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Functional Study of *GJB2* in Hereditary Hearing Loss

Yun Hoon Choung, DDS, MD, PhD; Sung-Kyun Moon, MD, PhD; Hong-Joon Park, MD, PhD

Objectives/Hypothesis: **The gene *GJB2* of the gap junction protein connexin 26 (Cx26) was found to be the main causative gene of autosomal recessive nonsyndromic hearing loss (DFNB1). Although 35delG has been known as the major mutation in Western countries, 235delC was reported to be a specific form of mutation in Asian populations. The objective of the study was to identify how 235delC and E114G changes found in the Korean population affected the function of *GJB2* using molecular biological techniques.** ***Methods:*** **Genes containing 235delC and E114G were cloned into the pcDNA3 vector, and HeLa cells were transfected with the recombinant DNA samples by the liposome complex method. The expression and subcellular localization of Cx26 were determined, using antibodies against amino acid sequences in the intracellular loop (IL) and N-terminal (NT) portions of Cx26. To analyze functions of the *GJB2* as a gap junction channel, we examined Lucifer yellow dye transfer between cells with a scrape-loaded technique. Wild-type *GJB2* (WT) with normal hearing was used as a positive control, and mock transfected cells were used as a negative control.** ***Results:*** **Immunocytochemical analysis showed that cells transfected with E114G and WT gave characteristic punctate patterns of reaction in the cell membrane with both antibodies. However, 235delC cells were not stained with anti-IL antibody but stained slightly just around the nucleus only with anti-NT antibody. In a functional study of *GJB2*, transfer of Lucifer yellow into contiguous cells was detected in both WT and E114G, but no transfer activity was observed in 235delC.** ***Conclusions:*** **The 235delC mutation showed a loss of targeting activity to the cell membrane and severe deterioration of gap junction activity. For the E114G, we did not find any difference from WT transfected cells.**
Key Words: **Hereditary hearing loss, gap junction, connexin 26, functional study.**

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INTRODUCTION

Hearing impairment affects 1 in 1000 infants and 4% of people under the age of 45 years.[1] Approximately 50% of congenital hearing loss is hereditary, and 80% of hereditary hearing loss is inherited in an autosomal recessive way.[2–5]

Mutations in the connexin26 (Cx26) gene (*GJB2*) are known as a major cause of autosomal recessive nonsyndromic sensorineural hearing loss (DFNB1). The most frequent mutation of *GJB2* associated with deafness is 35delG, which is common in white and European populations.[3,6] Other populations may have additional or different specific mutations, such as 167delT among Ashkenazi Jews, R143W in Africans, and 235delC in Asians.[7–9] In Korea, 35delG is rare, whereas 235delC is a frequent mutation form. These mutation patterns are similar to the pattern reported in the Japanese population.[9] 235delC was found only in patients with autosomal recessive hearing loss, and E114G was identified in both the normal population and the hearing-impaired group in Korea. Thus, these changes (235delC and E114G) could be related to the hearing loss. However, we do not have any reports about the mechanism and functional studies of these changes. The aim of the present study was to identify how these two changes (235delC and E114G) found in the Korean population affect the function of *GJB2* using molecular biological techniques.

MATERIALS AND METHODS

We used Cx26 genes (*GJB2*) detected in patients with autosomal recessive hearing loss in Korea. Both 235delC identified in the congenitally deaf group and E114G derived from the normal-hearing group and from the congenitally deaf group are included in the study. We obtained blood samples from patients with homozygous genes. Blood samples from newborns who showed normal hearing levels were used as wild-type, positive control. The present study was approved by the Institutional Review boards of the Ajou University School of Medicine (Suwon, Korea). Written informed consent was obtained for all participants. We performed polymerase chain reaction (PCR) with extracted genomic DNA samples and confirmed the sequence of *GJB2* as previously described.[9] Mock transfected cells (cells transfected with only vectors) were used as a negative control.

