

# Functional Evaluation of GJB2 Variants in Nonsyndromic Hearing Loss

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Mutations in the gap junction  $\beta 2$  (*GJB2*) gene, encoding the connexin26 (CX26) protein, are the most common cause of nonsyndromic hearing loss (HL) in many populations. In the East Asian population, two variants, *p.V27I* (c.79G>A) and *p.E114G* (c.341G>A), are considered benign polymorphisms since these variants have been identified in both HL patients and normal hearing controls. However, some studies have postulated that homozygotes carrying both *p.V27I* and *p.E114G* variants could cause HL. To elucidate possible roles of these variants, we used *in vitro* approaches to directly assess the pathogenicity of four haplotypes generated by the two polymorphisms: VE (wild type), I\*E (*p.V27I* variant only), VG\* (*p.E114G* variant only), I\*G\* (both variants). In biochemical coupling assays, the gap junctions (GJs) composed of VG\* and I\*G\* types displayed defective channel activities compared with those of VE wild types or I\*E types, which showed normal channel activities. Interestingly, the defect in hemichannel activity was a bit less severe in I\*G\* type than VG\* type, suggesting that I\* variant (*p.V27I*) may compensate for the deleterious effect of G\* variant (*p.E114G*) in hemichannel activities. Our population studies using 412 Korean individuals showed that I\*G\* type was detected at around 20% in both HL patients and normal controls, suggesting that I\*G\* type may not be a pathogenic polymorphism. In contrast, VG\* type was very rare (3/824) and detected only in HL patients, suggesting that VG\* homozygotes (VG\*/VG\*) or compound heterozygotes carrying VG\* type with other mutations may cause HL.

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## INTRODUCTION

Hearing loss (HL) is one of the most common sensory disorders, affecting 2–3 in 1,000 newborns. Mutations in the gap junction  $\beta 2$  (*GJB2*) gene encoding the connexin26 (CX26) protein, are responsible for approximately 50% of autosomal recessive nonsyndromic HL in many populations (1,2). To date, more than 150 different variants have been reported in *GJB2* gene (3; <http://davinci.crg.es/deafness/>) and the spectrum and prevalence of the mutations and polymorphisms in *GJB2* gene vary significantly among different ethnic groups.

The most frequent pathogenic mutations, *c.35delG* and *c.167delT*, account for 70% and 40% of *GJB2*-related HL in Caucasians and Ashkenazi Jews, respectively (4–7). In contrast, East Asian HL patients rarely display these mutations, but another single base pair deletion, *c.235delC*, is present in 7% to 12% of this group (8,9). Besides these mutations, *p.V27I*, *p.M34T*, *p.V37I* and *p.E114G* are reported most frequently in these populations, but the pathogenicity of these variants remains controversial (10–12). The *p.M34T* variant first was reported as a pathogenic mutation with a dominant ef-

fect, but this finding was questioned subsequently and the *p.M34T* was suggested to be a recessive mutation (13,14). The *p.V37I* was described originally as a nonpathogenic polymorphism, but recently this variant was proposed to be pathogenic and associated with mild HL (15,16). Recent biochemical, electrophysiological and genetic studies of *p.M34T* and *p.V37I* supported these suggestions (17,18).

The *p.V27I* and *p.E114G* variants are very common, with allele frequencies of 28.3% and 18.3%, respectively, in the East Asian population (19–21). The *p.V27I* variant has been found alone or together with the *p.E114G* variant in East Asian individuals. In contrast, the *p.E114G* variant has been found together with the *p.V27I* variant in most cases, and these variants generally occur in *cis* configuration (22). Each of the *p.V27I* and *p.E114G* variants have been considered benign

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polymorphisms, but when present together in *cis* configuration, homozygous *p.V27I/p.E114G* has been suggested to cause HL (23). However, in other studies, *p.V27I/p.E114G* variants have been considered benign polymorphisms, because *p.V27I/p.E114G* homozygous genotypes have been reported in unaffected individuals (21,24). To resolve these discrepancies, we have investigated the pathogenicity of different haplotypes of the *p.V27I* and *p.E114G* variants using *in vitro* approaches, and assessed the possible genotype-to-phenotype correlation of these variants with HL in the Korean population.

