

Functional Evaluation of *GJB2* Variants in Nonsyndromic Hearing Loss

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ABSTRACT

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 to 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 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.

Keywords: hearing loss, connexin26, p.V27I, p.E114G, polymorphism, function, gap junction, hemichannel

INTRODUCTION

Hearing loss (HL) is one of the most common sensory disorders, affecting 2~3 in 1000 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 (<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 (3-6). In contrast, East Asian HL patients rarely display these mutations, but another single base pair deletion, c.235delC, is present in 7-12% of this group (7-8). Besides these mutations, p.V27I, p.M34T, p.V37I and p.E114G are most frequently reported in these populations, but the pathogenicity of these variants remains controversial (9-11). The p.M34T variant was first reported as a pathogenic mutation with a dominant effect, but this finding was subsequently questioned and the p.M34T was suggested to be a recessive mutation (12-13). The p.V37I was originally described as a non-pathogenic polymorphism, but recently this variant was proposed to be pathogenic and associated with mild HL (14-15). Recent biochemical, electrophysiological and genetic studies of p.M34T and p.V37I supported these suggestions (16-17).

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 (18-20). 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 (21). Each of the p.V27I and p.E114G variants have been considered benign polymorphisms, but when present together in *cis*-configuration,

homozygous p.V27I/p.E114G has been suggested to cause HL (22). 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 (20, 23). 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-phenotype correlation of these variants with HL in the Korean population.

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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 soundproof 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 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 LASERGENE-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 which was previously reported by Yang *et al* (24).

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). 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 widely used for 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 cover-slips, placed in a 35 mm Petri dish, and cultured to 80% confluence one day 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 cells with GJ was patch-clamp using micro electrode filled with a

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1% Lucifer Yellow (LY, molecular weight 457; charge 2-) solution in intracellular solution (KCl 120 mM, MgCl₂ 1 mM, HEPES 10 mM; pH 7.4 with NaOH). The LY transfer was recorded 30 sec - 3 mins after injection through an electrophoresis method based on the technique described Haas *et al* (25). **Ionic coupling assay was performed as previously described (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 minutes at room temperature in PI solution prepared in HBSS containing 10 mM EGTA, 1 mM MgCl₂, and 0.15 mM 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 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, haplotypes 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.

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

In order 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 transiently transfected in HEK293 cells (Fig.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 (Fig.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 (Fig.2B, C, 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 (Fig.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 minute), slow transfer (transferred between 1 and 2 minutes), and no transfer. The p.D46E variant of CX26, a well known causative mutation in HL, was used as a negative control (27). 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 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 markedly delayed 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 co-transfected 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 performed the ability to transfer Ca²⁺ 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 [Ca²⁺]_o, which is known to keep VE type hemichannel in the closed state (28). It is also known that Cx hemichannels are opened by lowering the extracellular Ca²⁺ concentration or by omitting Ca²⁺ altogether (29). For our hemichannel functional studies, we used Ca²⁺ free HBSS containing 0.15 mM 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) (Fig.3A and supplemental Fig.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 (Fig.3A and supplemental Fig.2C, D).

We then co-transfected the VE with one of the I*E, VG*, or I*G* type, and examined the effect on the PI dye transfer (Fig.3B and supplemental Fig.2E, F, G). Co-transfection of VE/I*E (93.7%) showed similar PI loading efficiency to that of VE (92.3%) or I*E (90.0%)

alone. However, co-transfection of VE/VG* (43.0%) or VE/I*G* (62.1%) displayed lower PI loading efficiencies compared to that of VE alone, but higher efficiencies compared to those of VG* (12.4%) or I*G* (34.5%) alone. Interestingly, the PI loaded through hemichannel of I*G* type was more than loaded through cells transfected with the VG* type (VG* versus I*G*) and a similar pattern was observed when cells were co-transfected 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 (Fig.3B and supplemental Fig.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 co-existence 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 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 4).