The present study consisted of three steps (Fig. 1). In the first step, genomic DNA samples of Cx26 were extracted from patients with congenital hearing loss, cloned with PCR, and

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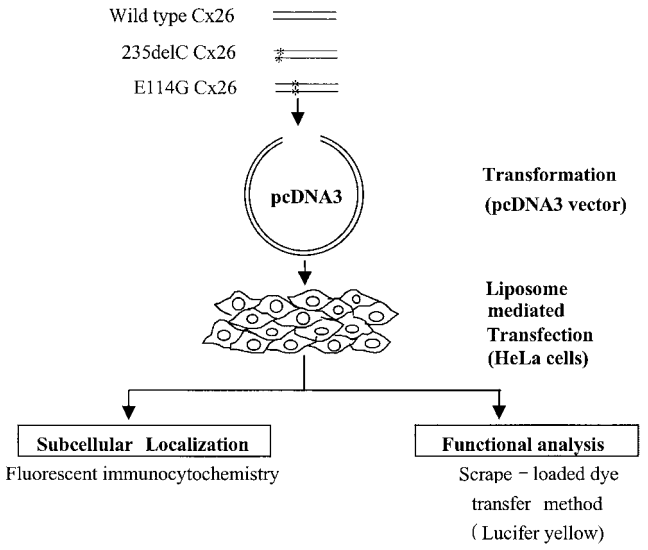

Fig. 1. Schema of experiment. The study was carried out by three steps, transformation, transfection, and subcellular localization of Cx26 as well as functional study of gap junction using 235delC, E114G, wild-type Cx26, and mock cells. The expression and subcellular localization of Cx26 was determined using antibodies against amino acid sequences in the intracellular loop and N-terminal of Cx26. To analyze functions of the gap junction, we examined Lucifer yellow dye transfer between cells with scrape-loaded technique.

transformed into pcDNA3 vectors. In the second step, cloned genomic DNA samples were transfected into HeLa cells that have no expression of Cx26. Third, we examined the expression and subcellular localization of Cx26 in transfected HeLa cells and performed a functional study of the intercellular dye transfer through gap junctions.

Cloning of Cx26

Polymerase chain reaction. The primers used were prepared by including the entire coding region of *GJB2* (691 bp) and the specific sites (italicized) of restriction enzymes as follows: Cx-BamH1: 5'-CAA*GGATCC*ATGGATTGGGGC-3' Cx-EcoR1: 5'-CGC*GAATTC*TTAAACTGGCTT-3'.

Samples were denatured at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute, and then processed for final extension at 72°C for 5 minutes with the AccuPower PCR Premix (Bioneer, Seoul, Korea).

Transformation. Wild-type, deletion (235delC), and missense (E114G) types of Cx26 DNA samples were inserted into pcDNA3 vectors. We mixed 15 μL PCR product, 2.5 μL universal buffer (10X), and 5.5 μL distilled water (DW) with 1 μL each of restriction enzymes BamH1 and EcoR1. The mixture was incubated at 37°C for 1 hour. A similar mixture was made, but instead, 3 μL of pcDNA3 vectors (33.65 μg/mL) was substituted for the PCR product to make vectors open-frame. Prepared PCR products were mixed with pcDNA3 vectors, T4 DNA ligase (Promega), ligase buffer (Promega, 10X) and 20 μL DW, then incubated for ligation at 16°C for 8 hours. Fifty microliters of competent cells (DH5α[*sup*E44Δ*lac*U169(FΦ80*lac*ZΔM15)*hsd*-R17*rec*A*end*1*gyr*a96*thi*-1*rel*A1]) were added to 5 μL of the ligation mixture, and this complex was electrophoresed with an electric pulse of 1.4 kV. We added 1 μL Luria-Bertani (LB) to this complex, then performed incubation at 37°C for 30 minutes. We smeared them on LB agarose medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, 10% bacto agar) containing 50 μg/mL ampicillin, then performed incubation at 37°C for 16 hours. We incubated the colonies in LB liquid medium containing ampicillin.

Plasmid DNA preparation. Wizard Genomic DNA Purification Kit (Promega) was used for purification of plasmid DNA according to the manufacturer's protocol.

