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ORIGINAL ARTICLE

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Clinical features of patients with *GJB2* (connexin 26) mutations: severity of hearing loss is correlated with genotypes and protein expression patterns

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Abstract Mutations in the *GJB2* (connexin 26, Cx26) gene are the major cause of nonsyndromic hearing impairment in many populations. Genetic testing offers opportunities to determine the cause of deafness and predict the course of hearing, enabling the prognostication of language development. In the current study, we compared severity of hearing impairment in 60 patients associated with biallelic *GJB2* mutations and assessed the correlation of genotypes and phenotypes. Within a spectrum of *GJB2* mutations found in the Japanese population, the phenotype of the most prevalent mutation, 235delC, was found to show more severe hearing impairment than that of V37I, which is the second most frequent mutation. The results of the present study, taken together with phenotypes caused by other types of mutations, support the general rule that phenotypes caused by the truncating *GJB2* mutations are more severe than those caused by missense mutations. The present in vitro study further confirmed that differences in phenotypes could be explained by the protein expression pattern.

Keywords Connexin 26 · *GJB2* · 235delC · V37I · Deafness · Phenotype · Genotype

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Introduction

Mutations of the *GJB2* (connexin 26, Cx26) gene have recently drawn much attention because they have been recognized as the most prevalent genetic cause of congenital hearing loss. A broad range of phenotypes, from mild to profound hearing loss, is associated with *GJB2* mutations (Cryns et al. 2004), and more than 90 different *GJB2* mutations are associated with recessive forms of nonsyndromic hearing loss (The Connexins-deafness Homepage: http://www.crg.es/deafness). Universal neonatal hearing screening programs are the current trend and have become popular in many countries (Govaerts et al. 2001; Joint Committee on Infant Hearing 2000; Mehl and Thomson 2002; National Institutes of Health 1993), because it is thought that optimum language development requires early identification of hearing loss and early intervention (Yoshinaga-Itano et al. 1998). Cochlear implantation has resulted in remarkable

improvement in auditory skills and development of speech production for patients with profound hearing loss associated with *GJB2* mutations (Fukushima et al. 2002; Matsushiro et al. 2002). It is clear that genetic testing to determine the cause of deafness facilitates prediction of the course of hearing loss and prognostication of language development. There is, however, some controversy regarding genotype/phenotype correlation (Cohn et al. 1999; Cryns et al. 2004; Denoyelle et al. 1999; Estivill et al. 1998; Murgia et al. 1999; Orzan et al. 1999). For example, prediction of the degree of hearing loss was difficult, and environmental factors as well as modifier genes may have been involved (Cohn et al. 1999; Murgia et al. 1999; Orzan et al. 1999). On the other hand, a series of reports have indicated that certain phenotypes are dependent on certain genotypes (Denoyelle et al. 1999; Estivill et al. 1998). A recent report of a multi-center-based study in Europe and the United States suggested that inactivating mutations, which include stop or frameshift mutations, show significantly severer phenotypes than those caused by noninactivating mutations (missense mutations) (Cryns et al. 2004).

We have recently shown that mutation spectrums are quite different between the Japanese population and populations with European ancestry and emphasized the importance of specific population-based genetic databases for genetic testing (Ohtsuka et al. 2003). In Japanese (who are one example of Asian populations), the most common mutation was an inactivating mutation, 235delC, which is comparative to the 35delG mutation known as the most prevalent mutation in those with European ancestry. Interestingly, the second most common mutation was the V37I mutation, which has recently been reported as a mild phenotype causative genotype (Cryns et al. 2004). Given this background, we attempted to: (1) compare the differences in phenotypes caused by the 235delC and V37I mutations, (2) test a hypothetical general rule that inactivating mutations show more severe phenotypes than those caused by noninactivating mutations, and (3) test whether the differences in phenotype could be explained by protein expression study.

