide intercellularly, suggesting disruption of both ionic and biochemical coupling. Heterozygous GJs also showed dysfunctional intercellular couplings and hemichannel opening, confirming the dominant-negative nature of the p.D46E mutation. The Cx26-T86R mutant protein did not form GJs, since the mutated protein was confined in the cytoplasm and not transported to the cell membrane. When Cx26-T86R was co-expressed with Cx26-WT, ionic and biochemical coupling was normal, consistent with the recessive nature of the mutation. These studies revealed distinct pathogenic mechanisms of two GJB2 mutations identified in Korean families. © 2009 Wiley-Liss, Inc.

KEY WORDS: hearing loss, connexin26, GJB2, gap junction, mutation, hemichannel

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INTRODUCTION

Gap junctions (GJs) are intercellular channels mediating cell-cell communication and tissue homeostasis. These structures allow passage of ions and other intracellular small molecules with a molecular weight of less than 1 kDa, such as second messenger signaling molecules, between the cytoplasm of neighboring cells (Bruzzone et al., 1996). A complete GJ channel consists of two hemichannels, which are called connexons. Each hemichannel is comprised of six connexin (Cx) subunits (Sosinsky and Nicholson, 2005), which all share a common topology consisting of four transmembrane domains (TM1-TM4), two extracellular loops (EC1 and EC2), a single intracellular loop (IC), and cytoplasmic amino- and carboxy-terminal domains. GJs assembled from the same Cxs are called homomeric GJs, while GJs assembled from different Cx subtypes form heteromeric hemichannels and hybrid GJs (Sosinsky, 1995). The exact biochemical assembly of mixed types of compatible Cxs determines the specific permeability of the GJ channels (Cao et al., 1998).

Mutations in Cx genes are associated with human disorders, mostly hearing loss (HL) and skin diseases (Arita et al., 2006; Laird, 2006). Mutations in three Cx genes, GJB2 (MIM# 121011), GJB3 (MIM# 603324), and GJB6 (MIM# 604418), are associated with hearing loss. Cx26 and Cx30 are expressed in the fibrocytes of the spiral ligament and spiral limbus, basal cells of stria vascularis, and all types of supporting cells in the organ of Corti. These proteins have been suggested to play an important role in the recycling of the K+ ion between the endolymph and perilymph (Kikuchi et al., 2000). Mutations in the gene encoding Cx26 (GJB2) are the most common cause of non-syndromic autosomal recessive hearing loss in humans (Zelante et al., 1997), which is typically pre-lingual (Petersen and Willems, 2006). A number of GJB2 mutations have also been associated with autosomal dominant hearing loss (Connexin-Deafness Homepage: World Wide Web URL: http://davinci.crg.es/deafness/). Most of the GJB2 autosomal dominant forms of hearing loss tend to be post-lingual, progressive, and often are associated with a syndromic phenotype (Van Laer et al., 1999; Welch et al., 2007; Yan et al., 2006).

Here, we report the identification of two missense mutations, p.D46E (c.138T>G) and p.T86R (c.257C>G) in the GJB2 gene. The two mutations, detected in two Korean pedigrees, exhibited different inheritance modes, such that the hearing loss associated with the p.D46E mutation was inherited in an autosomal dominant pattern, while the p.T86R mutations resulted in an autosomal recessive hearing loss. Using a variety of in vitro approaches, we assessed the pathogenic mechanism of the p.D46E and p.T86R mutations. Consistent with their inheritance patterns, Cx26 proteins with the p.D46E mutation exhibited a dominant-interfering nature on normal gap junction function, whereas the p.T86R mutant appeared to be functionally inactive.

