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Evaluation of the pathogenicity of *GJB3* and *GJB6* variants associated with nonsyndromic hearing loss

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ABSTRACT

A number of genes responsible for hearing loss are related to ion recycling and homeostasis in the inner ear. Connexins (Cx26 encoded by *GJB2*, Cx31 encoded by *GJB3* and Cx30 encoded by *GJB6*) are core components of gap junctions in the inner ear. Gap junctions are intercellular communication channels and important factors that are associated with hearing loss. To date, a molecular genetics study of *GJB3* and *GJB6* as a causative gene for hearing loss has not been performed in Korea. This study was therefore performed to elucidate the genetic characteristics of Korean patients with nonsyndromic sensorineural hearing loss and to determine the pathological mechanism of hearing loss by analyzing the intercellular communication function of Cx30 and Cx31 variants. Sequencing analysis of the *GJB3* and *GJB6* genes in our population revealed a total of nine variants, including four novel variants in the two genes. Three of the novel variants (Cx31-p.V27M, Cx31-p.V43M and Cx-30-p.I248V) and two previously reported variants (Cx31-p.V84I and Cx30-p.A40V) were selected for functional studies using a pathogenicity prediction program and assessed for whether the mutations were located in a conserved region of the protein. The results of biochemical and ionic coupling tests showed that both the Cx31-p.V27M and Cx31-p.V84I variants did not function normally when each was expressed as a heterozygote with the wild-type Cx31. This study demonstrated that two variants of Cx31 were pathogenic mutations with deleterious effect. This information will be valuable in understanding the pathogenic role of *GJB3* and *GJB6* mutations associated with hearing loss.

1. Introduction

A number of genes responsible for hearing loss (HL) are related to ion recycling and homeostasis in the inner ear [1]. Connexins (Cxs) are membrane proteins and core components of gap junctions (GJs), which are intercellular communication channels that are important for recycling potassium ions from the hair cells to the endolymph during auditory transduction [2–4]. GJs allow inorganic ions and small molecules with molecular weights of less than 1 kDa to pass between adjacent cells by nonspecific and passive diffusion [5]. Each GJ channel is composed of two hemichannels, termed connexons and each hemichannel is composed of six connexin (Cx) subunits that contain one cytoplasmic loop, two extracellular loops, and four transmembrane domains, with the amino- and carboxy-terminal ends facing the cytoplasm [6]. The transmembrane and extracellular loop domain sequences within the Cx gene family are conserved. The main differences between the Cx proteins are the lengths and sequences of the cytoplasmic loop and the carboxyl-terminal domain [7–11]. The hemichannel is formed from either identical (homomeric) or different (heteromeric) Cx proteins, and two hemichannels dock together with identical (homotypic) or different (heterotypic) connexons [12]. Because of the various possible combinations, each GJ channel shows considerable functional differences with distinct permeabilities [13,14].

To date, many studies have been performed on variants of Cx26 (gap junction beta-2 protein) which is encoded by the *GJB2* gene [15]. The *GJB2* gene accounts for approximately 50% of the autosomal recessive nonsyndromic HL cases in Caucasians [16]. However, it only accounts for 10–15% of the nonsyndromic HL cases in Koreans [17], suggesting an ethnic difference between Koreans and Caucasians. The gap junction beta-6 (Cx30, encoded by *GJB6*) is co-expressed with Cx26 in the fibrocytes of the spiral ligament, basal cells of stria vascularis, spiral limbus, and supporting cells in the organ of Corti [18]. However, expression of the gap junction beta-3 (Cx31, encoded by *GJB3*) and Cx26 only shows the similar pattern in fibrocytes of the spiral ligament and spiral limbus [19]. Furthermore, it has been

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determined that the variants of other Cx proteins in addition to Cx26 also alter the function of GJs and affect inner ear function [20]. However, only a few genetic and functional studies have been performed on Cx31 and Cx30 variants in other countries. Moreover, neither genetic nor functional studies of these proteins have been performed in Korea. Therefore, this study investigated the *GJB3* (encoding Cx31) and *GJB6* (encoding Cx30) genes that are expressed in the inner ear and associated with HL.

The work was performed to determine the genetic characteristics of Koreans with nonsyndromic sensorineural HL and the evaluation of the pathogenicity of *GJB3* and *GJB6* sequence variants through functional studies.

2. Materials and methods

2.1. Subjects and molecular genetic analysis

A total of 215 unrelated Korean patients with nonsyndromic sensorineural HL from the Kyungpook National University Hospital were included in this study. All participants provided written informed consent according to the protocol approved by the Institutional Review of Board of Kyungpook National University Hospital prior to the study. In total, 68 unrelated Koreans with normal hearing diagnosed by pure tone audiometry (PTA) test were recruited for use as control subjects. Genomic DNA was extracted from peripheral blood using a FlexiGene DNA extraction kit (Qiagen, Hilden, Germany).