Transfection

We seeded 70,000 to 100,000 HeLa cells in a six-well plate flooded with 4 mL growth medium supplemented with serum (Dulbecco's modified Eagle's medium [DMEM]–20% fetal bovine serum [FBS]) and incubated these cells at 37°C in a CO_2 incubator for 24 hours until the cells were 30% to 50% confluent. We prepared two solutions in 12 $\times$ 75-mm sterile tubes. One solution consisted of 100 μL Opti-MEMI medium diluted with 2 μg vector DNA; the other solution was made by adding 6 μL Lipofectin reagent into the same medium. We mixed these two solutions gently and incubated them at room temperature for 5 to 10 minutes. The cells were washed with 2 mL serum-free growth medium without antibacterial agents. We overlaid 1 mL lipid–DNA complex onto the cells, then incubated them for 5 hours. We added 1 mL DMEM–40% FBS and incubated the cells for 24 hours. We replaced the DNA-containing medium with 2 mL of DMEM–20% FBS and incubated the cells for another 24 hours. We removed all the culture medium again and added DMEM–20% FBS containing 5 ng/mL Genticin antibiotic (G418 sulfate) to the cells, then distributed these cells in a 24-well plate.

Expression and Subcellular Localization of Cx26 and Functional Study

Expression and subcellular localization of Cx26. Fluorescent immunocytochemistry was used to verify expression and subcellular localization of Cx26. We laid 1 $\times$ 1-cm cover glasses onto a 6-well plate and added HeLa cell suspension, then incubated it for 24 hours. These glasses were washed with phosphate-buffered saline (PBS) and fixed with neutral formalin for 10 minutes. We prepared cells with 0.15% Triton X-100 in PBS for 5 minutes. The cells were washed 3 times with PBS, then prepared with 1% BSA for 1 hour. We marked several areas on the parafilm in the plastic case. We dropped 20 μL (10 μg/mL) primary rabbit antibody directed against amino acid sequences in the intracellular loop of human Cx26 (Zymed, South San Francisco, CA) on the separated areas of the parafilm. On these antibody solutions, we overlaid cover glasses adhered with cultured cells upside down. After incubation for 1 hour, they were washed 3 times for 10 minutes with PBS in a 6-well plate. Fluorescin isothiocyanate (FITC)–conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (Jackson Immunoresearch, PA) in the amount of 1.5 mg/mL was diluted to 1:200 and added to each slide glass by the volume of 50 μL. They were incubated for 1 hour in a dark room, washed with PBS, and mounted with 10 μL glycerol. This was examined with a fluorescent microscope (high-performance cooled charge-coupled device (CCD) imaging systems, Apogee Instruments Inc). Using the same method, we used 20 μL primary goat polyclonal IgG reacted with amino acid sequences in the N-terminal of human Cx26 (Santa Cruz Biotechnology, CA) (30 μg/mL) and 50 μL FITC-conjugated rabbit anti-goat IgG secondary antibody (Jackson Immunoresearch) (1.5 mg/mL) that was diluted to 1:500.

Functional study of GJB2. Functionality of the mutated Cx26 was determined by the method of scrape-loaded dye transfer described by El-Fouly et al.[10] with some modification. The transfected HeLa cells were incubated in the 40-mm dishes for 24 hours. They were washed twice with PBS, flooded with PBS containing 0.5% Lucifer yellow, then scrape-loaded with a surgical scalpel. The dye solution was left on the cell monolayer for 10

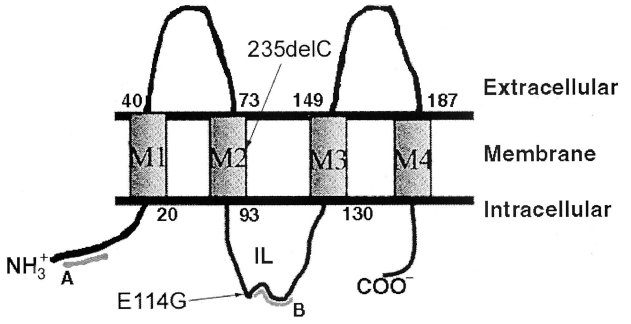

Fig. 2. The molecular structure of connexin 26. 235delC and E114G are located in M2 and IL, respectively. Epitopes that are used in the immunocytochemistry—(**A**) N-terminal, (**B**) intracellular loop. M1 to M4 = transmembrane domains; IL = intracellular loop.

minutes for spreading, and the cultures were rinsed once with PBS to remove unloaded dye and background fluorescence. We observed cell communication under fluorescent microscope (high-performance cooled CCD imaging systems).