Materials and methods

Subjects and clinical evaluation

Pure-tone audiometry results were available for 60 individuals from independent families in whom biallelic *GJB2* mutations were identified. These patients were from seven university hospitals (Hirosaki, Iwate, Gunma, Shinshu, Kokusai Iryoufukushi, Hamamatsu, and Kyushu) located in different regions in Japan. The age when the patients/parents noticed hearing impairment was from 0 to 49 (mean 8.00, SD 12.51) years of age. None of these patients had any other associated neurological signs. All subjects gave prior informed consent for participation in the project, which was approved by the ethical committee of each hospital.

Severity was classified by using a pure-tone average over 500, 1,000, 2,000, and 4,000 Hz in the better-hearing ear. Hearing impairment was classified as follows: normal hearing, <20 dB; mild hearing loss, 21–40 dB; moderate hearing loss, 41–70 dB; severe hearing loss, 71–95 dB; and profound hearing loss, greater than 95 dB. When the threshold exceeded the output limits of the audiometer, it was recorded as the output limit for air-conducted sounds plus 10 dBHL; i.e., if the output limit of the audiometer was120 dBHL, the threshold was described as 130 dBHL.

Mutation Analysis

To identify *GJB2* mutations, a DNA fragment containing the entire coding region was amplified using the primer pair Cx48U/Cx1040L, as described elsewhere (Abe et al. 2000). Polymerase chain reaction (PCR) products were sequenced and analyzed with an ABI sequencer 377XL (Perkin-Elmer, Wellesley, MA, USA). DNA samples from 147 unrelated Japanese who had normal hearing were used as controls.

Reverse transcription (RT)-PCR analysis

Total RNA was extracted from NCTC2544 cells with the Catrimox-14 RNA Isolation Kit Ver.2.11 (Iowa Biotechnology, Urbandale, IA, USA). The yield of total RNA was determined by Agilent 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). Reverse transcription (RT)-PCR assay was performed with the aid of an RNA PCR kit (Takara, Tokyo, Japan). The primers for human *GJB2* and the specific sites of restriction enzymes were added with the amplification step. The primers were sense *Xho* I-Cx26 5′-cccctcgaggatggattggggcacgctgcagacgatcctggg-3′ and antisense Cx26-*EcoR* I 5′-cccgaattcgttaaactggctttttgacttcccagaac-3′. These primers yield oligomer products of a distinctive size: 712 bp. PCR steps were denaturing at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then processing with a final extension at 72°C for 5 min. After amplification, expected sizes of PCR products were confirmed on 2% agarose gel, and the bands were visualized by ethidium bromide upon exposure to an ultraviolet transilluminator.

Transformation

Wild-type Cx26 PCR products were inserted into a pEGFP-C2 vector (Clontech, Palo Alto, CA, USA). The PCR products and vector were digested with *EcoR* I and *Xho* I. Prepared PCR products were inserted into vector. Ligation reactants were transformed into Escherichia coli DH5α. Positive colonies were incubated in Luria-Bertani (LB) liquid medium containing kanamycin. A QIAprep spin miniprep kit (Qiagen, Valencia, CA,

USA) was used for purification of plasmid DNA according to the manufacturer's protocol. Plasmid DNA was identified by restriction enzyme analysis. Selected constructs were sequenced and analyzed with an ABI sequencer 377XL (Perkin-Elmer).

