Deficient gap junction coupling of two common hearing loss-related variants in GJB2

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Author Contributions

Conceptualization: H J, Data curation: K C. Funding acquisition: K C and H J. Methodology: K C and H J. Validation: H J. Writing - original draft: K C. Writing - review & editing: H J.
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HIGHLIGHTS

- **GJB2** p.V37I causes deficient gap junction coupling.
- Ionic and biochemical coupling was severely defective in c.299-300delAT-transfected cells.
- Environmental factors may impact the functional consequences of **GJB2** p.V37I.

ABSTRACT

**Objectives.** To explore the functional consequences of two common variants, p.V37I and c.299-300delAT in hearing loss associated gene **GJB2**.

**Methods:** Connexin 26 expression and gap junctional permeability were studied in HEK 293T cells transfected with plasmids expressing **GJB2** wild-type, p.V37I, or c.299-300delAT CX26 proteins with fluorescent tags. Functional analyses of different **GJB2** haplotypes were performed to fully assess the alteration of ionic and small-molecule coupling.

**Results:** The p.V37I protein was localized at the plasma membrane, but failed to effectively transport intercellular propidium iodide or Ca²⁺ efficiently, indicating impairment of both biochemical and ionic coupling. The presence of **GJB2** p.V37I appeared to increase the sensitivity of cells to H₂O₂ treatment. In contrast, the known variant c.299-300delAT protein was not transported to the cell membrane and could not form gap junctions, instead being confined to the cytoplasm. Ionic and biochemical coupling was defective in c.299-300delAT-transfected cells.

**Conclusions:** The p.V37I and c.299-300delAT **GJB2** mutations resulted in deficient gap junction-mediated coupling. Environmental factors may impact the functional consequences of **GJB2** p.V37I. These results may inspire the development of molecular therapies targeting **GJB2** mutations for hearing loss.
**INTRODUCTION**

*GJB2* encodes connexin 26 (CX26), a member of the connexin family of highly structurally related gap junction (GJ) proteins. A complete GJ channel between adjacent cell membranes consists of two hemichannels. Both hemichannels and GJ channels allow diffusion of ions and small molecules (< 1200 Da) [1]. Mutations in *GJB2* accounted for almost 20% of non-syndromic hearing impairment (NSHI) in many populations [2, 3]. The mutational spectrum of *GJB2* varies among different ethnic groups and populations. In East Asia, the most common variants, namely c.109G>A (p.V37I), c.235delC, and c.299-300delAT [2, 4, 5], impact a large number of individuals with hearing loss.

The pathogenicity of c.235delC is well established [6, 7]. By contrast, despite clinical evidence indicating that c.299-300delAT is also pathogenic [2, 3, 8], functional analysis of this variant is lacking. Additionally, the variant p.V37I was initially recognized to benign polymorphism because of its high frequency in individuals with normal hearing [9]; however, it was subsequently proposed that homozygous p.V37I can lead to mild or moderate hearing impairment with incomplete penetrance [10]. So far, studies of these two variants have mostly focused on phenotypic analysis of patients. To our knowledge, the existing data on the functional effects of p.V37I are conflicting [7, 11, 12].

The extent of impairment of intercellular coupling due to the p.V37I mutation has not been well correlated with the severity of hearing loss observed in some clinical cases [7, 10, 11, 13]. Interestingly, the homozygous p.V37I mutation of *GJB2* has also been linked to postnatal childhood hearing impairment and age-related deafness [13, 14]. In view of these findings, a possible modifying
effect of environmental factors on the clinical phenotype of p.V37I should be investigated. In this study, we performed functional evaluations of two common alleles of GJB2: p.V37I and c.299-300delAT.

MATERIALS AND METHODS

Ethics approval

The experiments in this study were conducted following the Code of Ethics of the World Medical Association, and were approved by the Institutional Review Board of the ** Hospital, *** University. We obtained informed consent from the patients or their guardians.

CX26 variants construction and transfection of HEK 293T cells

DNA samples were extracted from peripheral blood donated from individuals with and without hearing impairment as previously described [4]. The GJB2 coding region was amplified by PCR of both wild-type (WT) and variant CX26 genes using the following primers: F, 5'-CCGGAATTCCGCCACCATGGATTGGGGCACGCTG-3' and R, 5'-CGCGGATCCGGATCGGTGTTTTTGACTTCCCA-3'. The PCR products were subcloned into vectors pEGFP-C2 and pmCherry-C1 (Clontech Inc., USA) to create fusion proteins of CX26 with enhanced green fluorescent protein (EGFP) or mCherry, respectively (Table 1). The targeted sequences were confirmed by Sanger sequencing, to exclude additional unintended mutations.