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DISCUSSION

Mutations in the *GJB2* gene are the major cause of nonsyndromic HL in many populations (30-32). The p.V27I and p.E114G variants in *GJB2*, which occur frequently in East Asian populations, have been reported as either polymorphisms or mutations (9, 22, 33-34). 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 (Fig. 2 and Table 1). In our hemichannel study, there were 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 to 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 (Fig. 3 and supplemental Fig.1). Interestingly, the hemichannels composed of p.E114G with p.V27I have milder effect than that hemichannel composed of p.E114G with 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 4 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 activities, but did not entirely lose their function, as shown in previously reported p.D46E mutation (Table 1 and Fig.3) (27). Thus, it can be postulated that the VG* and I*G* types can also cause mild defects in channel function *in vivo*. It has been reported that other connexins co-expressed with CX26 in the cochlea, such as Cx30 and Cx31, can compensate for defective function of other connexins (26, 35-36). 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* 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 non-pathogenic 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 statistically significant 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 (37). 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 2063 Chinese patients with nonsyndromic hearing impairment. These observations supports the view that the VG* type is more strongly associated with hearing loss (38).

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 (39-41). However, in *in vitro* functional studies, including electrophysiological analysis in *Xenopus* oocytes or biochemical coupling analysis, these variants showed total loss of function phenotype (42-44). Other studies have also 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 (11, 45-47). 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 co-exist 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 (37) suggest that one copy of the VG* form with VE type (VE/VG*) may be non-pathogenic, 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.

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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 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 red asterisks indicate amino acid substitution in the positions.

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. Asterisks mark the injected cells in the cell pair tested. These results are monitored 2 min after the injection.

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 co-transfection of I*E, VG*, and I*G* with the VE and I*E, respectively (B). Each bar represents the mean (\pm S.E.M.). * Indicates statistically difference to hemichannel loaded compare to VE type. # Indicates statistically difference in between the two types.

Supplemental Figure 1. Ionic permeability by GJ of four haplotypes. The fura-2 signals show mechanically elicited intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) response in the stimulated cell (#1) and its spread to GJ-coupled cells (#2). The untransfected cell (#3) is used as a control cell. (A) In the VE type, the $[\text{Ca}^{2+}]_i$ was consistently followed by the intercellular spreading of a calcium wave (n=10/10). (B) The I*E type was similar to that of VE type, showing normal $[\text{Ca}^{2+}]_i$ transfer (n=11/11). (C) The VG* type exhibited delayed and weak transfer $[\text{Ca}^{2+}]_i$ from the cell to neighboring cell via the GJ (n=15/21), in other cases, normal transfer (n=5/21) and no transfer (n=1/21). (D) The I*G* type showed delayed and weak transfer in most cases (n=15/24), in a few cases, either normal transfer (n=6/24) or no transfer (n=3/24). The calcium permeability of cells co-transfected with VE and VG* (n=7) or VE and I*G*(n=6) was similar to that of the cells transfected with VE type (data not shown)

Supplemental Figure 2. Hemichannel dye loading assay with different types of haplotype. Loading of membrane impermeable fluorescent dye 0.15 mmol/L PI in Ca^{2+} -free HBSS. (A) VE, (B) I*E, (C) VG*, (D) I*G*, (E) VE/I*E, (F) VE/VG*, (G) VE/I*G*, (H) I*E/VG*, and (I) I*E/I*G*

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)

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

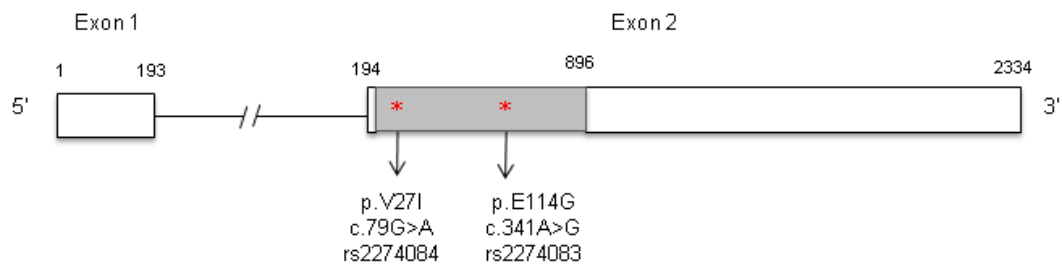
Variables	Amino acid	Controls (%)	NSHL patients (%)	<i>p</i> -value	Odds ratio [95% CI]
rs2274084 (p.V27I)					
Allele					
C	V	166 (59.7)	339 (62.1)	0.508	1.105 [0.822~1.484]
T	I	112 (40.3)	207 (37.9)		
Genotypes					
CC	VV	55 (39.6)	109 (39.9)	0.501	-
CT	VI	56 (40.3)	121 (44.3)		
TT	II	28 (20.1)	43 (15.8)		
rs2274083 (p.E114G)					
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)		