The *GJB3* and *GJB6* coding regions were amplified by polymerase chain reaction (PCR) using the following designed primers: F1-5′CCCAG TTCCC AGTGT CAAAA3′, R1-5′TACGA CAACG CAGGC AAGA3′, F2-5′ TAGGT CGGGC AATGT AGCA3′ and R2-5′GAACT CAGAA CACTG CCTGG T3′ (for *GJB3*) and F-5′GGTTG GTATT GCCTT CTGGA3′ and R-5′TAGGG ATAAA CCAGC GCAAT3′ (for *GJB6*). An ABI 3130XL DNA sequencer was used to assay the products, and the data were analyzed with the Chromas Pro program and the Basic Local Alignment Search Tool (BLAST) on the NCBI website (http://www.ncbi.nlm.nih.gov/).

The variants for the functional studies were evaluated for potential pathogenicity using SNPs3D (http://www.snps3d.org) and PhD-SNP (http://gpcr.biocomp.unibo.it/~emidio/PhD-SNP/PhD-SNP.htm), and protein conservation was evaluated using CLC Sequence Viewer (v.6.0.1), a multiple sequence alignment program.

2.2. Molecular cloning and transfection

The human Cx31 and Cx30 cDNAs were subcloned into the pmCherry-N1 and pEGFP-N1 vectors, respectively (Clontech Inc., Mountain View, CA, USA), and each variant of Cx31 and Cx30 was generated according to the site-directed mutagenesis manual. The sequences of the constructed vectors were confirmed using Sanger sequencing.

The human embryonic kidney cell line, HEK293, and the human cervical cancer cell line, HeLa, were used for transfection of the Cx31 and Cx30 constructs, respectively, and were obtained from the Korean Cell Line Bank (Seoul, Korea). These cell lines are widely used for GJ studies because they lack endogenous expression of Cxs. The cells were seeded on a cover slip in 24-well plate and cultured to 80% confluence one day before transfection. The cells were transfected using the Fugene HD reagent (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer's instructions.

2.3. Assays for measuring biochemical and ionic coupling

GJ biochemical coupling was measured using a single cell dye transfer assay. One of the two adjoining cells forming a GJ in Hanks' balanced salt solution (HBSS) containing 1.2 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, adjusted to pH 7.4 with NaOH (external solution) was analyzed by patch-clamp using a micro electrode filled with a 1% Lucifer Yellow (LY, molecular weight 457, charge −2; Sigma) solution or with 0.15 mM propidium iodide (PI, molecular weight 650, charge +2; Invitrogen) in internal solution (120 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES in HBSS). The LY transfer was recorded for 0.5–3 min after injection by electrophoresis using a method based on the technique described by Haas [21] and the PI transfer was recorded for 1–5 min.

Transfected cells grown on cover slips were loaded with the calcium indicator dye, fura-2 acetoxymethyl ester (fura-2AM, 5 μM), and pluronic F-127 which was used to help disperse fura-2AM in HBSS (containing 1.2 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES). The cells were then incubated for 30 min at room temperature under constant agitation. The cells were then placed in HBSS including 200 μM suramin (external solution), which blocks intercellular Ca^{2+} signaling through a purinergic receptor [18]. This solution was also used as the pipette solution. Mechanical stimulation was performed by lightly touching one cell membrane, and calcium image was analyzed using Axon Imaging Workbench software (version 6.0, Axon Instruments, CA).

3. Results

3.1. Sequencing analysis and selection of variants used in the functional study

From the results of the analysis of the *GJB3* and *GJB6* gene sequences in Korean patients with nonsyndromic HL, seven variants in the *GJB3* and two variants in the *GJB6* were identified, and two novel variants were detected in each gene (Table 1). The p.V27M and p.V84I variants in *GJB3* and the p.I248V variants in *GJB6* were not observed in normal Korean controls. These variants also contained nonsynonymous substitutions; therefore, it is likely that they affected the tertiary structure of the Cx protein and the formation of a hemichannel or a GJ. To evaluate the evolutionary conservation of the amino acid substitutions, the amino acid sequences of Cx genes obtained from seven species were aligned (Fig. S1). The p.V27M and p.V84I variants in *GJB3*, but not the p.I248V variant in *GJB6*, are located in evolutionarily conserved position of the Cx gene family across the seven species, and these variants are also predicted to be pathogenic by SNPs3D and PhD-SNP (Table 2). These three variants in addition to Cx31-p.V43M used as a control and Cx30-p.A40V on which no functional studies have been performed were selected for functional studies. Thus, five variants (p.V27M, p.V43M and p.V84I of Cx31 and p.A40V and p.I248V of Cx30) were assessed to clarify their possible roles in HL.