HEK 293T cells do not express the endogenous GJB2 or CX26 protein, and have been commonly used in GJ studies [15, 16]. Cultured HEK 293T cells were dissolved and transferred onto glass coverslips, and cultured to average 80% confluence 1 day before transfection. Lipo2000 (Invitrogen Inc., USA) was used for cells transfection following the manufacturer’s instructions. Functional analyses were conducted 48–72 hours post transfection.
Assays for measurement of biochemical coupling and hemichannel permeability

GJ-mediated biochemical coupling was assessed by examining the diffusion of a membrane-impermeable dye using the single-cell dye transfer method [16]. Single HKE 293T cells with GJs were microinjected with propidium iodide (PI; molecular weight, 650 Da; Invitrogen Inc., USA) dissolved in intracellular solution (120 mM KCl, 1 mM MgCl$_2$, 10 mM HEPES with pH 7.4). Localization of CX26-EGFP in transfected cells and the transfer of PI were detected as green and red signals, respectively, at 5 minutes post injection under fluorescence microscopy. The incidence of coupling among cells transfected with the CX26 expression constructs is reported here as the percentage of microinjections in which the GJ tracer was transferred from the injected cell to its neighbor cell.

To measure the unitary gating properties of the hemichannels in cells transfected with different CX26 expression constructs, we use a PI dye loading assay [15, 16]. HKE 293T cells were cultured in PI solution (0.15 mM in a Ca$^{2+}$-free HBSS) for 20 minutes at room temperature, in which the PI dye could enter into cells through hemichannels comprising functional CX26 subunits. The residual PI solution was then removed and washed with PBS three times, and the cells were fixed with 4% paraformaldehyde and visualized under a fluorescence microscope (Olympus, Japan). The percentage of cells visually confirmed to be expressing CX26-GFP and containing PI (red signal) among the CX26-expressing cells was calculated.

Measurement of GJ-mediated intercellular ionic coupling

Ionic coupling assays were completed with the Ca$^{2+}$ fluorescence indicator fluo-3 AM following a detailed procedure that was previously described [17]. Specifically, HEK 293T cells were incubated in a 6-well plate and the targeted CX26 expression constructs tagged with mCherry were transfected into cells using liposomes. At 48 hours post transfection, cells were incubated with HBSS containing
5 μM Fluo-3 AM and 20 μM ATP for 1 hour at room temperature. Successful transfection of CX26 was directly visualized by mCherry protein. The CX26-targeted cells were gently stimulated with a glass microelectrode under the scanning mode of an LSM 710 confocal laser scanning microscope (Zeiss, Germany). Fluorescence was visualized and recorded with the LSM 710. Zen 2009 software was used for data analysis.

**Tolerance of cells expressing GJB2 p.V37I to H$_2$O$_2$ intervention**

After transfection with CX26 expression constructs for 36–48 hours, HEK 293T cells were incubated with a range of H$_2$O$_2$ concentrations (20 μM, 40 μM, 80 μM) for 8 hours. Cellular apoptosis assays were conducted using an annexin-V detection kit (KeyGen Inc., China) following the manufacturer’s instructions. Cells were harvested and sent for flow cytometry on a FACS machine. Cells expressing CX26-mCherry and/or the apoptosis marker annexin V were recognized and counted. The cell apoptosis rate was calculated as the ratio of mCherry-positive cells expressing both mCherry and Annexin V. Additionally, the biochemical hemichannel permeability following different H$_2$O$_2$ insults (10 μM, 20 μM, 40 μM, 80 μM) were measured via PI dye loading assay described above.

**Statistical tests**

GJ percentages, PI loading percentages, and cellular apoptosis rates were recorded as mean values ± standard deviations. One-way analysis of variance was used to analyze these parameters using SPSS 23.0 (SPSS Science, USA). Statistically significant difference was defined when $P$ value was < 0.05.