RESULTS

The 235delC mutation is present in the second transmembrane domain of Cx26 and introduces a frameshift within codon 79 resulting in premature termination at codon 81. In the intracellular loop of Cx26, E114G is located where glutamate is substituted by glycine at amino acid 114 (Fig. 2).

Expression and Subcellular Localization of GJB2

The expression and subcellular localization of Cx26 protein were analyzed in HeLa cells transfected with Cx26 DNA samples using fluorescent immunocytochemistry with site-specific antibodies. In the staining of primary antibody reacted with intracellular loop of Cx26, cells transfected with wild-type and E114G gave characteristic punctuate patterns of reaction in the cell membrane. However, cells expressing 235delC did not show any staining on the cell membrane and cytoplasm as mock transfected cells (Fig. 3). Using primary antibody directed against the amino acid portion of N-terminal of Cx26, we detected punctuate staining in the cell membrane of cells expressing wild-type and E114G, but only a weak reaction around

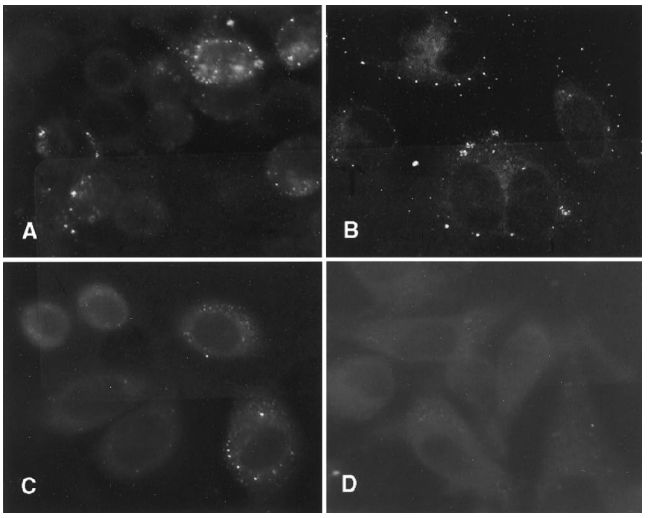

Fig. 4. Immunocytochemistry of HeLa cells using primary antibody against N-terminal portion of Cx26. (**A**) Wild-type (WT), (**B**) E114G, (**C**) 235delC, and (**D**) mock transfected cells. Both WT and E114G transfected HeLa cells showed positive immunofluorescent reactions in the cell membranes. However, 235delC transfected cells showed weak reactions just around the nucleus regions. Mock transfected cells showed no reaction.

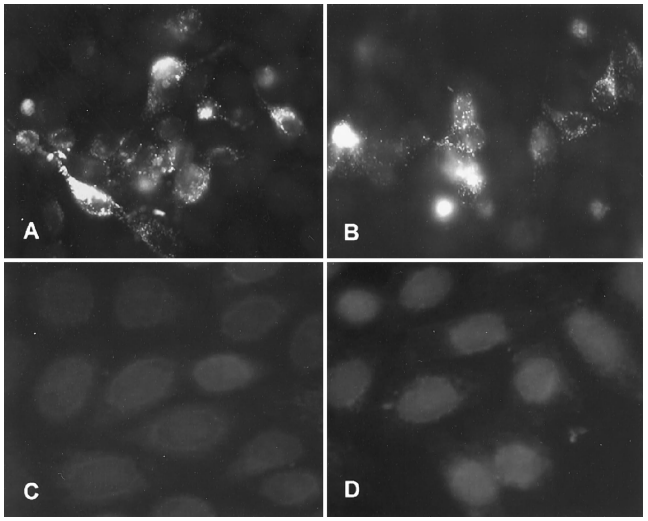

Fig. 3. Immunocytochemical analysis of HeLa cells using primary antibody against intracellular loop portion of Cx26. (**A**) Wild-type (WT), (**B**) E114G, (**C**) 235delC, and (**D**) mock transfected cells. Both WT and E114G transfected HeLa cells showed positive immunofluorescent reactions in the cell membranes. However, 235delC transfected cells showed no reaction like mock transfected cells.