Mutagenesis of the *GJB2* gene

The following primers were used to produce the *GJB2* mutations: V27I sense 5′-ctggctcaccatcctcttcatt-3′, V37I sense 5′-tatgatcctcattgtggctgcaa-3′, and 235delC sense 5′-ccggctatgggcctgcagctgatct-3′. First, PCR reactions (100 µl) were prepared containing 4 µg of the plasmid DNA (see above), 1.0 µM of mutation primer, 1.0 µM of Cx26-*EcoR* I primer, 2.5 U of Takara Ex taq Hot Start Version (Takara, Tokyo, Japan), and Ex-taq buffer (10x) consisting of 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2, and 1 mM deoxynucleoside triphosphate mixture. These PCR reactions were denatured at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then processed with a final extension at 72°C for 5 min. Second PCR reactions (100 µl) were prepared containing 10 µl of first PCR products, 1.0 µM of *Xho* I-Cx26 primer, 2.5 U of Takara Ex taq Hot Start Version (Takara), and Ex-taq buffer (10x) consisting of 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2, and 1 mM deoxynucleoside triphosphate mixture. Second PCR conditions were the same as above. These PCR products were inserted into a pEGFP-C2 vector with the same techniques as transformation (see above). The plasmid DNA containing Cx26 mutations were sequenced and analyzed with a sequencer and identified by restriction enzyme analysis.

Transfection and visualization

COS-7 cells grown on glass cover slips were transfected with the cloned plasmid vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after the transfection, cells were fixed by 4% formaldehyde and stained by DAPI and TRITC-conjugated phalloidin (Chemicon, Temecula, CA, USA). Cover slips were mounted onto glass slides and visualized under a Leica confocal microscope TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany).

Results

Mutation spectrums

Among thirteen mutations that have been reported in Japanese (Ohtsuka et al. 2003), 11 were identified in our 60 biallelic patients. These biallelic mutations were found to be either four different homozygous or 14 different compound heterozygous mutations. These included five inactivating mutations and six missense mutations. The five inactivating mutations were one stop mutation (Y136X), three deletion frameshift mutations (235delC, 176-191del16, 299-300delAT), and one insertion frameshift mutation (605ins46). The six missense mutations were V37I (109G → A), G45E (134G → A), T86R (257C → G), T123N (368C → A), R143W (427C → T), and F191L (570T → C). T123N and F191L were categorized as changes with unknown relation to disease (The Connexin-deafness Homepage: http://www.crg.es/deafness); however, we included both mutations as missense mutations in the present report because both were found among the hearing-loss patients in either a homozygous or compound heterozygous state. The nonsense mutation, Y136X (408C → A), converts a tyrosine residue (TAC) at codon 136 to a stop codon (TAA). Three deletion frameshift mutations, 235delC, 176-191del16, and 299-300delAT, and one insertion frameshift mutation, 605ins46, were found. The 235delC mutation causes a frameshift at codon 79 resulting in a truncated polypeptide and was found in two of the 147 controls (294 alleles). The 176-191del16 mutation, present in four subjects, causes a frameshift leading to an altered amino-acid sequence from codon 59 followed by a stop at codon 76. The 299-300delAT deletion, seen in two subjects, causes a frameshift leading to an altered amino-acid sequence from codon 100 followed by a stop at codon 113. The 605ins46 mutation has a tandem repeat of 46 nucleotides (corresponding to the positions 559–604 of the Cx26 DNA sequence) at the position 605. A stop codon (TGA) is produced at the 202nd amino acid, leading to the premature truncation in the series of polypeptide synthesis. Three previously described common sequence changes, V27I (79G → A), E114G (341A → G), and I203T (608T → C), which were thought to be nonpathological polymorphic changes (Abe et al. 2000), were frequently found in patients as well as controls.

Audiometric evaluation of the patients with biallelic *GJB2* mutations

Audiometric results were obtained from 60 patients with biallelic *GJB2* mutations. Fig. 1 shows a collection of overlapping audiograms from subjects bearing 18 combinations of mutations. Although the severity of hearing impairment in individuals varied according to the combinations of mutations, there seemed to be certain phenotypes determined by each combination. First, the hearing levels of the patients homozygous for 235delC mutations were comparatively severe to profound (Fig. 1). In addition, 235delC/299-300ATdel, G45E/G45E/Y136X/Y136X, G45E/Y136X/R143W, 235delC/R143W, and R143W/T86R also showed severe hearing impairment. In contrast, the patients homozygous for V37I had significantly mild-to-moderate hearing impairment (Fig. 1). Similarly, relatively milder phenotypes were found in the patients with 235delC/V37I, V37I/R143W, F191L/F191L, T123N/176-191del16, and