**RESULTS**

**Intracellular protein expression and localization of two connexin variants (Fig. 1)**

CX26-p.V37I was localized at the cell membrane and directly formed GJs, based on the typical
plaques observed between two neighboring cells (hereafter referred to as cell pairs). In contrast, CX26-c.299-300delAT molecules were scattered in the cytoplasm and unable to form GJ plaques with normal appearance. Quantification did not show a significant difference in the GJ formation rates between CX26-WT (73.4 ± 3.87%, n = 872 cell pairs), CX26-WT/CX26-p.V37I (68.8 ± 3.56%, n = 878 cell pairs), and CX26-p.V37I (70.0 ± 8.62%, n = 909 cell pairs). These results indicated that each mutant had different impacts on GJ formation.

**Impairment of GJ-mediated biochemical coupling in GJs containing variant CX26**

The biochemical permeability of variant GJ channels was first tested by probing the cell-to-cell transfer of PI, which is a GJ-permeable fluorescent dye (Fig. 2). Cells transfected with CX26-WT (n = 40 cell pairs tested) or CX26-WT/p.V37I (n = 34 cell pairs tested) showed high efficiency of PI transfer through the GJ channel (85.0% and 88.2%, respectively) after microinjection. By contrast, PI diffusion via GJ channels composed of CX26-p.V37I or CX26-c.299-300delAT was markedly impaired or absent, accounting for 9.5% efficiency (n = 42 cell pairs tested) in the CX26-p.V37I variant and 0% (n = 20 cell pairs tested) in the CX26-c.299-300delAT variant (Fig. 2). Non-transfected HEK 293T cells, which served as negative controls (n = 15 cell paired tested), failed to probe intercellular dye diffusion (data not shown). These findings indicated that GJ channels containing CX26-p.V37I subunits had less severe effects on biochemical coupling than those containing CX26-c.299-300delAT.

Next, we assessed the biochemical permeability of hemichannels by evaluating the transfer rate of extracellular PI dye into HEK 293T cells (Fig. 3). The rates of PI transfer into cells co-transfected with CX26-WT and CX26-p.V37I (2587/3124 cells, 82.9 ± 3.06%) or CX26-WT alone (2689/3216 cells, 83.7 ± 1.71%, p > 0.05) were similar. In contrast, PI transfer through hemichannels composed of either CX26-p.V37I (781/3022 cells, 25.9 ± 2.94%, p < 0.05 compared with CX26-WT) or CX26-c.299-300delAT
(35/1421 cells, 2.46 ± 0.41%, $p < 0.01$ compared with CX26-WT) alone was impaired. These results suggested that both mutations damaged the biochemical permeability of the hemichannels.

**Deficient GJ-mediated intercellular ionic coupling in mutant GJs**

Intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+]_i]$) increases following a mechanical stimulation in the affected cell, and is followed by a secondary $[\text{Ca}^{2+]_i]$ increase in neighboring cells coupled through functional GJs. Non-transfected HEK 293T cells (#3 in Fig. 4) were considered as a negative control. Cells transfected with CX26-WT showed a consistent increase in the $[\text{Ca}^{2+]_i]$, with an intercellular wave of calcium signal (14/14 cell pairs, 100%; Fig. 4A). Cells transfected with both CX26-WT and CX26-p.V37I had a similar response to that of CX26-WT, exhibiting normal $[\text{Ca}^{2+]_i]$ transfer (13/13 cell pairs, 100%). By contrast, transfection of CX26-p.V37I alone led to weaker transfer of intracellular $\text{Ca}^{2+}$ to neighboring cells via the GJs (5/17 cell pairs, 29.4%). No transfer of intracellular $\text{Ca}^{2+}$ (0/14 cell pairs) was detectable in cells transfected with CX26-c.299-300delAT (Fig. 4D). These results indicated that GJs comprising the CX26-p.V37I and CX26-c.299-300delAT variant proteins had partial to complete loss of GJ-mediated ionic coupling ability.