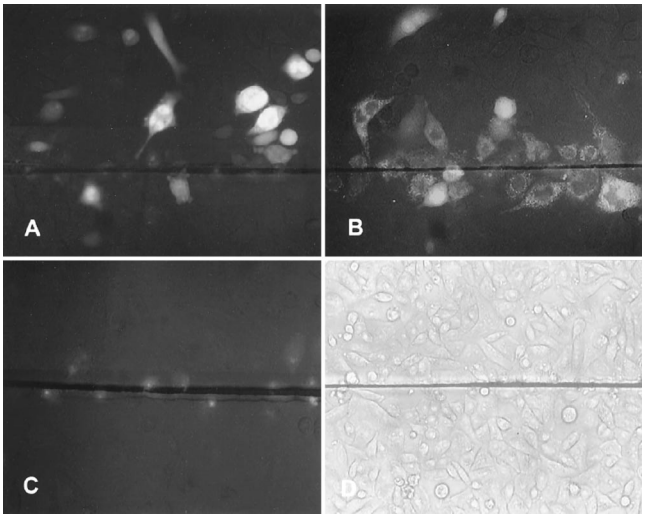

Fig. 5. Functional study of *GJB2* using scrape-loaded dye (Lucifer yellow) technique. (**A**) Wild-type (WT), (**B**) E114G, and (**C**) 235delC transfected cells (fluorescent microscopic view). Transfer of Lucifer yellow into contiguous cells was detected in WT and E114G but not in 235delC transfected HeLa cells. (**D**) Light microscopic view of HeLa cells with scrape-loaded dye technique.

the nucleus of 235deIC cells and no reaction in their cell membrane. Mock transfected cells were not stained (Fig. 4).

Functional Study of GJB2

To examine whether the altered Cx26 could form functional gap junctions, the intercellular transfer of Lucifer yellow was observed in HeLa cells expressing Cx26 using scrape-loaded dye transfer technique. Cells expressing E114G showed dye transfer to contiguous cells from loaded scrape line similar to that observed in the wild-type cells. However, no dye transfer was observed in cells expressing 235delC. Mock cells were not stained (Fig. 5).

DISCUSSION

Gap junctions contain channels that connect neighboring cells. The channels are relatively nonspecific but allow molecular movements based on molecular size by passive diffusion. The molecules smaller than 1000 Da such as cyclic adenosine monophosphate (cAMP) and inorganic ions (Na^+, K^+, Ca^{2+}, etc) can pass through gap junctions, but proteins or nucleic acids cannot. The cells communicate with adjacent cells by transfer of these small molecules. This is an important mechanism for regulating events between cells during embryogenesis and normal organ function.[11]

Gap junctions are thought to play an important role for maintaining hearing function by the local circulation of potassium between the fluids of the inner ear.[12,13] The blockage of K^+ circulation causes hearing impairment. *KCNQ1*, *KANQ4*, *KCNE1*, *GJB2* (Cx26) and *GJB3* (connexin 31) have recently been found as deafness genes encoding ion channels regulating K^+ recycling pathway.[14] Cx26 is expressed in the epithelial cells surrounding the hair cells and also in the adjacent connective tissue system of fibrocytes positioned under and around the marginal cells of the stria. Thus, mutations in Cx26 can result in severe hearing loss.[15]

Connexins are the protein subunits of gap junctions; hexameric connexin oligomers are arranged in the plasma membrane as connexon hemichannels that dock with partners in adjacent cells to generate a direct intercellular communication pathway.[16,17] Cx26 is one of 14 isoforms located on chromosome 13q12 and has molecular weight of 26.5 kD.[12,18]

Connexin has a similar topological distribution with cytoplasmic and carboxyl terminal regions, a single intracellular loop and two extracellular loops (Fig. 2).[19] The N-terminal domain, together with the transmembrane domain M1 border, forms a charge complex that may act as a voltage sensor. Transmembrane domain M2 is an essential portion for oligomerization to form connexon hemichannel. M3 has an amphipathic character, suggesting that it contributes to the lining of the channel. The two extracellular loops (E1 and E2) are thought to determine the heterotypic compatibility. Each extracellular loop has a set of three cysteine residues, which helps maintain the rigid tertiary structure that enables two opposing connexons to dock with each other. Because the intracellular loop domain and the C-terminal domain may be involved in pH gating of the channel and are highly variable among the different connexins, they are thought to be important for regulation.[17] Therefore, the change of gap junction proteins from connexin gene mutations may cause severe defects of the structure and function of the gap junction.