3.2. Assays for measuring biochemical coupling

HEK293 cells were transfected with expression plasmids encoding wild-type (WT) Cx31 and three mutated (MT) Cx31 constructs tagged with mCherry, a red fluorescent protein marker, to identify the location of the Cx proteins in HEK293 cells. We observed that the Cx31-WT-mCherry and the three Cx31-MT-mCherry proteins were localized on the cell membrane and formed GJs as shown by the characteristic

Table 1
The *GJB3* and *GJB6* variations detected in Koreans.

Gene	Nucleotide change	Amino acid change	Genotype distribution[a]		dbSNP[b]
			Patients	Controls	
GJB3	c.79G>A	p.V27M	0/1/211	0/0/63	–
	c.127G>A	p.V43M	0/1/211	0/1/62	–
	c.250G>A	p.V84I	0/7/205	0/0/63	rs145751680
	c.357C>T	p.N119N	1/34/177	0/11/52	rs41310442
	c.580G>A	p.A194T	0/7/208	0/2/66	rs117385606
	c.813 + 43A>C	–	200/15/0	67/1/0	rs41266429
	c.813 + 53A>G	–	149/53/13	47/16/5	rs476220
GJB6	c.261A>T	p.P87P	0/1/197	0/0/57	–
	c.742A>G	p.I248V	0/1/197	0/0/57	–

[a] Genotype distribution: numbers indicate homozygous/heterozygous/wild type.
[b] dbSNP is Single Nucleotide Polymorphism database and the reference ID number for the National Center for Biotechnology Information (NCBI) is presented.

Table 2
The Cx31 and Cx30 variants selected for functional study.

Protein	Amino acid change	Domain[a]	Pathogenicity prediction		Reference
			SNPs3D[b]	PhD-SNP[c]	
Cx31	p.V27M	TM1	−0.44	Disease 3	This study
	p.V43M	EC1	−0.44	Neutral 0	This study
	p.V84I	TM2	−1.02	Disease 1	Chu et al. (2001)
Cx30	p.A40V	TM1	−0.67	Neutral 2	Wang et al. (2011)
	p.I248V	COOH	1.34	Neutral 3	This study

[a] Abbreviations: TM — transmembrane, EC — extracellular loops, COOH — carboxy-terminal.
[b] SNPs3D: positive score indicates a non-deleterious variant and a negative score indicates a deleterious variant.
[c] PhD-SNP (Predictor of human Deleterious Single Nucleotide Polymorphisms) classifies variants as disease-related (Disease) or as neutral polymorphisms (Neutral).

plaques between two adjacent cells. The three variants of Cx31 formed GJs regardless of the protein domain in which the mutations occurred. The biochemical coupling test of WT and mutated GJ channels was performed by examining the cell-to-cell diffusion of Lucifer Yellow (LY), a cell membrane-impermeable fluorescent dye. The diffusion of dye was recorded as follows: immediate transfer (less than 30 s), delayed transfer (30 s to 3 min) and no transfer (more than 3 min). In the GJ channels composed of Cx31-WT subunits, LY was almost immediately transferred after injection (Fig. 1A; n = 18/21). The Cx26-p.D46E negative control [22] did not display intercellular dye transfer (data not shown; n = 24/25). Similarly, the LY dye did not diffuse through any GJ channels that were composed of Cx31-p.V27M or Cx31- p.V84I (Fig. 1B; n = 36/40 and Fig. 1C; n = 41/47, respectively). For the Cx31-p.V43M variant, LY was either immediately transferred (n = 18/29) or showed partly delayed transfer (Fig. 1D; n = 9/29). These data indicate that GJ channels composed of either Cx31-p.V27M or Cx31-p.V84I proteins (homozygotes) cannot function in the transfer of substances. We performed an additional biochemical coupling test to assess the heterozygous conditions using Cx31-WT with Cx31-p.V27M or Cx31-p.V84I which greatly influenced the ability to transfer substances as homozygotes, because they were all found as heterozygotes in the patients. The HEK293 cells were co-transfected with equal amounts of Cx31-WT and Cx31-p.V27M (or Cx31-p.V84I) plasmid DNA. The hybrid GJ consisting of Cx31-WT/Cx31-p.V27M transferred LY slowly (Fig. 1E; n = 31/35) and occasionally did not transfer the dye at all (n = 4/35). In the Cx26-WT/Cx26-p.D46E which served as a control and Cx31-WT/Cx31-p.V84I hybrid GJ, LY was transferred slowly and weakly (data not shown and Fig. 1F; n = 10/23 and n = 17/37, respectively) or not at all (n = 13/23 and n = 17/37, respectively).