MATERIALS AND METHODS

Samples

Two Korean families with cases of hereditary non-syndromic hearing loss (NSHL) were studied. All participants provided written informed consent according to the protocol approved by the Ethics Committee of Kyungpook National University Hospital. The SR-349 family demonstrated a pattern of inheritance most consistent with dominant NSHL, while hearing loss in the KNUF-13 family most closely followed a recessive pattern of segregation. No evidence for any other clinical symptoms was found, and environmental causes such as ototoxic drugs and infectious diseases were excluded by interviews. After a complete physical and otoscopic examination, audiological studies including pure tone audiometry, tympanometry, and/or auditory brainstem response were performed in a sound-proofed room. The pure-tone audiogram (PTA) was calculated as an average of the threshold measured at 0.5, 1.0, 2.0, and 3.0 KHz. Genomic DNA was extracted from peripheral blood using the FlexiGene DNA extraction kit (QIAGEN, Hilden, Germany). One hundred unrelated Koreans with normal hearing were collected for use as controls.

Molecular analysis

The GJB2 coding region was amplified by polymerase chain reaction (PCR) using the following primers: F, 5'-TCT TTT CCA GAG CAA ACC GC-3' and R, 5'-GGG CAA TGC GTT AAA CTG GC-3'. The PCR conditions
consisted of an initial denaturation step at 95 °C for 2 min followed by 35 cycles of denaturation (30 sec at 95 °C), annealing (30 sec at 55 °C), and extension (1 min at 72 °C). A final extension step at 72 °C for 10 min concluded the cycle. Sequences were read using an ABI 3130XL DNA genetic analyzer (Applied Biosystems, Foster City, CA, USA). The resultant sequences were compared with the coding sequence of \textit{GJB2} (GenBank Accession No. NM_004004.5). Nucleotide numbering reflects cDNA numbering with +1 corresponds to the A of the ATG translation initiation codon. Names of all variants were checked with Mutalyzer (http://www.LOVD.nl/mutalyzer/) (Wildeman et al., 2008). The residue conservation analysis was performed with ClustalX (www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html).

\textbf{Molecular cloning and transfection of HEK 293 cells}

WT human Cx26 cDNA was subcloned into the pEGFP-N1 vector (Clontech Inc., Mountain View, CA, USA) to create a Cx26-EGFP fusion protein. The p.D46E mutation in the Cx26 coding sequence was introduced using the QuikChange site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA, USA). The following forward and reverse primers containing the \textit{GJB2} point mutation (mutated bases underlined) were synthesized: p.D46E, F: 5’-GGT GTG GGG AGA G\_GA GCA GGC CGA CTT TG-3’ and p.D46E, R: 5’-CAA AGT CGG CCT GCT CCT CTC CCC ACA CC-3’. The Cx26 cDNA sequence containing the p.T86R mutation (Cx26-T86R) was subcloned into the pEGFP-N1 vector using genomic DNA of the proband carrying a homozygous mutation (p.T86R/p.T86R). The Cx26-T86R sequence was amplified using PCR with the following primers, which contain restriction sites for the \textit{Bam}HI and \textit{Eco}RI enzymes: F: 5’-CTC AAG CTT CGA ATT CAT GGA TTG GGG CAC G-3’ and R: 5’-GGC GAC CGG TGG ATC CCG ATA AAC TGG CTT TTT-3’. Cx26-D46E and Cx26-T86R sequences were confirmed by DNA sequencing.

The human embryonic kidney HEK 293 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). This cell line was cultured in the recommended media consisting of minimum essential medium (Cellgro, Herndon, VA, USA), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), and 0.5% penicillin and streptomycin (Cellgro, Herndon, VA, USA). Cells were then transfected using the FuGene6 transfection agent (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer’s instructions. Transfection with a plasmid containing the mCherry-tagged-Cx26 WT cDNA was used as a control and in studies examining the properties of heterozygous mutations. The original plasmid containing the mCherry sequence was obtained from Dr. Roger Tsien’s laboratory in the Department of Pharmacology at the University of California San Diego (La Jolla, CA, USA). The overexpression of the Cx26-T86R mutant protein was induced by adding 5 mM of sodium butyrate (Sigma, Saint Louis, MO, USA) to the culture media.