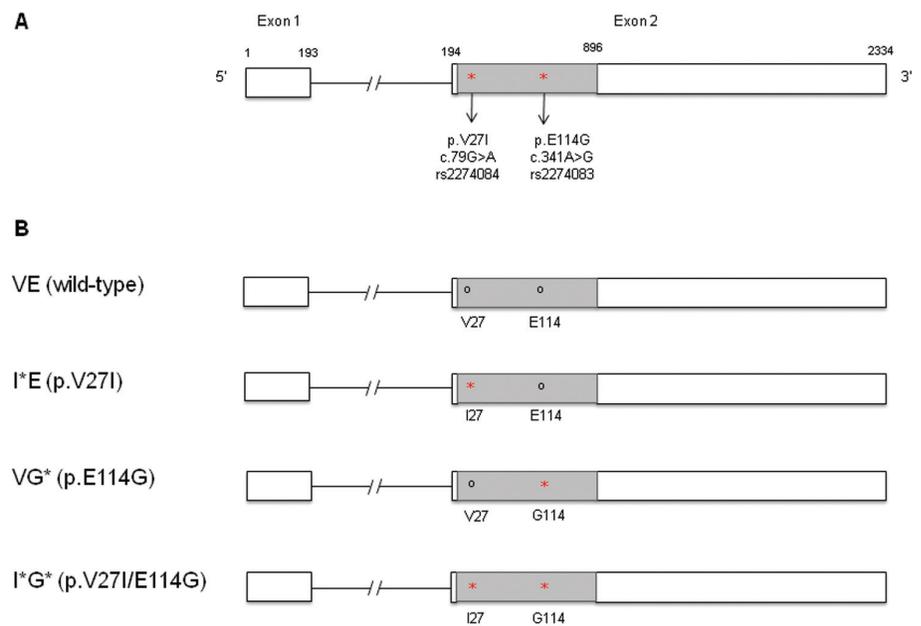
## MATERIALS AND METHODS

### Subjects

A total of 273 subjects with nonsyndromic HL were recruited from the Department of Otolaryngology, Kyungpook National University Hospital and Soree Ear clinic. No evidence of any other clinical symptoms was found, and environmental causes of HL such as ototoxic drugs and infectious diseases were excluded by interviews. One hundred thirty-nine unrelated Koreans with normal hearing were collected for use as controls. Hearing sensitivity was assessed by pure tone audiogram (PTA) in a sound-proof room using a diagnostic audiometer. All participants provided written informed consent according to the protocol approved by the Ethics Committee.

### Molecular Analyses

Genomic DNA of subjects was extracted from peripheral blood using the FlexiGene DNA extract kit (QIAGEN, Hilden, Germany). The *GJB2* gene (GenBank Accession No. NM\_004004.5) coding region was amplified by polymerase chain reaction (PCR) using the following primers: forward, 5'-TCT TTT CCA GAG CAA ACC GC-3'; reverse, 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 57°C, depending on



**Figure 1.** Schematic representation of the *GJB2* gene and the four amino acid haplotypes of *p.V27I* and *p.E114G*. (A) The boxes represent the two numbered exons, and protein coding region are marked in gray. The *p.V27I* and *p.E114G* are marked with an arrow; the first line gives the amino acid substitution, the second line is the position and base substitution starting from the start codon, and the third line is the SNP name (rs-number) from NCBI. (B) The amino acid haplotypes of *p.V27I* and *p.E114G* are shown; the circles indicate wild-type amino acid; and the asterisks indicate amino acid substitution in the positions.