HeLa cells were transfected with Cx30-WT and two Cx30-MT constructs tagged with EGFP, a green fluorescent protein marker, and PI was used as the dye for visualizing transfer. The Cx30-WT-EGFP and two Cx30-MT-EGFP proteins were localized on the cell membrane and formed GJs. For the biochemical coupling test, PI was relatively easily transferred through GJ channels composed of Cx30-WT subunits (Fig. 2A; n = 25/30), but was not completely transferred through the Cx26-p.D46E channels (data not shown; n = 20/20). PI transfer through GJ channels consisting only of Cx30-p.A40V subunits (Fig. 2B; n = 32/40) occurred at a ratio similar to that of Cx30-WT, whereas approximately half of the GJ channels composed of Cx31-p.I248V transferred PI (Fig. 2C; n = 17/31).

3.3. Assays for measuring ionic coupling

After identification of the GJs expressed in transfected cells, the ability of the GJs to intercellularly transfer Ca^{2+} ions was examined using calcium imaging analysis. The mechanically stimulated cell was referred to as cell #1, its GJ-forming neighboring cell was cell #2, and an untransfected neighboring cell used as a control was cell #3. The fura-2 signals showed the mechanically elicited intracellular Ca^{2+} concentration in the stimulated cell (#1) and neighboring cells (#2) in real time. Untransfected cells did not display intercellular Ca^{2+} transfer to neighboring cells from mechanically stimulated cells.

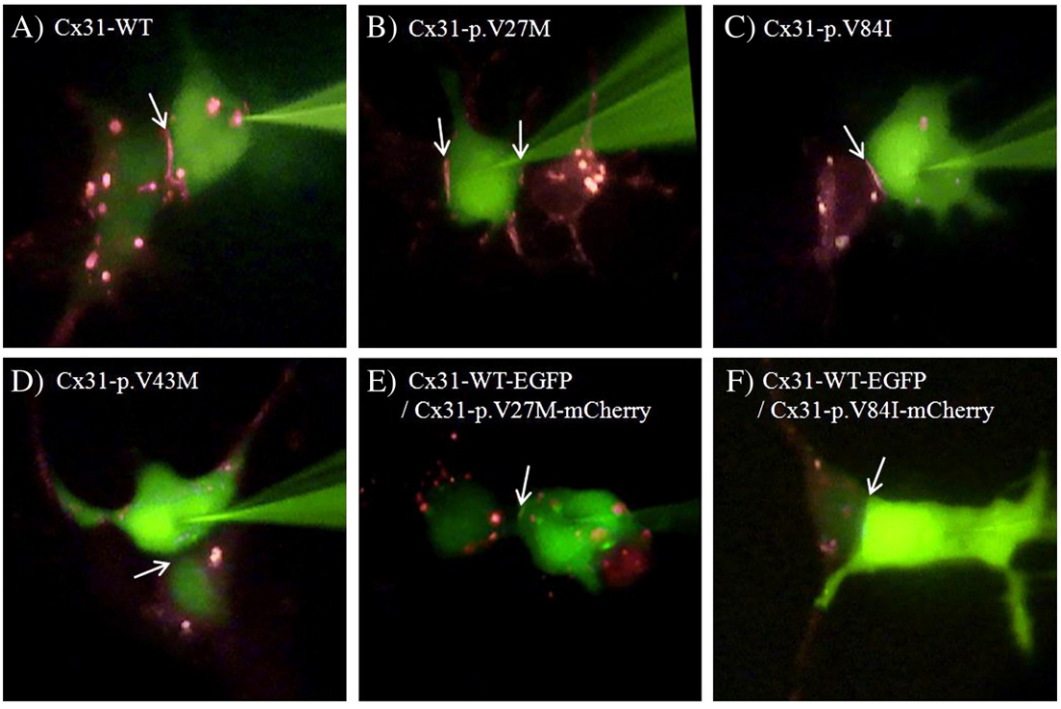

Fig. 1. Diffusion of LY through the GJ of Cx31-WT, Cx31 variants and hybrid of Cx31-WT and Cx31 variants in HEK293 cells. Arrows indicate the presence of GJs at the cell membrane bordering the two cells in HEK293 cells expressing mCherry. Individual cells were microinjected with LY, and intercellular diffusion of dye was monitored for 3 min after injection of LY. The Cx31-WT (A) transferred LY through GJs from the injected cell to the neighboring cell, whereas neither Cx31-p.V27M (B) nor the Cx31-p.V84I (C) variant transferred LY. The Cx31-p.V43M (D) variant transferred LY slowly. Both the Cx31-WT-EGFP/Cx31-p.V27M-mCherry (E) and Cx31-WT-EGFP/Cx31-p.V84I-mCherry (F) hybrid GJs showed delayed transfer.