\textbf{Gap junction functional studies}

\textit{Assays for measuring ionic coupling}

Transfected HEK 293 cells grown on cover-slips were loaded with the membrane-permeant form of the calcium indicator dye fura-2 acetoxyethyl ester (fura-2 AM, 5 µM) for 1 hour at room temperature under constant agitation. Details of the method used to assess ionic coupling by imaging were described previously (Sun et al., 2005). The Axon Imaging Workbench software (version 2.2, Axon Instruments, CA) was used for the image analysis.

\textit{Assays for measuring biochemical coupling}

GJ-mediated biochemical coupling was measured by examining the diffusion of a membrane-impermeable dye from the injected cell to neighboring cells connected by GJs. Dye injection was accomplished by forming the whole cell recording configuration with a patch-clamp electrode filled with 1.5 mM of propidium iodide (PI, molecular weight 650; charge 2+, Invitrogen) in the intracellular solution (120 mM KCl, 1 mM MgCl$_2$, 10 mM HEPES; pH 7.4 with NaOH). The PI transfer was recorded 5 minutes after whole cell recording mode was established. In some experiments, the single electrode electrophoresis method described by Haas was used for the microinjections (Haas et al., 2001).
Assays for measuring the hemichannel permeability

Cells were bathed in normal HBSS, which also contained 1.2 mM [Ca\(^{2+}\)], to keep wild-type Cx26 (Cx26-WT) hemichannels in the closed state (Thimm et al., 2005). Cx hemichannels were opened by lowering the extracellular Ca\(^{2+}\) concentration (Muller et al., 2002). Dye loading through hemichannels was accomplished by incubating cells in a Ca\(^{2+}\)-free HBSS solution supplemented with 0.15 mM of PI. Percentage of cells among cells visually confirmed to be transfected with Cxs was counted. Data are expressed as mean ± standard error (S.E.M.). The data were tested for significance with a two-way ANOVA using the SPSS software (v. 12.0), and only results with \(p\)-values of <0.05 were considered statistically significant. Details of these methods have been described previously (Stong et al., 2006).

RESULTS

Subjects and Genetic analysis

The coding region of Cx26 was sequenced from members of two Korean families segregating NSHL. Family SR-349 displayed progressive post-lingual hearing loss (Fig. 1). The proband, a twelve-year-old girl, had bilateral moderate sensorineural loss. Her average thresholds of pure tone audiometry (PTA) measured were 64 dB in the left ear and 55dB in the right ear. Other affected members in the family also showed similar moderate sensorineural hearing loss. The ages of onset of hearing loss among the affected members in the family were variable. Hearing loss started at ten in the proband, thirties in the patient II:4, forties in the patient II:2, and the fifties in the patient I:2. Yet, the hearing loss was progressive in all the affected members. No one displayed any other obvious abnormalities including skin manifestations such as thickening or eruption, or abnormalities in the eyes or hairs through physical examinations by physicians. Sequence analysis in this family revealed a T to G substitution at nucleotide position 138 (c.138T>G). This nucleotide change converts an aspartic acid to a glutamic acid at the amino acid position 46 (p.D46E). All affected individuals (I-2, II-2, II-4, and III-1) were heterozygous for this mutation, but normal hearing members in the family did not exhibit this change. Therefore, the p.D46E substitution was regarded as an autosomal dominant mutation. In the KNUF-13 family, the proband showed a profound congenital hearing loss (reviewed but not shown). Her ABR (auditory brainstem response) measurements at 6 months of age showed the wave V at 80 dB from both ears, and her auditory steady-state responses displayed bilateral profound hearing loss in all frequencies at 12 months of age. No other clinical signs of abnormalities or disease symptoms were found. Sequencing analysis of this family revealed a homozygous missense mutation at nucleotide position 257 (c.257C>G) that resulted in the replacement of an arginine by a threonine residue at codon 86 (p.T86R). The patient’s father, mother, grandmother, and uncle were all heterozygous for the mutation. Therefore, the p.T86R substitution was regarded as an autosomal recessive mutation. Neither of these mutations was observed in 100 unrelated Korean control subjects. Therefore, we propose that these GJB2 mutations cause the hearing loss phenotype observed in these two families. In order to evaluate the evolutionary conservation of the amino acids affected by these mutations, the amino acid sequences of Cx genes obtained from four mammalian species were aligned. Both of the amino acids at the mutation sites corresponded to conserved amino acids in the Cx gene family across the four species (reviewed but not shown).
In order to examine the effect of p.D46E and p.T86R mutations on the cellular localization of Cx26, we transfected HEK 293 cells with expression plasmids encoding wild-type or mutated Cx26 tagged with fluorescent protein markers. These fluorescent proteins allowed us to track the intracellular location of the Cx proteins (Fig. 2). Cx26-WT was localized at the cell membrane and formed GJs as seen by the characteristic plaques between two adjacent cells (Fig. 2A). The Cx26-D46E mutant was also expressed at the cell membrane and retained the ability to form GJs (Fig. 2B). In contrast, the Cx26-T86R mutant was not transported to the cell membrane, expression of this mutant was only observed in the cytoplasm of transfected cells, and no formation of GJs was observed (Fig. 2C). The Cx26-T86R mutant was never observed as GJ plaques at the cell-cell contact areas even after several days in culture or after connexin overexpression induced by sodium butyrate addition to the culture media (George et al., 1998; Wilkinson and Akrigg, 1992). Therefore, we conclude that the mechanism for hearing loss caused by Cx26-T86R mutation stems from its inability to be localized to the cell membrane.