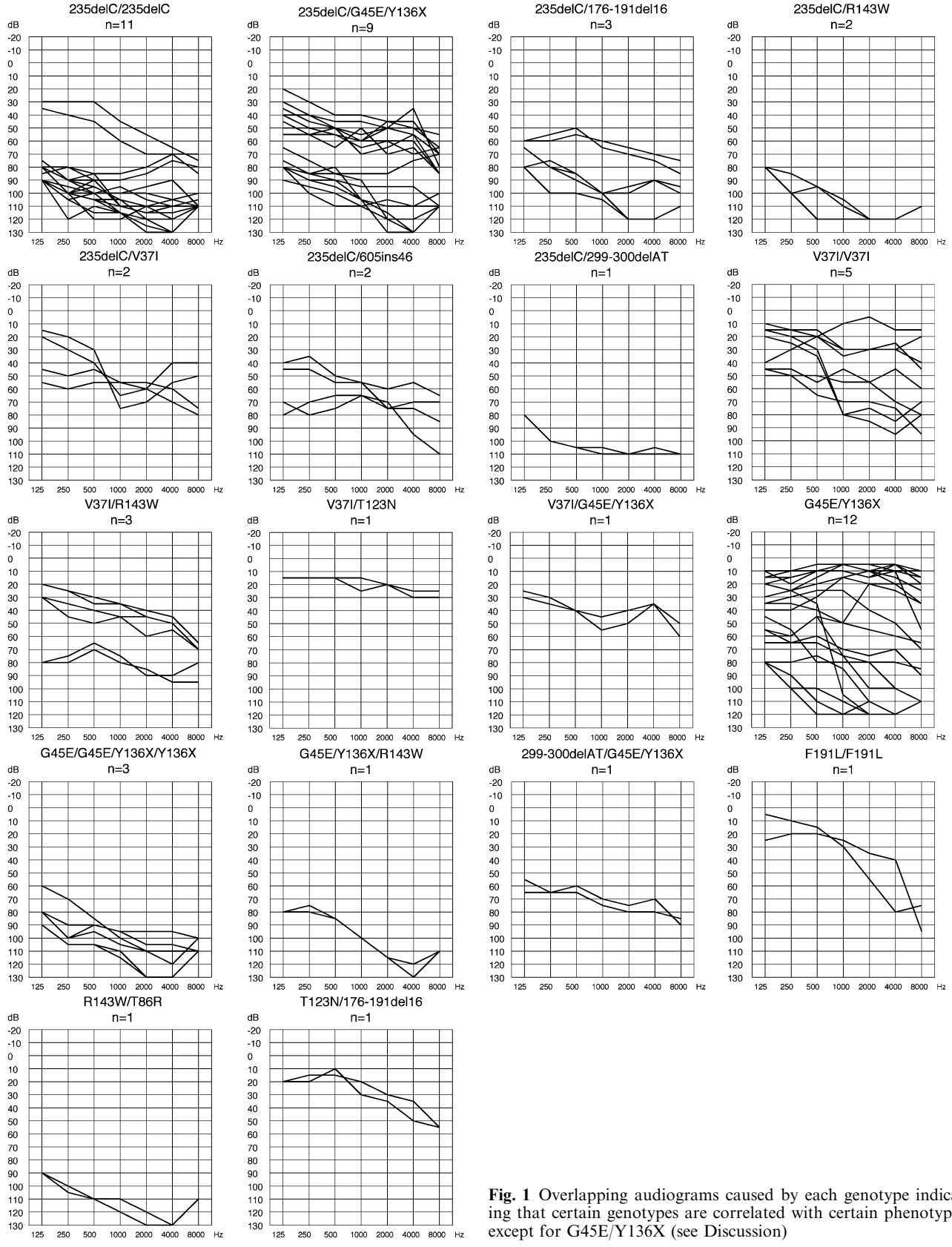

Fig. 1 Overlapping audiograms caused by each genotype indicating that certain genotypes are correlated with certain phenotypes, except for G45E/Y136X (see Discussion)

V37I/G45E/Y136X. Hearing of one patient associated with V37I/T123N was within normal range (Fig. 1).