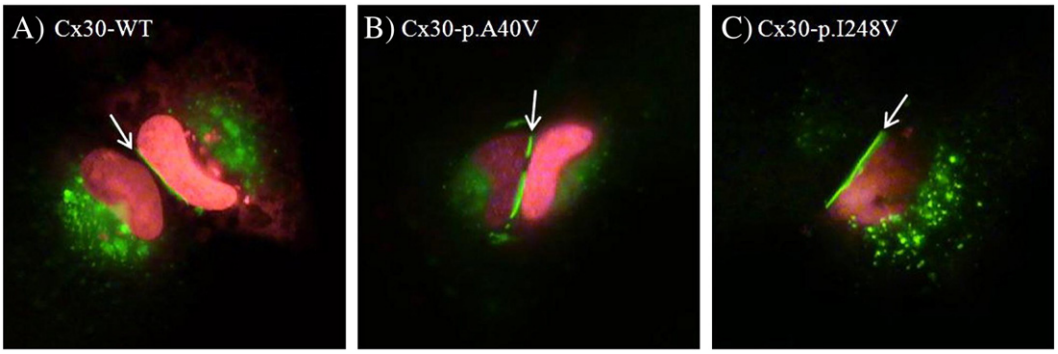

Fig. 2. Diffusion of PI through GJ of Cx30-WT and Cx30 variants in HeLa cells. Arrows indicate the GJs as identified by EGFP in the HeLa cells, which were monitored for 5 min after injection of PI. Intercellular diffusion of the dye is shown between two cells expressing Cx30-WT-EGFP (A) and Cx30-p.A40V-EGFP (B). The Cx30-p.I248V (C) variant transferred PI in about half of all cells.

In the GJ composed of Cx31-WT, the increased concentration of intracellular Ca^{2+} in the mechanically stimulated cells led to an increase in the Ca^{2+} concentration in the coupled neighboring cell (Fig. 3A; n = 44/56). In GJs composed of the Cx31-p.V43M variant, the incidence of no transfer (n = 19/32) was slightly higher than that of transfer (Fig. 3B; n = 13/32). In cells expressing the Cx31-p.V27M or Cx31-p.V84I variants, Ca^{2+} was rarely transferred (Fig. 3C; n = 15/20 and Fig. 3D; n = 35/50). This result indicates that GJs composed of either the Cx31-p.V27M or the Cx31-p.V84I variant lost GJ-mediated ionic coupling. Additional ionic coupling tests were performed for heterozygotes composed of the Cx31-WT and Cx31-p.V27M or Cx31-p.V84I variant. The Cx31-WT/Cx31-p.V27M heterozygote slowly transferred Ca^{2+} in half of the experiments (Fig. 3E; n = 11/22), and some GJs did not transfer Ca^{2+} at all (n = 2/22). For the Cx31-WT/