When the Cx26-WT protein that was tagged with mCherry (Cx26-WT-mCherry) was transfected together with the Cx26-D46E-EGFP mutant, these proteins co-localized to the gap junctional plaques, suggesting that the Cx26-D46E mutant is able to co-assemble with the Cx26-WT (Fig. 2D). The Cx26-T86R mutant, however, was unable to interact with Cx26-WT to co-assemble into the same GJ plaques (Fig. 2E). Following co-transfection, Cx26-T86R-EGFP (green fluorescence) remained in the cytoplasm, while the Cx26-WT-mCherry (red fluorescence) was observed in the GJ plaques (Fig. 2E).
Functional Consequences of Two Missense Mutations in the GJB2 Gene

Figure 2. Intercellular localization of the wild-type Cx26 and the mutant Cx26 proteins in HEK 293 cells. (A) Cx26-WT traffics to cell membranes forming GJ-like aggregates. (B) The Cx26-D46E protein traffics to the membrane to form plaques between adjacent cells. (C) The Cx26-T86R mutant was not targeted to the plasma membrane as indicated by the diffuse green fluorescence signal in the cell cytoplasm. (D) Co-expression of Cx26-WT-mCherry and Cx26-D46E-EGFP resulted in co-localization of the two proteins at junctional plaques. (E) Co-expression of Cx26-WT-mCherry and Cx26-T86R-EGFP did not result in co-assembly, and only the Cx26-WT-mCherry formed GJs, while Cx26-T86R-EGFP was expressed in the cytoplasm. Scale bar: 10 µm for all panels.