The comparison between patients homozygous for 235delC or V37I, which are the most and the second most prevalent mutations in Japanese (Ohtsuka et al. 2003), showed significant differences in phenotype (Figs. 1, 2). Those homozygous for the 235delC mutation ($n = 11$, mean 100.68 dB, SD 21.25 dB) exhibited a significantly severer phenotype than that caused by V37I ($n = 5$, mean 37.75 dB, SD 23.09 dB) ($P = 0.003$ Fisher's exact test). Those compound heterozygous for the 235delC mutation ($n = 19$, mean 78.75 dB, SD 27.76 dB) were significantly different from those compound heterozygous for V37I ($n = 7$, mean 47.14 dB, SD 18.35 dB) ($P = 0.021$ Fisher's exact test). Concerning the comparison between a combination of inactivating mutations and a combination of noninactivating mutations, the former ($n = 30$, mean 88.33 dB, SD 25.67 dB) showed a severer phenotype than that caused by the latter ($n = 11$, mean 47.39 dB, SD 31.19 dB) ($P = 0.0003$ Fisher's exact test).

Localization of Cx26 and its mutants

The inherent fluorescence of GFP determined the intracellular localization of the recombinant fusion proteins. Transfected GFP-Cx26 wt (wild type) were found to be localized as labeled puncta, which may be representative of gap junctions along the plasma membrane. In contrast, GFP- Cx26 235delC was not recognized at the plasma membrane but was retained within the cytoplasm close to the nucleus. Both GFP-Cx26 V27I and GFP- Cx26 V37I were found to be localized along the plasma membrane as well as being dispersed in the cytoplasm, which is a similar pattern to that shown in the wild type. (Fig. 3.)

Discussion

The present study, using different spectrums of *GJB2* mutations (Ohtsuka et al. 2003), confirmed that certain genotypes are correlated with certain phenotypes in *GJB2* deafness. The most common mutation, 235delC, exhibited severer hearing impairment whereas V37I, which is the second most common mutation, showed significantly mild hearing impairment. Audiometric data revealed an additional comparatively severe phenotype as well as a relatively mild phenotype.

Among more than 90 different *GJB2* mutations, 35delG, accounts for up to 75% of mutated alleles in populations with European ancestry (Estivill et al. 1998; Gasparini et al. 2000; Van Laer et al. 2001). A series of reports has described that patients associated with