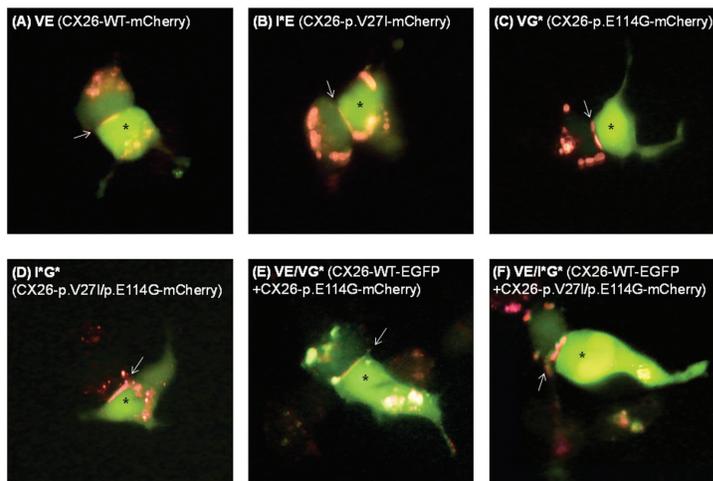
the primers); extension (1 min at 72°C) and a final extension step at 72°C for 10 min. An ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, CA, USA) was used for direct sequencing, and data was analyzed by using ABI sequencing Analysis (v.5.0) and LASER-GENE-SeqMan software. The samples were also tested for the presence of mutations in the coding region of *GJB3*, *GJB4* and *GJB6* gene by the method previously reported by Yang *et al.* (25).

### Construction of CX26 Variants and Transfection of HEK 293 Cells

The single coding region of human CX26 was amplified by PCR from genomic DNA of normal hearing subject was subcloned into the pEGFP-N1 and pmCherry-N1 vectors (Clontech Inc, Mountain View, CA, USA) to create CX26-EGFP and CX26-mCherry fusion proteins. The *p.V27I* and *p.E114G* variants in CX26 were created with the

QuikChange site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA, USA). Flowing forward and reverse primer containing the CX26 point mutation (mutated bases underlined) were synthesized: *p.V27I* F, 5'-GAA AGA TCT GGC TCA CCA TCC TCT TCA TTT TTC GC-3'; R, 5'-GCG AAA AAT GAA GAG GAT GGT GAG CCA GAT CTT TC-3'; *p.E114G* F, 5'-GGA GAT AAA GAG TGG ATT TAA GGA CAT CG-3'; R, 5'-CGA TGT CCT TAA ATC CAC TCT TTA TCT CC-3'. The correct introduction of intended mutations and the absence of unintended additional mutations in the CX26-*p.V27I* and CX26-*p.E114G* vector sequences were confirmed by DNA sequencing.

The human embryonic kidney HEK293 cell line, which is used widely for gap junction (GJ) studies because of its lack of endogenous expression of connexins, was obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were detached, seeded onto glass coverslips, placed in a



**Figure 2.** Lucifer Yellow (LY) permeability by GJ of four haplotypes. Arrows indicate the presence of GJs shown by pmCherry and pEGFP at the cell membrane bordering the two cells. Intercellular coupling was present between cells expressing VE (CX26-WT-mCherry) (A), I\*E (CX26-p.V27I-mCherry) (B), VE and VG\* (CX26-WT-EGFP + CX26-p.E114G-mCherry) (E), and VE and I\*G\* (CX26-WT-EGFP + CX26-p.V27I/p.E114G-mCherry) (F). Cell transfected with VG\* (CX26-p.E114G-mCherry) (C) and I\*G\* (CX26-p.V27I/p.E114G-mCherry) (D) were uncoupled. \* Marks the injected cells in the cell pair tested. These results are monitored 2 min after the injection.

35-mm Petri dish, and cultured to 80% confluence 1 d prior to transfection. The cells were transfected using the Fugene HD transfection kit (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer's instructions.

**Assays for Measuring Biochemical Coupling, Ionic Coupling and Hemichannel Permeability**

GJ biochemical coupling was measured with the single cell dye transfer assay. For dye transfer assays, one of the two cells with GJ was injected by microelectrode filled with a 1% Lucifer Yellow (LY, molecular weight 457; charge 2-) solution in intracellular solution (KCl 120 mmol/L, MgCl<sub>2</sub> 1 mmol/L, HEPES 10 mmol/L; pH 7.4 with NaOH). The LY transfer was recorded 30 s to 3 min after injection through an electrophoresis method based on the technique described Haas *et al.* (26). Ionic coupling assay was performed as described previously (26).