**Effects of the CX26-p.V37I mutation on cellular tolerance to H$_2$O$_2$ intervention**

Transfected HEK 293T cells were treated with a range of H$_2$O$_2$ concentrations. After 8 hours of exposure, obvious cell shrinkage and condensation was observed (Fig.5A). We quantified the early cellular apoptosis marker annexin V-allophycocyanin using flow cytometry. CX26-p.V37I haplotype cells showed higher apoptosis rates than CX26-WT and CX26-WT/p.V37I cells at 0 μM and 20 μM H$_2$O$_2$ (Fig. 5B-C). The cellular biochemical hemichannel function was impaired greatly over 40 μM H$_2$O$_2$ (Fig.5D), probably due to an increase in intracellular $\text{Ca}^{2+}$ that induced GJs to close.
DISCUSSION

Our study demonstrated that CX26 proteins produced by GJB2 variants p.V37I and c.299-300delAT may be partly or completely devoid of function, inducing deficient GJ-mediated intercellular coupling. Notably, our observations suggest that the p.V37I variant may increase cellular susceptibility to environmental stress. These results may inspire the development of molecular therapies targeting GJB2 mutations for hearing loss.

Studies have reported that GJB2 p.V37I is strongly related to hearing loss. Experiments in transgenic knock-in mice indicate that this hearing loss is probably due to lowered endocochlear potential and excitotoxicity of hair cells caused by potassium accumulation around the cells [18, 19]. However, the molecular mechanism of this allele remains unclear. Our study fully demonstrated that GJB2 p.V37I leads to loss of function of CX26 in both ionic and biochemical coupling, [7, 11, 12], albeit with less severe results than that caused by c.299-300delAT. Similar to a study in Hela cells by Jara et al. [11], we found that cells expressing CX26-p.V37I transferred PI to a small proportion of neighboring cells (25.9 ± 2.94%), indicating a partial deficiency in biochemical hemichannel coupling of GJs comprising this variant. However, the effect of p.V37I on biochemical GJ coupling (average, 9.5%) was less severe in this study than in the study by Jara et al., who found that none of the seven cells tested showed transfer of the PI tracer [11]. Conflicting results were also seen in a study by Kim et al.[12], who reported less severe biochemical GJ coupling (average 31.3% for PI transfer between cells) and worse hemichannel coupling (average 11.2% for PI loading). It is reasonable to speculate that there might be deficiency in the transfer of glucose and small metabolite reactive oxygen species (ROS) within inner ear supporting cells carrying CX26-p.V37I or CX26-c.299-300delAT variant proteins, leading to cellular dysfunction, intoxication, or apoptosis. This extends previous studies on p.V37I [7, 11, 12, 18-20] and supports the
clinical hypothesis that p.V37I, similar to c.299-300delAT, is pathogenic.

The ionic coupling defect of p.V37I has been observed in previous studies, with varying results. In Hela cells, GJ conductance in p.V37I cell pairs was 1.3 ± 0.2 nS, compared with 4.4 ± 0.63 nS in WT cell pairs [11]. In contrast, injection of p.V37I RNAs into *Xenopus* oocytes resulted in almost complete loss of junctional channels (0.013 ± 0.003 nS), compared with 3.646 ± 0.817 nS recorded in WT cells [7]. In this study, we applied the fluorescent Ca²⁺-specific tracer fluo-3 AM to monitor the activity of ionic coupling through CX26-mediated GJs. Cellular Ca²⁺ from Golgi bodies and endoplasmic reticulum is released into the cytoplasm to increase the [Ca²⁺]ᵢ in response to stress. Fluctuation in the [Ca²⁺]ᵢ can be recorded and quantified through the fluorescence intensity of fluo-3. As seen in Fig. 4, after cellular stimulation of cells carrying either CX26-WT or CX26-WT/p.V37I, the [Ca²⁺]ᵢ of adjacent cells increased, indicating normal flow of Ca²⁺. By contrast, weakening or loss of Ca²⁺ influx was observed in cells with expression of CX26-p.V37I (29.4% of cell pairs) or CX26-c.299-300delAT (0% of cell pairs). Taken together, these results suggested the loss of function of ionic coupling in the CX26-p.V37I and CX26-c.299-300delAT variant proteins.