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

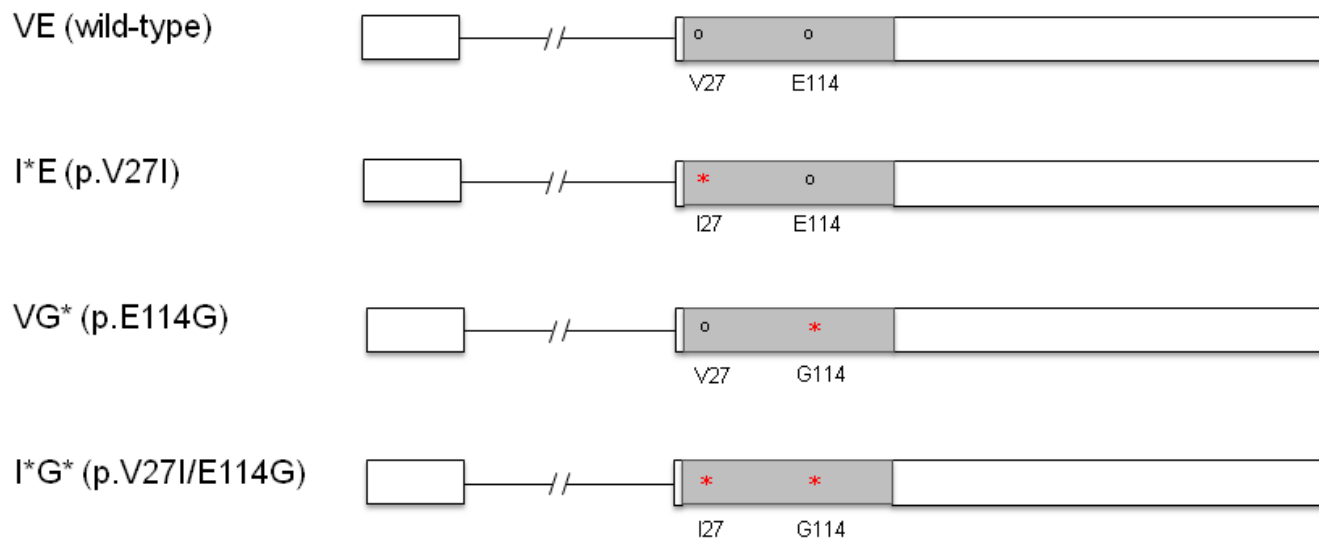
Haplotypes	Controls (%)	Patients (%)	<i>p</i> -value	Odds ratio [95% CI]
VE	166 (59.7)	336 (61.5)	0.554	1.093 [0.814~1.470]
I*E	60 (21.6)	92 (16.8)	0.115	0.747 [0.520~1.074]
VG*	0 (0.0)	3 (0.5)	-	-
I*G*	52 (18.7)	115 (21.1)	0.420	1.162 [0.807~1.675]