The hemichannel assay used Propidium Iodide (PI, molecular weight 650; charge + 2, Invitrogen) for measurement of the connexon unitary gating properties

with a dye loading assay. Cells were incubated for 40 min at room temperature in PI solution prepared in HBSS containing 10 mmol/L EGTA, 1 mmol/L MgCl<sub>2</sub>, and 0.15 mmol/L PI. The hemichannel assay was performed using cells transfected with different vectors plus the pEGFP vector. Data are expressed as mean and standard deviation. The data were tested for significance by using one-way analysis of variance (ANOVA), and only results with *P* values of < 0.05 were considered statistically significant.

**Statistical Tests**

The SHEsis program (<http://analysis.bio-x.cn/>) was used to calculate and estimate allele frequencies, genotype frequencies, haplotype frequencies, and linkage disequilibrium in controls and patients. The Statistical Package for the Social Sciences (SPSS for Windows, version 12.0K, SPSS Inc., Chicago, IL, USA) was used for analysis of the hemichannel assay data.

*All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).*

**RESULTS**

We performed *in vitro* functional analyses and population study for *p.V27I* and *p.E114G* variants to clarify the possible roles of these variants in HL. This study examined different combinations of alleles of *p.V27I* (substitution of valine for isoleucine in codon 27, c.79G>A) and *p.E114G* (substitution of glutamic acid for glycine in codon 114, c.341A>G). These two alleles at two different sites can comprise four amino acid haplotypes, VE (wild type), I\*E (*p.V27I* variant only), VG\* (*p.E114G* variant only), and I\*G\* (both variants), as shown in Figure 1.

**Protein Localization, Biochemical and Ionic Coupling Test, and Hemichannel Permeability Test**

To examine to which degree haplotypes affected the level of protein expression and their cellular localization, the four vectors with different amino acid haplotypes were transfected transiently in HEK293 cells (Figure 2). The VE form of CX26 was localized at the membrane of HEK 293 cells and forms GJs as seen by the characteristic plaques between two adjacent cells (Figure 2A, arrow). Similarly, the I\*E, VG\*, and I\*G\* were expressed at the membrane region and retained the ability to form normal-appearing GJ plaques (Figure 2B–D, arrows).

To assess the biochemical permeability of the different haplotype GJ channels, we examined the cell-to-cell transfer of the impermeant dye Lucifer Yellow (LY), which was loaded into one of the cells forming the GJs with a patch pipette (see Figure 2). Dye transfer rate was evaluated by observing the amount of dye transferred into the other cells and categorized into three groups; fast transfer (transferred within 1 min), slow transfer (transferred between 1 and 2 min), and no transfer. The *p.D46E* variant of CX26, a well known causative mutation in HL, was used as a negative control (28). This variant displayed highly defective intercellular dye transfer (Table 1). The cells transfected with the VE type showed a high efficiency of LY transfer through GJ channel, 88.9% of the cells examined

**Table 1.** Percentage of the LY dye transfer through GJ channels.

Type	Dye transfer (%)		No transfer (%)
	Fast transfer	Slow transfer	
VE	24 (88.9)	1 (3.7)	2 (7.4)
p.D46E	2 (7.4)	5 (18.5)	20 (74.1)
I*E	23 (88.5)	2 (7.7)	1 (3.8)
VG*	17 (38.6)	21 (47.7)	6 (13.6)
I*G*	25 (44.6)	21 (37.5)	10 (17.9)
VE/VG*	12 (92.3)	1 (7.7)	0 (0.0)
VE/I*G*	15 (93.8)	1 (6.3)	0 (0.0)

showed fast transfer (Table 1). The LY transfer rate of the I\*E type was similar to that of VE type, showing fast transfer in 88.5% of the cells examined. In contrast, the LY transfer through GJ channels composed of VG\* and I\*G\* types was either delayed markedly or failed, accounting for 61.3% (n = 27/44) in the VG\* type and 55.4% (n = 31/56) in the I\*G\* type, respectively. When the assay was carried out with the cells cotransfected with VE and VG\* or VE and I\*G\*, the LY transfer was comparable to that of VE alone (Table 1). These findings indicate that VG\* and I\*G\* type GJs display a moderate deficit in GJ-mediated biochemical coupling on their own, but in combination with the VE form in hybrid GJs, they have no significant effect. We also tested the ability to transfer Ca<sup>2+</sup> ions intercellularly across the GJs in four haplotypes and showed a consistent result with biochemical assay (Supplementary Figure 1).