H₂O₂ is often used to simulate environmental stress for research on susceptibility following metabolism of O₂⁻, HO⁻, and OH⁻ [7, 20]. Interestingly, in our study, cells expressing CX26-p.V37I had a higher apoptosis rate than those expressing CX26-WT, even without H₂O₂ (0 µM) exposure. We suspect that this result may be a reflection of the vulnerability of HEK 293T cells to the impaired GJs, exposure to liposomes during transfection, or disposal of cellular debris during culture. The biochemical hemichannel permeability in cells expressing only CX26-p.V37I was significantly impaired when exposure to 40µM to 80µM H₂O₂. The functional defects in GJs containing CX26-p.V37I may lead to failure to effectively discharge ROS and thus apoptosis. This might partly
explain the clinical observation that GJB2 p.V37I carriers may have increased susceptibility to environmental stress [6, 14, 18, 19, 21, 22]. Interestingly, Liu et al. found that heterozygous carriers of GJB2 p.V37I or c.235delC also show increased vulnerability to noise [23]. Ftoni et al. reported a similar result, showing that partial loss of GJB2 (c.35delG) caused accelerated presbycusis, probably due to redox imbalance and dysregulation of the Nfr2 pathway [24]. Nevertheless, further research is necessary to confirm our hypothesis.

This study has potential limitations. Although functional analysis of transfected cells has been widely adopted in studies of hereditary hearing loss [15, 16, 20], the evaluation of GJ couplings in cochlear supporting cells in the animal model would likely provide compelling evidence to support our conclusion. The defect in GJ function at the cellular level was not sufficient to explain the mild to moderate hearing loss observed in some cases [4, 10, 13]. It is possible that additional unknown modifiers, such as CRYL1 rs14236 variant [25] or other GJ subunits encoded by GJB3 and GJB6, could alleviate the damaging outcome of p.V37I in the cochlea. This issue requires further investigation.

In conclusion, our findings showed that two common hearing loss-related variants in GJB2, p.V37I and c.299-300delAT, resulted in deficient GJ-mediated coupling. Environmental factors may impact the functional consequences of GJB2 p.V37I.
REFERENCES


FIGURE LEGENDS

**Fig. 1.** (A-F) Immunofluorescence staining results after various CX26 vectors transfection. Compared with the massive plaques in HEK 293T cells expressing CX26-c.299-300delAT (white arrows in panels E and F) or empty vector (D), intercellular gap junctions (yellow arrows in panels A–C) were observed. (G) The percentages of gap junctions formed in three haplotype were shown; ns: not significant.

**Fig. 2.** Propidium iodide (PI) permeability by gap junctions formed by the four CX26 haplotypes in HEK 293T cells. Asterisks indicate cells that were injected with PI. (A–D) Representative images of cells expressing CX26-WT (A) or CX26-WT/p.V37I (C) showing the detection of PI dye in neighboring cells with gap junctions (green), in contrast to leaky PI transfer capacity in cells expressing CX26-p.V37I (B) or c.299-300delAT (D). (E) Cell count analysis supporting the severe alteration in PI loading CX26-p.V37I cells; *P < 0.01, ns: not significant.

**Fig. 3.** Hemichannel dye loading assay of HEK 293T cells expressing the four connexin 26 (CX26) haplotypes. (A–D) Representative images of cells loaded with the membrane-impermeable fluorescent dye propidium iodide. (E) Quantification of the PI loading rate, showing a significant reduction in cells expressing CX26-p.V37I compared with that in cells expressing CX26-WT; *P < 0.01; ns: not significant.

**Fig. 4.** (A-B) Ionic permeability of gap junctions (GJs) in HEK 293T cells were functional in cells expressing the CX26-WT and CX26-WT/p.V37I haplotypes. (C–D) Impaired ionic GJs permeability were detected in CX26-p.V37I and CX26-c.299-300delAT. (E) The percentages of Ca²⁺ transfer rates were shown; *P < 0.01.
Fig. 5. (A) Representative results showing the morphological changes of HEK 293T with three haplotypes after H\textsubscript{2}O\textsubscript{2} incubation, (B) The tendency of apoptosis in HEK 293T cells expressing CX26-WT and/or CX26-p.V37I, then treated with 20 μM to 80 μM H\textsubscript{2}O\textsubscript{2} in the experiments. (C) Apoptosis rate calculated; (D) The biochemical hemichannel function showed the dynamic changes in cells carrying three vectors. \textasteriskcentered P < 0.05 when compared the values in CX26-p.V37I with the values in CX26-WT or CX26-WT/p.V37I haplotypes. ns: not significant.
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<th>Plasmid</th>
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<tr>
<td>pEGFP-C2</td>
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<td>GFP</td>
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<tr>
<td>pmCherry-C1</td>
<td>Empty vector for plasmid construction, Cellular apoptosis assay (control)</td>
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Fig. 5.