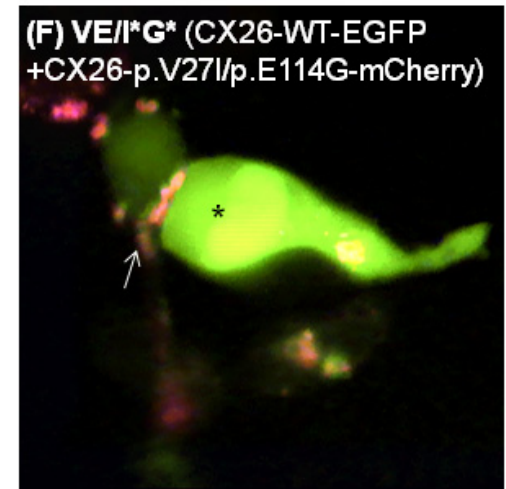
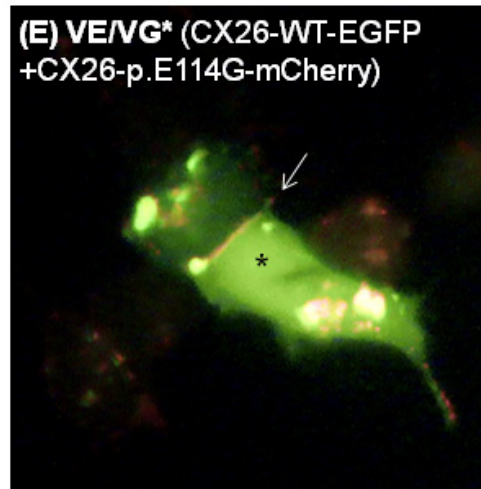
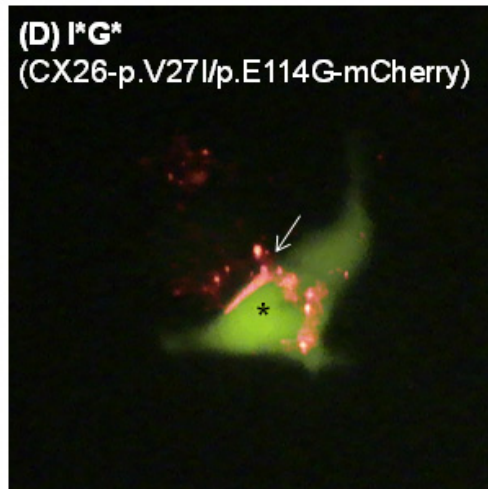
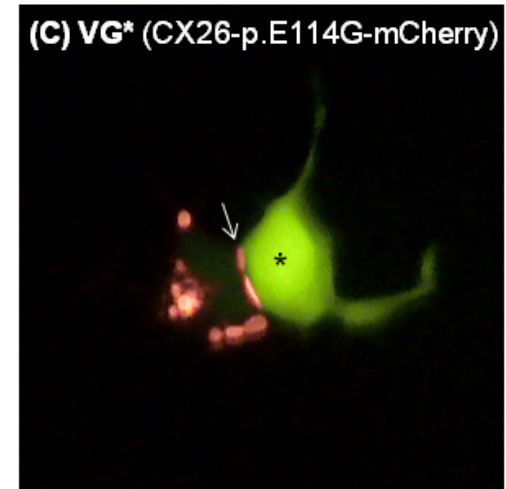
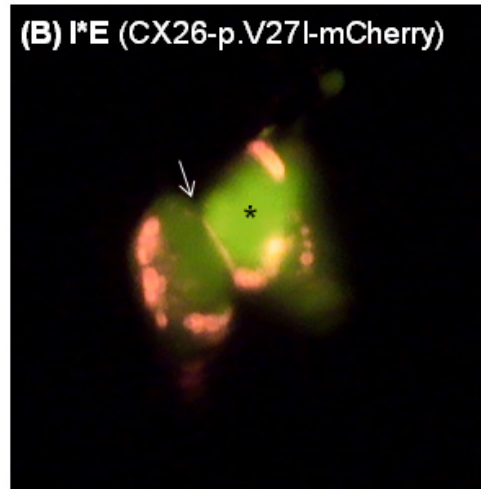
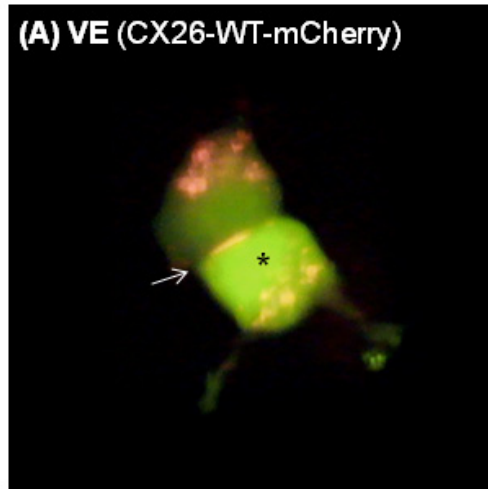
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(A)