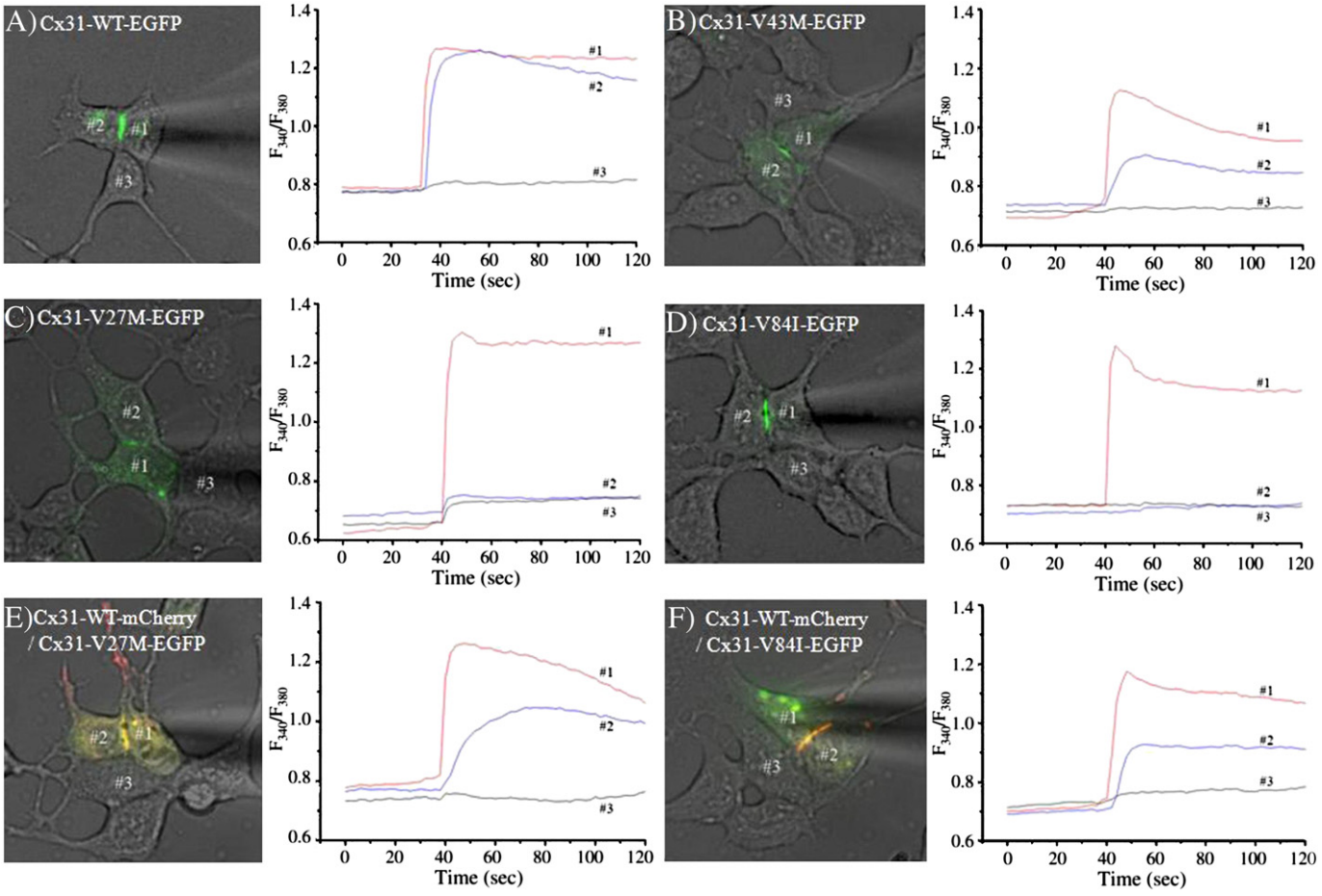

Fig. 3. Diffusion of calcium ions through GJs of Cx31-WT, Cx31 variants and hybrids of the Cx31-WT and Cx31 variants in HEK293 cells. Cell #1 is the mechanically stimulated cell, cell #2 is the cell that forms a GJ with cell #1 and cell #3 is an untransfected cell used as a control. The Cx31-WT-EGFP (A) construct showed that as the intracellular Ca^{2+} concentration of cell #1 was increased, that of cell #2 immediately increased. The Cx31-p.V43M-EGFP variant did not transfer calcium ions at all or transferred them slowly (B). In the cells transfected with Cx31-p.V27M-EGFP (C) or Cx31-p.V84I-EGFP (D), the intracellular Ca^{2+} concentration of cell #1 increased in response to mechanical stimulation, but that of cell #2 rarely increased. In both the Cx31-WT-mCherry/Cx31-p.V27M-EGFP (E) and Cx31-mCherry/Cx31-p.V84I-EGFP (F) hybrid GJ, the intracellular Ca^{2+} concentration cell #1 was increased, and that of cell #2 was increased slowly.

Cx31-p.V84I heterozygote, intercellular Ca^{2+} was slowly transferred (Fig. 3F; n = 9/20) or not transferred at all (n = 5/20).

In the Cx30-WT and the Cx30-p.A40V and Cx30-p.I248V variants, intercellular Ca^{2+} was easily transferred (Fig. 4A; n = 11/12, Fig. 4B; n = 13/15 and Fig. 4C; n = 18/18, respectively).

4. Discussion

The *GJB3* and *GJB6* genes, which are expressed in the inner ear, are involved in nonsyndromic HL with a dominant or recessive inheritance pattern. Many studies of the *GJB2* gene, which is a major causative gene for autosomal recessive nonsyndromic HL in Caucasians, have been performed to date, but little is known about the contributions of *GJB3* and *GJB6* to genetic HL in humans. The *GJB3* and *GJB6* genes are evolutionarily close to *GJB2*, and the expression pattern of *GJB6* is very similar to that of *GJB2* in the inner ear. Considering the relationships among these genes in terms of evolution and development, it is possible that *GJB3* and *GJB6* are associated with HL in humans. Thus, we performed functional and molecular genetic analyses with *GJB3* and *GJB6*.

The molecular genetic analysis of the *GJB3* and *GJB6* genes in 215 Korean patients with nonsyndromic HL identified nine variants, including four novel variants. Four of these variants were identified only in the patient group and not the control group. The p.V84I variant in the *GJB3* gene was detected with a particularly high allele frequency in our patients (1.65%, 7/424). The p.V84I variant (c.250G>A) was first identified as having a 0.87% (1/114) allele frequency in the USA [23]. The p.V84I was also detected at low allele frequencies (0.35%, 2/568 and 0.20%, 1/506, respectively) in two studies in China [24,25]. These data indicate that p.V84I in the *GJB3* gene occurs with a high relative frequency in Korean patients compared with patients in other countries.