Effects of Cx26-D46E and Cx26-T86R mutations on ionic coupling of GJs

To test the ionic coupling of the reconstituted GJs in transfected cells, we first examined their ability to transfer Ca\(^{2+}\) ions intercellularly across the GJs. The intracellular Ca\(^{2+}\) concentration increase ([Ca\(^{2+}\)]) in response to a mechanical stimulation in the touched cell was followed by a delayed [Ca\(^{2+}\)] increase in cells coupled by GJs composed of Cx26-WT (Fig. 3A). In all cells tested (n=23), the increases of [Ca\(^{2+}\)] in the stimulated cell (referred to as cell #1) were transferred to a neighboring cell (cell #2) that had been visually confirmed to be coupled by GJs. In the third cell (cell #3) that was not coupled with cell #1 through GJs, no [Ca\(^{2+}\)] increases were observed. These data were consistent with previously published results (Sun et al., 2005) and support the notion that GJs transferred the [Ca\(^{2+}\)] increases. In cells transfected with the Cx26-D46E mutant, no intercellular Ca\(^{2+}\) transfer was observed (Fig. 3B; n=15). The results indicated that GJs composed of the Cx26-D46E mutant completely lost the GJ-mediated ionic coupling. Results obtained from cells co-transfected with a 1:1 ratio of Cx26-WT and mutant Cx26-D46E plasmid DNA demonstrated that the function of hybrid Cx26-WT/Cx26-D46E GJs was affected by ionic coupling in some cases. The ionic coupling of GJs that were confirmed to contain both Cx26-WT and the Cx26-D46E mutant by double-colored fluorescent signals exhibited normal [Ca\(^{2+}\)] transfer in most cases (Fig. 3C; n=14/19). In a few cases, either delayed transfer (n=4/19) or no transfer (n=1/19) was also observed. In all cases examined, the [Ca\(^{2+}\)] transfer through GJs composed of Cx26-WT/Cx26-T86R hybrid channels was not significantly different from WT GJs (Fig. 3D, n=15).
Figure 3. Effects of Cx26 mutations on ionic permeability of GJs reconstituted in HEK 293 cells. HEK 293 cells expressing the specified constructs were analyzed by calcium imaging 24 h post-transfection. The fura-2 signals show mechanically elicited intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) response in the source cell (#1) and its spread to neighboring cells. Cell 2 (#2) is the cell that forms a GJ with cell #1. The untransfected cell (#3) is used as a control cell. Scale bars are 10 µm for all panels. (A) In the Cx26-WT transfected cells, the [Ca\(^{2+}\)] was consistently followed by the intercellular spreading of a calcium wave (n=23/23). (B) The Cx26-D46E mutation failed to transfer [Ca\(^{2+}\)] from the cell to neighboring cell via the GJ (n=15/15). (C) Co-expression of Cx26-WT/Cx26-D46E showed [Ca\(^{2+}\)] transfer in most cases (n=14/19), although some cases exhibited delayed or no transfer (n=5/19). (D) The GJ formed with Cx26-WT and Cx26-T86R exhibited a virtually wild-type response (n=15/15).

Biochemical coupling through Cx26-D46E and Cx26-T86R mutant GJs

The biochemical permeability of mutated GJ channels was tested by examining the cell-to-cell dye diffusion of PI, which is a cell membrane impermeable fluorescent dye (Fig. 4). Untransfected HEK 293 cells, which served as negative controls (n=11), did not display intercellular dye transfer (data not shown). PI transfer through GJ channels composed of Cx26-WT subunits was readily observed (Fig. 4A). In contrast, PI diffusion through GJ channels composed of Cx26-D46E mutants was not observed (Fig. 4B, n=12). In HEK 293 cells co-transfected with equal amounts of Cx26-WT and Cx26-D46E plasmid DNA, marked reduction in dye transfer was observed, suggesting that the hybrid Cx26-WT/Cx26-D46E GJs had reduced biochemical coupling (Fig. 4C, n=10). In contrast, when the PI transfer assay was carried out with cells co-transfected with Cx26-WT and the Cx26-T86R mutant, the presence of the mutant subunit did not significantly reduce the dye transfer (Fig. 4D, n=5). These findings indicate that T86R in combination with Cx26-WT in hybrid GJs doesn’t affect GJ-mediated biochemical coupling. These differences in biochemical coupling between the two mutations may account for the different inherited patterns of hearing loss.
Figure 4. PI permeability by Cx26-WT and Cx26 mutants. Individual cells expressing EGFP and mCherry were microinjected with PI, and the intercellular diffusion of the dye was monitored 5 min after the injection. Intercellular coupling was present between cells expressing Cx26-WT (A), Cx26-WT and Cx26-D46E (C), and Cx26-WT and Cx26-T86R (D). Cells transfected with Cx26-D46E alone (B) were uncoupled. Cells co-transfected with Cx26-WT and Cx26-D46E (C) showed weaker PI transfer than cells with only Cx26-WT (A). Scale bars are 10 µm for all panels.