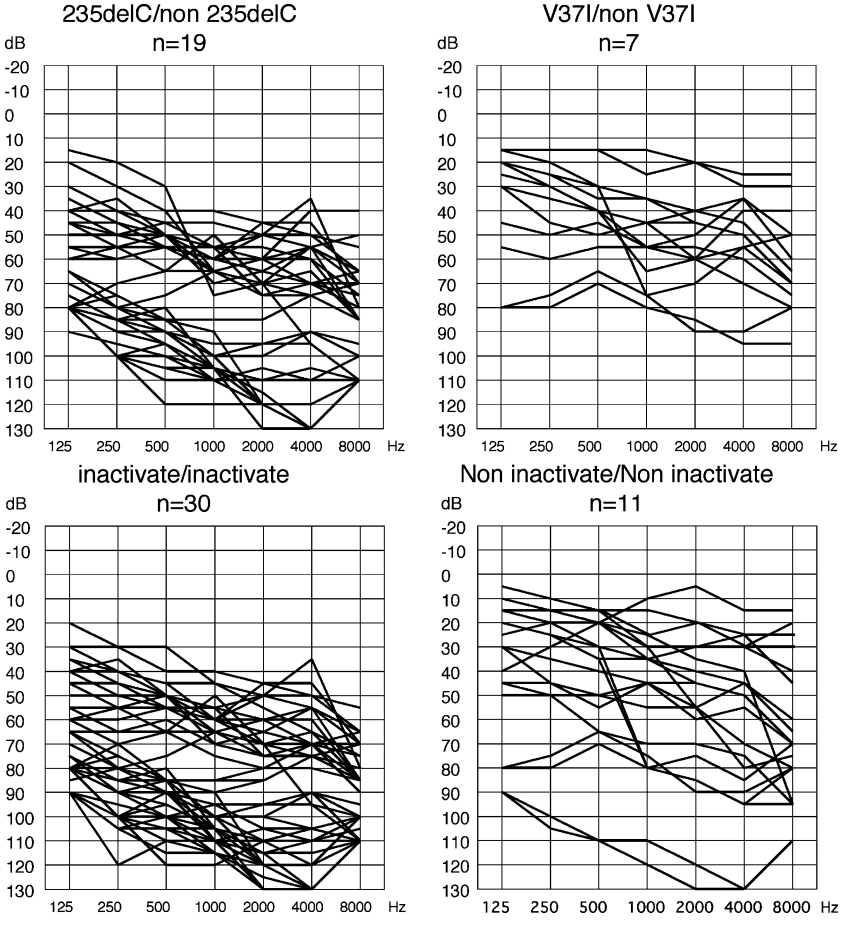

Fig. 2 Overlapping audiograms caused by 235delC/non 235delC, V37I/non V37I, inactivating mutation/inactivating mutation, and noninactivating mutation/noninactivating mutation. Note that patients associated with 235delC show relatively severer hearing loss whereas V37I-involved patients show a relatively mild phenotype. It is also evident that patients associated with inactivating mutation/inactivating mutation showed a severer phenotype than patients with noninactivating mutation/noninactivating mutation