For hemichannel studies, we bathed cells in normal HBSS which contained 1.2 mmol/L [Ca<sup>2+</sup>]<sub>o</sub>, which is known to keep VE type hemichannel in the closed state (29). It is also known that Cx hemichannels are opened by lowering the extracellular Ca<sup>2+</sup> concentration or by omitting Ca<sup>2+</sup> altogether (30). For our hemichannel functional studies, we used Ca<sup>2+</sup>-free HBSS containing 0.15 mmol/L PI dye. In most of the cells transfected with VE or I\*E types, the entry of the PI dye through hemichannels was opened by free calcium (92.3%, n = 1290 and 90.0%, n = 1079, respectively) (Figure 3A, Supplemental Figure 2A, B). In contrast, low percentages of the cells transfected with VG\* or I\*G\* (12.4%, n = 889 and

34.5%, n = 997, respectively) were loaded with the PI dye (see Figure 3A and Supplemental Figure 2C, D).

We then cotransfected the VE with one of the I\*E, VG\* or I\*G\* types, and examined the effect on the PI dye transfer (Figure 3B and Supplemental Figure 2E–G). Cotransfection of VE/I\*E (93.7%) showed similar PI loading efficiency to that of VE (92.3%) or I\*E (90.0%) alone. However, cotransfection of VE/VG\* (43.0%) or VE/I\*G\* (62.1%) displayed lower PI loading efficiencies compared with that of VE alone, but higher efficiencies compared with those of VG\* (12.4%) or I\*G\* (34.5%) alone. Interestingly, the PI loaded through hemichannel of I\*G\* type was more than that loaded through cells transfected with the VG\* type (VG\* versus I\*G\*), and a similar pattern was observed when cells were cotransfected with VE (VE/VG\* versus

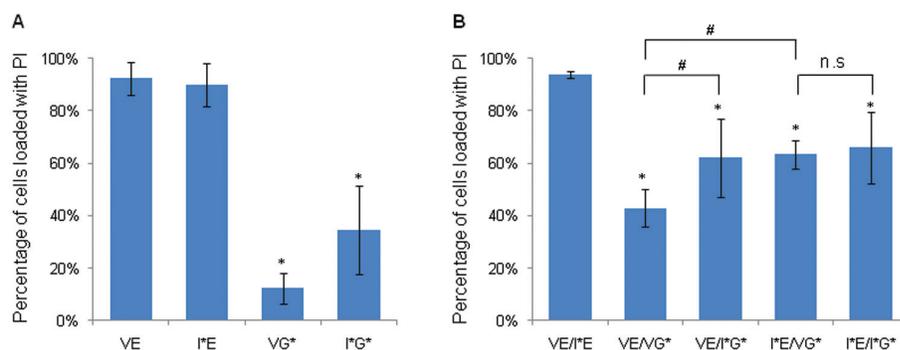
VE/I\*G\*). We also tested hemichannel activities in cells expressing I\*E types together with VG\* or I\*G\* types (Figure 3B and Supplemental Figure 2H, I). The I\*E/VG\* type displayed a greater percentage of cells with PI dye loading than VE/VG\* type (63.4% and 43.0%, *P* < 0.05), but no significant difference with I\*E/I\*G\* type (63.4% and 65.9% respectively). This result suggests that isoleucine residue of *p.V27I* exerts a much milder effect on hemichannel function than valine residue of *p.V27I* in coexistence with glutamic acid of *p.E114G*.

These results suggest that the VG\* type greatly reduces the hemichannel activity, but when complexed with the VE or I\*E types, their hemichannel permeabilities were improved, resulting in higher PI loading efficiencies (*P* < 0.05, one-way ANOVA test).

### Population Study

We evaluated the presence of two variants of the *GJB2* gene (GenBank Accession No. NM\_004004.5), *p.V27I* (rs2274084) and *p.E114G* (rs2274083), in 273 Korean nonsyndromic HL patients and 139 normal hearing controls. Patients carrying any other mutations in the *GJB2*, *GJB3*, *GJB4* and *GJB6* genes were excluded by sequencing analysis.