Effect of Cx26-D46E and Cx26-T86R mutations on biochemical permeability of hemichannels

In control experiments, only 0.8% (n=1340) of untransfected cells and 1.1% (n=349) of cells transfected with the pEGFP vector alone displayed PI loading after hemichannels were opened with the external free Ca²⁺ solution (Fig. 5A). Whereas, about 95.9% of cells (n=874) transfected with Cx26-WT exhibited PI loading through hemichannels when opened by an external free Ca²⁺ solution. In contrast, only a low percentage of the cells transfected with both Cx26-D46E and Cx26-T86R (0.8%, n=235 and 1.5%, n=381, respectively) exhibited hemichannel loading with PI. These results indicate that the two mutants abolished the biochemical permeability of the hemichannels. In cells co-transfected with Cx26-WT and Cx26-D46E, the PI loading was weaker compared to that in cells transfected with Cx26-WT alone. These data suggest that the Cx26-D46E mutant has a dominant-negative effect on dye diffusion through hemichannels, which is consistent with the dominant nature of the inherited hearing loss caused by the p.D46E mutation. PI loading of HEK 293 cells transfected with Cx26-WT and Cx26-T86R was similar to that of cells transfected with Cx26-WT alone. These results are also consistent with the recessive nature of the p.T86R mutation observed in the inherited hearing loss in the Korean patients.

To quantify the effect of the Cx26 mutants on hemichannel activities, we performed a time-dependent hemichannel dye loading assay. Dye loading in HEK 293 cells transfected with Cx26-WT, Cx26-D46E, or Cx26-WT and Cx26-D46E was recorded at various time points. After 10 minutes, about 90% of the cells transfected with Cx26-WT were already loaded. Cells transfected with the Cx26-D46E mutation only exhibited no dye loading for the entire experiment (60 minutes). In contrast, cells co-transfected with Cx26-WT and Cx26-D46E showed a time-dependent increase in dye loading (Fig. 5B). The hemichannels containing Cx26-WT and
Cx26-D46E was loaded with PI more slowly than hemichannels comprised of only Cx26-WT. A two-way ANOVA indicated a significant difference in dye loading among Cx26-WT/Cx26-WT, Cx26-WT/Cx26-D46E, and Cx26-D46E/Cx26-D46E hemichannels (p<0.05) at time points from 10 min to 60 min.

![Figure 5.](image.png)

**DISCUSSION**

Mutations in *GJB2*, which are one of the major causes of non-syndromic hearing loss, can follow a dominant or recessive inheritance pattern (Melchionda et al., 2005; Primignani et al., 2003; Welch et al., 2007). The major causative alleles, c.35delG and c.235delC, have been well characterized in many studies, and the carrier frequencies of these mutations vary in different ethnic populations (Park et al., 2000; Green et al., 1999; Han et al., 2008; Zelante et al., 1997). In addition, a number of novel *GJB2* variants that contain amino acid substitutions have been identified in numerous genetic studies (Martinez et al., 2009, Connexin-Deafness Homepage: World Wide Web URL: http://davinci.crg.es/deafness/). These variants were predicted to be pathogenic since the mutations were only found in patients and the residues were highly conserved among different species. The effects of these mutations on Cx function have been shown to be different depending on the position and type of amino acid substitution. Furthermore, the same mutation can cause different phenotypes in different individuals. For example, a p.V37I mutation in Cx26 causes various phenotypes from mild to severe hearing loss. Therefore, functional studies using cell lines are critical to understand the pathogenicities of the *GJB2* mutations (Han et al., 2008; Mani et al., 2008; Pollak et al., 2007).