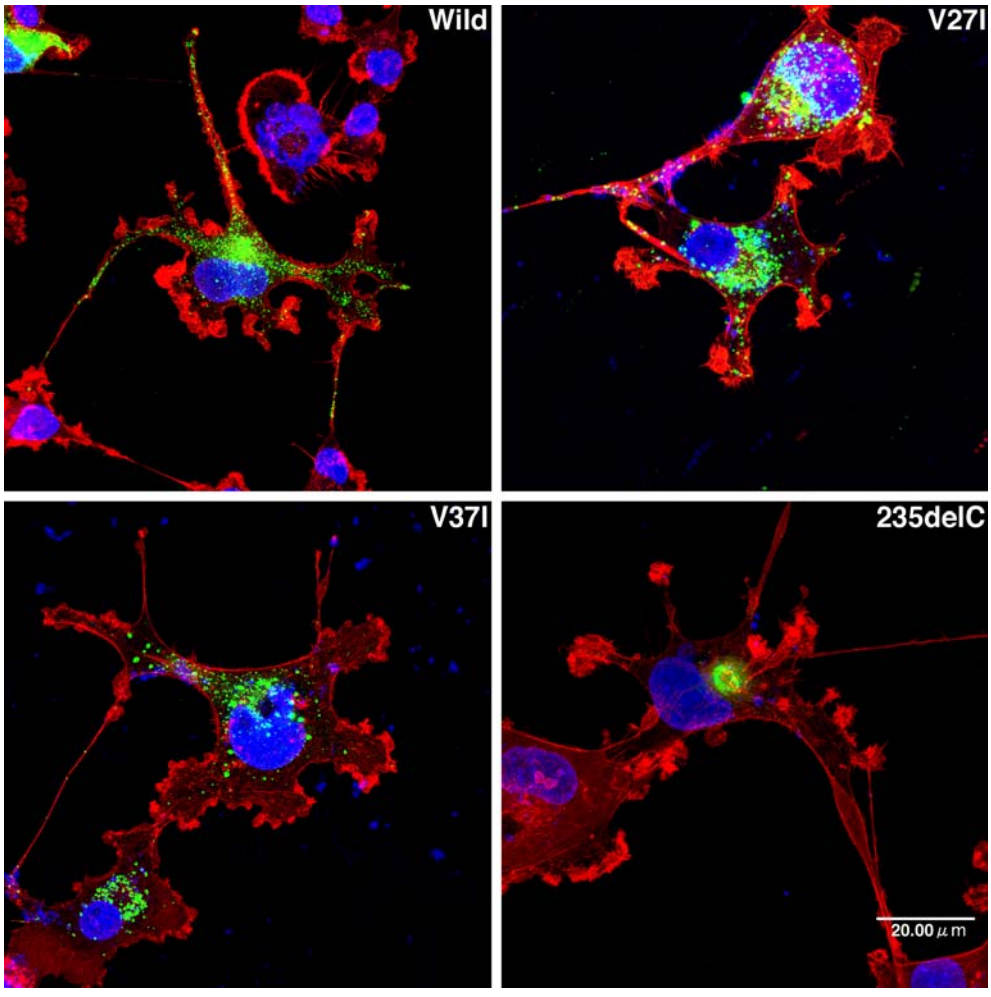

Fig. 3 Protein expression in transfected COS-7 cells. COS-7 cells transfected with GFP-Cx26 wt, GFP-Cx26 V27I, and GFP-Cx26 V37I, which were associated with normal–mild phenotypes, showed a characteristic puncta along the membrane. In contrast, only perinuclear staining was seen in GFP-Cx26 235delC. *Red* actin filament (TRITC- conjugated phalloidin): cell membrane, *Blue* DAPI: nucleus, *Green* Green fluorescent protein: chimeric protein

35delG exhibit severe-to-profound hearing impairment (Cohn et al. 1999; Cryns et al. 2004; Denoyelle et al. 1997, 1999; Green et al. 1999; Marlin et al. 2001; Wilcox et al. 2000). The status of the 235delC mutation, which seems to be a unique mutation in populations with Asian ancestry, is comparable to the 35delG mutation in Caucasoid populations. High prevalence of 35delG and 235delC mutations in the respective populations are due to a founder effect (Ohtsuka et al. 2003; Van Laer et al. 2001). Patients homozygous or compound heterozygous for the 235delC mutation exhibit a comparatively severer phenotype (Fig. 2), indicating that this frequent mutation should be the first to be considered when genetic screening for congenitally deaf patients is performed in Asian populations.

Several reports have indicated the existence of less-severe phenotypes correlated with certain specific mutations, especially in association with V37I (Bason et al. 2002; Cryns et al. 2004; Marlin et al. 2001; Rabionet et al. 2000; Wilcox et al. 2000). The exact phenotype has been rather difficult to prove because of the relatively small number of patients with V37I. The V37I mutation was originally reported as a polymorphism (Kelley et al. 1998), but the fact that valine 37 residue is highly conserved among different connexins, and that a series of reports identified homozygous or compound heterozygous V37I deafness patients (Abe et al. 2000; Bason et al. 2002; Marlin et al. 2001; Rabionet et al. 2000; Wilcox et al. 2000), indicate that it may be a disease-causing mutation. There seem to be ethnic differences in the allele frequency of V37I, as it was not detected in the control subjects from Italy, Spain, Germany, Greece, Israel, Ghana, or Austria (see Discussion in Bason et al. 2002) in spite of a high prevalence in the Japanese population (Abe et al. 2000; Kudo et al. 2000; Ohtsuka et al. 2003). The reported patients in whom the ethnic background was known were all of eastern-Asian origin (Abe et al. 2000; Bason et al. 2002; Kudo et al. 2000; Ohtsuka et al. 2003). In Japanese, V37I is the second most frequent mutated allele, and in this study, it was possible to collect a significant number of patients, and the present data confirmed a less severe phenotype caused by V37I. Due to such a mild phenotype, timing of presentation at clinics and diagnosis may be comparatively delayed. For patients with V37I/V37I, hearing impairment was noticed at the age of 20.6 (range 7–49, SD 17.08) years of age in contrast with 0.33 (range 0–3, SD 1.00) years for patients with 235delC/235delC. It