Table 2 shows allele and genotype frequency distributions of each variant. The



**Figure 3.** Percentage of transfected HEK293 cells loaded with PI through hemichannels. Percentage of transfected cells with VE, I\*E, VG\*, and I\*G\* types (A) and cotransfection of I\*E, VG\*, and I\*G\* with the VE and I\*E, respectively (B). Each bar represents the mean ( $\pm$  SEM). In the image, the asterisk (\*) indicates statistically different to hemichannel loaded compared to VE type; the number sign (#), statistically different in between VE/VG\* versus VE/I\*G\* and VE/VG\* versus I\*E/VG\*.

distributions of genotypes and alleles of *p.V27I* and *p.E114G* were all in Hardy-Weinberg equilibrium in both patients and controls. These variants displayed no significant difference in frequencies of both alleles ( $P = 0.501$  and  $P = 0.331$ ) and genotypes ( $P = 0.508$  and  $P = 0.330$ ) between patients and controls. To evaluate the extent of linkage disequilibrium (LD),  $D'$  values between *p.V27I* and *p.E114G* were calculated. The  $D'$  value between these two variants was 0.966, demonstrating that there is strong linkage disequilibrium between them. No significant difference in haplotype frequencies existed between patients and controls (Table 3).

**DISCUSSION**

Mutations in the *GJB2* gene are the major cause of nonsyndromic HL in many populations (31–33). The *p.V27I* and *p.E114G* variants in *GJB2*, which occur frequently in East Asian populations, have been reported as either polymorphisms or mutations (10,23,34,35). In this study, using *in vitro* approaches and a population study, we assessed the pathogenicities of the *p.V27I* and *p.E114G* variants in channel function and HL.

The biochemical permeability conferred by I\*E type channel was similar to that of wild type (VE), but CX26 carrying the VG\* or I\*G\* haplotypes showed delay or failure of the transfer of dye. However, the VG\* and I\*G\* forms of CX26 in combination with the VE or I\*E forms in hybrid GJs did not affect GJ-mediated biochemical coupling (see Figure 2, Table 1). In our hemichannel study, there was no significant difference between VE, I\*E and VE/I\*E, while VG\*, I\*G\*, VE/VG\*, VE/I\*G\*, I\*E/VG\* and I\*E/I\*G\* all displayed a significant difference in dye transfer compared with that mediated by the VE type. In all types, the hemichannels composed of only VG\* type had the most defective hemichannel activity, and the I\*G\* type displayed the next one (Figure 3 and Supplemental Figure 1). Interestingly, the hemichannels composed of *p.E114G* with *p.V27I* have milder effect than that hemichannel composed of *p.E114G* with

**Table 2.** Allele and genotype frequencies (%) of *p.V27I* and *p.E114G* variants in NSHL patients and normal hearing controls.

Variables	Amino acid	Controls (%)	NSHL patients (%)	P value	Odds ratio (95% CI)
<i>rs2274084 (p.V27I)</i>					
Allele					
C	V	166 (59.7)	339 (62.1)	0.508	1.105 (0.822~1.484)
T	I	112 (40.3)	207 (37.9)		
Genotypes					
CC	VV	55 (39.6)	109 (39.9)	0.501	-
CT	VI	56 (40.3)	121 (44.3)		
TT	II	28 (20.1)	43 (15.8)		
<i>rs2274083(p.E114G)</i>					
Allele					
T	E	226 (81.3)	428 (78.4)	0.330	0.835 (0.580~1.201)
C	G	52 (18.7)	118 (21.6)		
Genotypes					
TT	EE	96 (69.1)	171 (62.6)	0.331	-
TC	EG	34 (24.5)	86 (31.5)		
CC	GG	9 (6.5)	16 (5.9)		

<sup>o</sup>CI: confidence interval.

*p.V27V* (I\*G\* versus VG\*, I\*E/VG\* versus VE/VG\*). These results suggest that *p.V27I* may be capable of compensating for the loss of hemichannel activity. The results of our *in vitro* cell-based assays indicate that the VG\* and I\*G\* haplotypes of *GJB2* may play a role as pathogenic variants in hereditary hearing impairment.