In this study, we identified two different *GJB2* mutations from Korean hearing loss families. To characterize the pathological mechanism of the mutations, we employed several in vitro approaches, including double-colored labeling of reconstituted hybrid GJs. The novel mutation p.D46E was associated with dominantly inherited non-syndromic hearing loss and was not found in normal hearing controls from the Korean population, indicating that this mutation is a pathogenic variant. This idea was further supported by analysis of protein conservation, which
revealed the conservation of aspartic acid at position 46 across four different mammalian species (reviewed but not shown). The p.D46E mutant localized to the membrane normally but exhibited defective gap junction channel activity. In agreement with its inheritance pattern, heterologous gap junctions reconstituted with Cx26-WT and Cx26-D46E demonstrated a dominant-negative effect. The p.D46E mutation is located in the first extracellular domain (EC1), which is critical for hemichannel docking to form gap-junctional channels, voltage gating, and connexin-connexin interactions (Foote et al., 1998; Hua et al., 2003; Stong et al., 2006). Therefore, the dominant-negative effect of the p.D46E mutation may be due to defective docking of the two opposing connexin hemichannels. In many cases, mutations in extracellular domains are associated with dominant inheritance and syndromic phenotype, such as skin disorders and other ectodermal abnormalities (Deng et al., 2006; Martinez et al., 2009; Marziano et al., 2003; Matos et al., 2008; Melchionda et al., 2005; Sun et al., 2005). Two different mutations in the EC1 (p.W44C and p.W44S) have been reported to be associated with dominantly inherited non-syndromic hearing loss and to exert a dominant-negative effect on gap junction permeability (Martin et al., 1999; Marziano et al., 2003). The chemical properties of amino acid substitution caused by these three mutations (p.W44C, p.W44S and p.D46E) were different than those reported for the nearby mutations p.G45E, p.E47K, and p.D50N/p.D50Y. Since the EC1 domain has been suggested to play an important role in the hemichannel gating property, modification of this domain with different amino acid substitutions may alter the functionality of Cx via modification of the three-dimensional structure of the protein. Different mutations that occur in close proximity in the EC1 domain may, therefore, cause different clinical manifestations (Gomez-Hernandez et al., 2003; Stong et al., 2006).

The p.T86R mutation, on the other hand, was associated with recessive inheritance. This mutation has been reported previously in Japanese and Korean hearing loss patients, but assays of the cell biological function of this mutation have not yet been reported (Lee et al., 2008). In our studies, Cx26-T86R was expressed in a predominantly reticular pattern in the cytoplasm and was defective in oligomerization. Upon co-expression with Cx26-WT, intercellular trafficking was restored although Cx26-WT and Cx26-T86R did not co-localize and only the Cx26-WT was transported to the cell membrane. Cx26-T86R did not obviously affect the normal function of Cx26-WT, since ionic and biochemical coupling were normal in cells co-transfected with Cx26-WT and Cx26-T86R, consistent with the recessive inheritance pattern of the mutation. The p.T86R mutation is located in the second transmembrane domain of Cx26 and converts an uncharged amino acid to a positively charged amino acid. The Cx26-T86R phenotype is consistent with previous studies suggesting that transmembrane domains are important for oligomerization and membrane targeting of Cxs (Mani et al., 2008). In addition, several mutations in the transmembrane domains have been shown to cause recessively inherited patterns of hearing loss (Bruzzone et al., 2003; Man et al., 2007) in accord with the p.T86R mutation and its observed recessive inheritance in Korean family.

In summary, functional assays in combination with genetic analysis have demonstrated distinctly different pathogenic mechanisms for the two GJB2 mutations identified in Korean families. The p.D46E mutation, which is associated with dominantly inherited hearing loss, produces a dominant-negative variant, while the p.T86R mutation, which is associated with recessive hearing loss, produces a functionally null protein that does not interfere with normal GJ function in the presence of wild-type Cx26. This information will be valuable for understanding the pathogenic role of GJB2 mutations associated with hearing loss.

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