The 235delC mutation in the present study is deletion of cytosine in codon 79 in transmembrane M2 resulting in frameshift and premature termination (TGA) on codon 81. This mutation produces a short, defective protein of 80 amino acids, not the normal 226 amino acids. E114G causes missense mutation converting codon 114 from glutamate (E) to glycine (G), resulting in a change from negatively charged residual to nonpolar aliphatic residual. This change is thought to affect the tertiary structure and function of the connexin protein. We expected 235delC mutation to result in a more critical defect of Cx26 protein, especially since the mutated 235delC was identified only in patients with autosomal recessive forms of hearing impairment.

We immunostained the transfected HeLa cells to analyze expression and subcellular localization of Cx26 protein. The HeLa cells expressing the 235delC did not show any staining on the cell membrane and cytoplasm as mock cells in the staining of primary antibody reacted with intracellular loop of Cx26, but showed weak staining just around nucleus region in cytoplasm using primary antibody directed against amino acid portion of N-terminal of Cx26. These findings suggest that this is the result of a short amino acid, that is, the absence of the intracellular loop and more. This is also considered defective trafficking to the cell membrane from the endoplasmic reticulum or Golgi apparatus. In addition, the 235delC cells showed weaker staining than normal, which is considered a defect of oligomerization and stability. For E114G, the HeLa cells showed characteristic punctuate patterns of reaction in the cell membrane as wild-type HeLa cells in both immunocytochemical analyses using primary antibodies directed against amino acid portions of N-terminal and intracellular loop of Cx26. Martin et al.[8] reported targeting efficiency of mutated connexins. Wild-type Cx26 was efficiently targeted to the gap junction with 53.3% of total immunofluorescence detected at the cell periphery, but missense mutant M34T was less targeted in 26.9% and missense mutant W77R was reduced to 9.8%. Even though quantification of staining was not analyzed in the present study, a similar pattern of staining was observed in this study that E114G stained weaker than wild-type cells.

Gap junction–mediated intercellular communication has been measured by three types of assays in vitro (i.e., metabolic cooperative assay, fluorescent dye transfer, and electrophysiological methods). Metabolic cooperative assay is usually used for toxicologic analysis for various chemicals and has the disadvantage of lengthy experimentation.[20] A recently developed technique of intracellular dye injection allows quantitative data in a more rapid fashion, but this method requires a specialized microinjection apparatus that is not widely available to researchers.[8,21] We adapted scrape-loading dye transfer of Lucifer yellow introduced by El-Fouly et al.[10] in 1987, to measure the gap junction–mediated cell communication. This method is simple, and the level of cell communication is easily quantitated by counting the number of fluorescent

cells. This technique introduces macromolecules into cells by a transient perturbation of the cell membrane. It was reported that the viability of the scraped cells was 50% to 60% immediately after scrape-loading, but 90% of those viable cells on the line of scraping were able to take up the fluorescent dye. In the present study, transfer of Lucifer yellow into three or more contiguous cells was detected in HeLa cells expressing E114G. In the report of Martin et al.[8] using the microinjection technique, 20% of cells expressing wild-type Cx26 were coupled and mutations M34T and W77R showed low dye transfer of only 5.7% and 4.1%, respectively. In this functional study of *GJB2*, the E114G HeLa cells showed dye transfer similar to wild-type cells, but no transfer activity was observed in 235delC like mock transfected cells. These data suggest E114G mutation of *GJB2* has gap junction function similar to wild-type cells, but 235delC mutation has a severe defect of gap junction activity. Even though the in vitro data strongly suggest that E114G mutation of *GJB2* has gap junction function similar to wild-type cells, additional studies on the subjects segregating E114G are required to confirm the exact role of this variation on the deafness as M34T variant.[22]

CONCLUSION

The 235delC and E114G alterations of *GJB2* were found in the Korean patients with hearing loss. The 235delC mutation caused frameshift and premature termination on codon 81 and showed loss of targeting activity resulting in severe deterioration of gap junction activity. For the E114G, there is missense converting codon 114 from glutamate (E) to glycine (G) suggesting a change of tertiary structure and function of connexin protein. However, in our study, the E114G did not show any different dye transfer through the gap junction from wild-type transfected cells; therefore, it was considered as of polymorphic change, not a disease causing mutation.

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