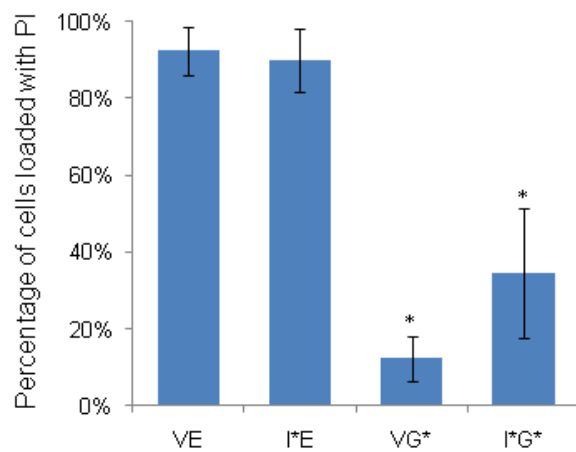


(B)





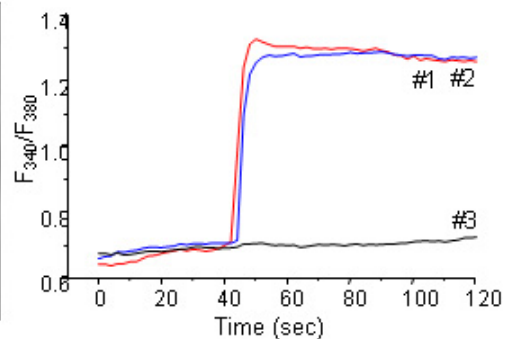
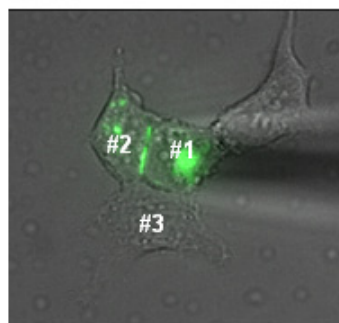
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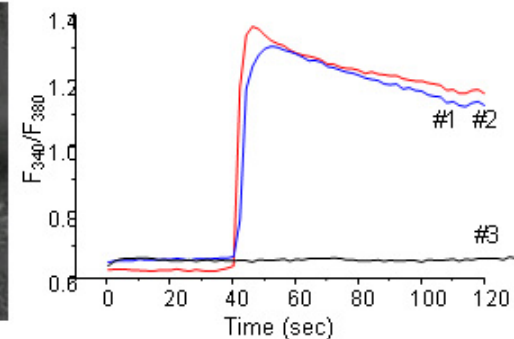
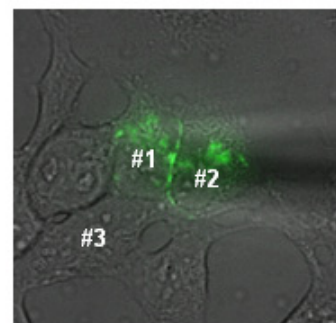
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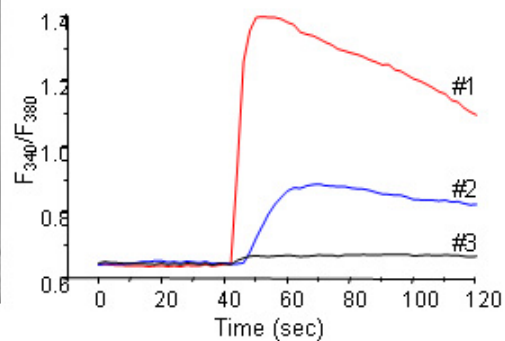
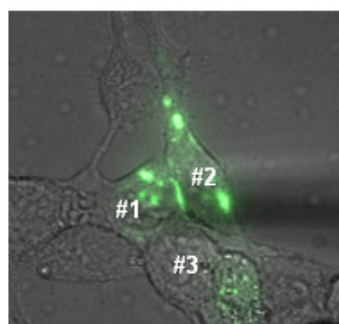
(A) VE



(B) I*E



(C) VG*



(B) I*G*