Five different variants were selected for functional studies, and three of these variants (Cx31-p.V27M, Cx31-p.V84I and Cx30-p.A40V) were predicted to be pathogenic because 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

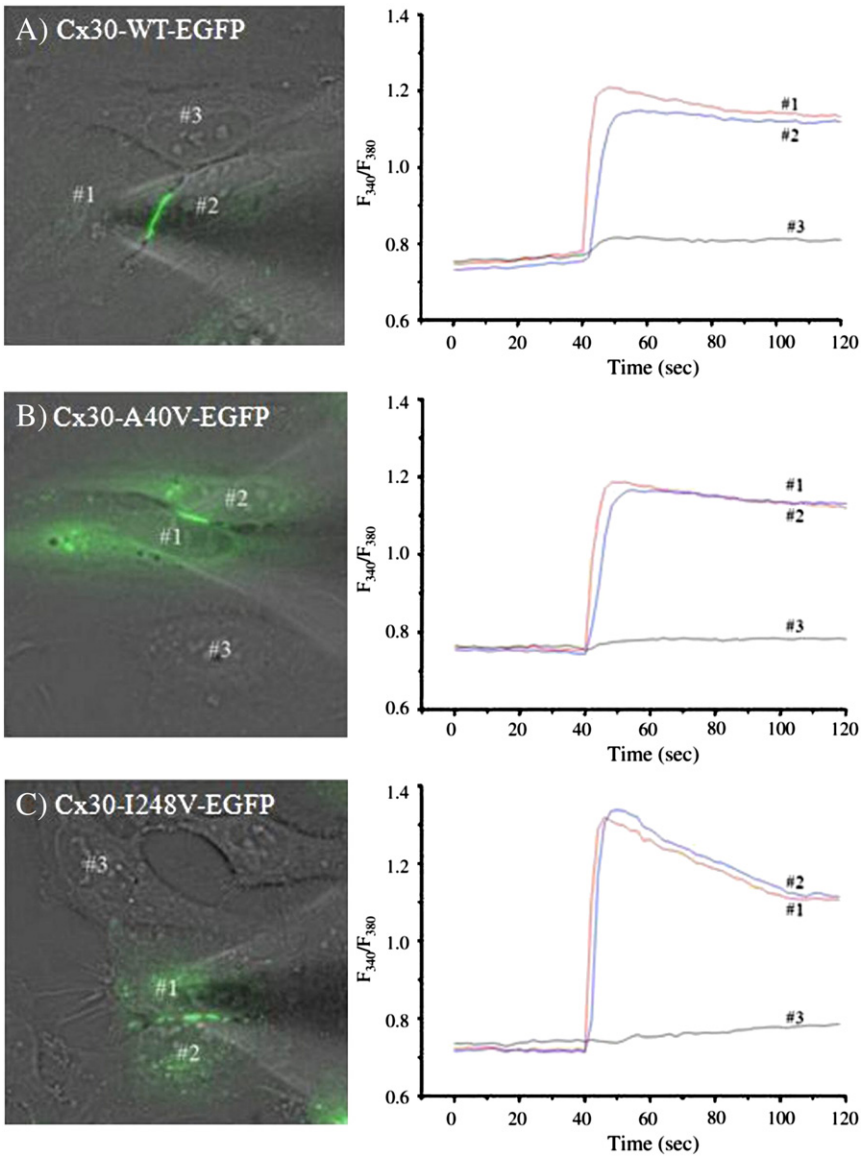

Fig. 4. Diffusion of calcium ions through the GJ of Cx30-WT and Cx30 variants in HeLa cells. Cell #1 is the mechanically stimulated cell, cell #2 forms a GJ with cell #1 and cell #3 is an untransfected cell used as a control. The Cx30-WT-EGFP (A), Cx30-p.A40V-EGFP (B) and Cx30-p.I248V-EGFP (C) constructs all showed that when the intracellular Ca^{2+} concentration of cell #1 increased, the Ca^{2+} concentration in cell #2 immediately increased.

shown to be different depending on the position and type of amino acid substitution. Therefore, functional studies are critical in understanding the pathogenic potential of the Cx30 and Cx31 mutations.

The results of the two functional studies indicated that the homozygous Cx31-p.V27M GJ was not very efficient at transferring either Ca^{2+} ions or LY dye. The hybrid GJ composed of the Cx31-WT/Cx31-p.V27M heterozygote showed a more delayed transfer capacity compared to Cx31-WT in both the biochemical and ionic coupling tests. The Cx31-p.V27M variant, first discovered in the current study is located in the equivalent position of p.V27I in the Cx26. It is considered that p.V27I in the Cx26 is a non-pathogenic variant in many previous studies [26,27], but p.V27 was substituted with the different amino acid in the Cx31. The size of methionine is larger than valine and isoleucine, and this can affect the formation of the GJ. It has been reported that the variants in the same position are substituted with different amino acids in the case of p.W44C and p.W44S, p.R75W and p.R75Q, p.M34T and p.M34A of Cx26, each variant showed different effects on function of the GJ [28,29]. The p.R75Q had gap junction plaques at the cell borders, but the p.R75W had smaller plaques and more intracellular staining. In the co-expressing Cx30, p.W44C and p.R75W completely inhibited neurobiotin transfer to neighboring cells, but the p.W44S and p.R75Q partially inhibited. The p.M34T and p.M34A both formed gap junction plaques, but dye transfer assays showed that the p.M34T transfer dye through the gap junctions and the p.M34A had a significantly reduced permeability. The p.M34T did not interfere with the efficient formation of stable connexon hexamer, but the p.M34A reduced channel permeability because of substitution of the original residue with a smaller residue. Reducing the size of the side chain at this position, it would lead to a partially closed channel. Different variants in the same position or domain may alter different functional effect of Cx through modification of the three-dimensional structure of protein.