However, in our study of the hearing impaired Korean population, we did not observe a significant difference in the frequencies of these four variant haplotypes between patients and controls. There are several possibilities regarding this discrepancy.

First, the *in vitro* function of the CX26 GJ channels observed in cell lines can be different from their *in vivo* function. In our *in vitro* assays, the VG\* and I\*G\* type channels displayed reduced channel ac-

tivities, but did not entirely lose their function, as shown in previously reported *p.D46E* mutation (Table 1 and see Figure 3) (28). Thus, it can be postulated that the VG\* and I\*G\* types also can cause mild defects in channel function *in vivo*. It has been reported that other connexins coexpressed with CX26 in the cochlea, such as Cx30 and Cx31, can compensate for defective function of other connexins (27,36,37). Therefore, it is possible that other connexins may complement the defect in channel function caused by the VG\* and I\*G\* variants.

Second, the VG\* type may be associated with HL, but the I\*G\* type may represent a benign polymorphism. In our functional study, the VG\* type displayed the most serious defect in channel activity among the four haplotypes, while the I\*G\* type led to a milder defect than VG\*

**Table 3.** Haplotype frequencies of *p.V27I* and *p.E114G* in NSHL patients and normal hearing controls.

Haplotypes	Controls (%)	Patients (%)	P value	Odds ratio (95% CI)
VE	166 (59.7)	336 (61.5)	0.554	1.093 (0.814~1.470)
I*E	60 (21.6)	92 (16.8)	0.115	0.747 (0.520~1.074)
VG*	0 (0.0)	3 (0.5)	-	-
I*G*	52 (18.7)	115 (21.1)	0.420	1.162 (0.807~1.675)

<sup>o</sup>CI: confidence interval.

type. The I\*G\* type is detected in both patients and controls at similar frequencies in Korean population, suggesting that the I\*G\* type represents a nonpathogenic polymorphism. Although the VG\* type was detected in heterozygous form (VE/VG\*) in three HL patients and not in normal hearing controls, such difference was not significant statistically because of the small number of the patients carrying this type. Nevertheless, we think, based on our results, that the VG\* type is associated with HL, which requires future studies. Recently, the VG\* type was detected in both patients and controls in the Chinese population, but the frequency of the VG\* type was much higher in patients than that in controls, which also supports the view that the VG\* type is associated with hearing impairment (38). Moreover, the genetic study of *GJB2* mutation by Dai *et al.* (2010) only found two patients with c.155\_c.158delTCTG and VG\* and one patient with c.235delC and VG\* out of 2,063 Chinese patients with nonsyndromic hearing impairment. These observations support the view that the VG\* type is more strongly associated with hearing loss (39).

The association of the *p.V37I* and *p.M34T* variants with HL has been similarly controversial, because these variants were detected in both patients and controls (40–42). However, in *in vitro* functional studies, including electrophysiological analysis in *Xenopus* oocytes or biochemical coupling analysis, these variants showed total loss of function phenotype (43–45). Other studies also have shown that these variants are associated with a mild-to-severe form of HL when they exist as homozygotes or compound heterozygotes with other mutations, such as c.35delG, c.167delT or c.235delC (12,46–48). Therefore, as with the results from our *p.V37I* and *p.M34T* studies, we speculate that the VG\* type is associated with a mild-to-severe form of HL, which depends on the mutations segregating in the second allele at this locus.

In summary, our cell-based functional assays and population studies of the four

haplotypes generated by the *p.V27I* and *p.E114G* variants in *GJB2*, indicate that the *p.V27I* variant may compensate the defective channel activities when it coexists with *p.E114G* type. Although our *in vitro* experiments of I\*G\* type showed a defect in channel activity, the similar frequencies of I\*G\* haplotype in patients and controls suggest that the I\*G\* variant is not associated with HL. The data from Korean (current study) and Chinese populations (38) suggest that one copy of the VG\* form with VE type (VE/VG\*) may be nonpathogenic, but the VG\* homozygous form (VG\*/VG\*) or compound heterozygote carrying the VG\* type with other mutations may be associated with HL. To clarify the exact role of the VG\* type in HL, careful follow-up genetic studies and clinical evaluation are needed.

#### ACKNOWLEDGMENTS

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#### DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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