For Cx31-p.V43M, the homozygote was not able to function normally in the transfer of substances, especially in the ionic coupling test, whereas the heterozygote functioned almost normally. These results indicate that homozygous Cx31-p.V43M can be pathogenic, but heterozygous Cx31-p.V43M is nonpathogenic, which is the form detected in the patients of this study.

In the case of Cx31-p.V84I, the results of biochemical and ionic coupling tests were similar to those of Cx31-p.V27M under homozygous conditions, but results from the hybrid Cx31-WT/Cx31-p.V84I GJ were somewhat different than the results from the hybrid Cx31-WT/Cx31-p.V27M GJ. The Cx31-WT/Cx31-p.V84I heterozygote showed a lack of transfer or delayed transfer in both the biochemical and ionic coupling tests, whereas the Cx31-WT/Cx31-p.V27M heterozygote primarily showed a delayed transfer or transfer. The Cx31-p.V84I variant is more functionally deficient than Cx31-p.V27M, and Cx31-p.V84I has also been shown to have a deleterious effect in HL. The Cx26-p.V84L mutation, which is at the equivalent amino acid position as the Cx31-p.V84I mutation, deregulates the IP3 cascade, which is important in the Ca^{2+} pathway [30]. This previous study supports the idea that Cx31-p.V84I is a pathogenic variant. The p.V84I (c.250A>G) mutation in *GJB3* was detected in several previous studies, but functional studies were not conducted. Our study was therefore the first to assess the function of the Cx31-p.V84I variant.

The Cx30-p.A40V variant functioned normally in the biochemical and ionic coupling tests, indicating that Cx30-p.A40V is a nonpathogenic variant that produces a functionally null protein that does not interfere with normal GJ function. However, it has recently been shown that Cx30-p.A40V causes Cx30 protein accumulation in the Golgi body rather than in the cytoplasmic membrane [31]. These differences in putative function may be due to the different experimental methods. The recently reported study employed the Tet-On system to regulate gene expression, and this system might yield different results than those found in our study in terms of the expression level of the Cx gene and the effect on HeLa cells.

The Cx30-p.I248V variant functioned normally in the ionic coupling test and had a small effect on biochemical coupling. GJs allow small molecules with molecular weights of less than 1 kDa to be transferred. The molecular weight (MW) of the PI dye used to test the biochemical coupling is 650 Da, whereas the MW of the calcium ions used to test the ionic coupling is 40.078 Da. This discrepancy in the results between the two functional studies for Cx30-p.I248V can be caused by differences of MW and characteristics of the substances that passed between the cells through GJs. Additional studies using a substance that is smaller than the PI dye such as inositol 1,4,5-trisphosphate (IP_3) [32], are needed to confirm the effects of the Cx30-p.I248V variant in biochemical coupling tests. However, the effect of the Cx30-p.I248V homozygote was weak in the biochemical coupling test and absent in the ionic coupling test using calcium ions. The calcium ions have a MW similar to that of potassium (MW: 39.098 Da), which is recycled through the GJs during auditory transduction. We conclude from these studies that Cx30-p.I248V is a nonpathogenic variant.

Based on our data, the variants of the *GJB6* gene are all nonpathogenic variants. To date, there have been several studies involving various combinations of Cx30 and Cx26 [28,33]. A 309-kb deletion in the *GJB6* gene is the second most frequent mutation that causes prelingual deafness in the Spanish population, and mutations in the *GJB2* and *GJB6* complex can result in a monogenic or digenic pattern of inheritance of prelingual deafness [34,35]. Our study identified only two variants of the *GJB6* gene, and none of our patients were carrying a deletion in the *GJB6* gene. Until now, there have been no reports of mutations in the *GJB6* gene in Korean patients with HL. Together, our studies indicate that the *GJB6* gene is not the main causative gene associated with HL in Korean patients. This information will be valuable in understanding the pathogenic roles of Cx31 and Cx30 mutations associated with HL and in applying gene therapy or early diagnosis.

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