should therefore be noted that patients with *GJB2* mutations can also be found among less-severe hearing-impaired populations.

A recent multi-center-based genotype–phenotype correlation study clearly showed that severity of hearing impairment is correlated with some particular genotype and proposed a hypothetical general rule that inactivating mutations (stop or frameshift mutations) cause more severe phenotypes than those caused by noninactivating mutations (Cryns et al. 2004). Concerning the comparison between combinations of inactivating mutations and combinations of noninactivating mutations, the present study also showed that the former cause a severer phenotype than that caused by the latter. Therefore, our study supports the above hypothetical general rule.

Overlapped audiograms showed high-frequency-predominant sensorineural hearing loss regardless of genotype. Overall, there seemed to be certain rules regarding genotype and phenotype correlations. Particular genotypes tended to have similar audiograms with minor exceptions (Fig. 1). Therefore, genotype is a fundamental factor to predict phenotype. However, variations among the same phenotypes still exist (Fig. 1). These variations may be explained by the following factors involved in phenotypes: (1) alterations in promoter regions, (2) additional genes such as *GJB6* (del Castillo et al. 2002), (3) modifier genes (Abe et al. 2001), (4) environmental factors. Concerning patients with G45E/Y136X, there was great variability in their phenotypes, ranging from normal to profound. A segregation study indicated that either G45E or Y136X situated on the same allele or different alleles. Our subcloning experiments confirmed the existence of two types of allele: cis allele and trans allele (data not shown). When two mutations are on different alleles (compound heterozygous state), the patients may exhibit severe-to-profound hearing impairment.

The present study further investigated whether the differences in phenotype could be explained by protein-expression study. In contrast to transfected GFP-Cx26 wt, which were found to be localized as labeled puncta along the plasma membrane (Fig. 3), the localization of transfected GFP-Cx26 235delC was not seen on the cellular membrane but mainly cohered at or around the nucleus. Such abnormal subcellular localization of mutated Cx26 protein with 235delC is consistent with a previous study (Choung et al. 2002). From these results, truncated mutations at the transmembrane domain, such as 235delC, were considered to lead to loss of function, resulting in serious hearing impairment. In the case of V37I, which is categorized as a noninactivating mutation, transfected GFP-Cx26 V37I was found along the membrane as in the wild type, indicating that the V37I protein may retain its function and therefore results in a rather mild phenotype. As expected, V27I, a known polymorphism, showed a similar distribution pattern to the wild type and V37I. To summarize, in the present study, the results indicate that protein expression patterns are well correlated with clinical phenotypes. A series of in vitro studies, including protein expression study, cell-to-cell communication properties, or physiological conductance experiments, sometimes provided discrepant results when compared to the phenotypic results, and limitations have been suggested (see discussion in Cryns et al. 2004). In the case of V37I, a complete loss of junctional properties has been reported (Bruzzone et al. 2003) in spite of a rather mild phenotype shown in a series of studies. The protein expression experiments in the current study, however, were in line with the phenotype associated with this mutation.

In conclusion, the present genotype–phenotype correlation results supported the view that phenotypes caused by the truncating *GJB2* mutations are severer than those caused by missense mutations. Anticipating severity of hearing impairment is sometimes difficult, but if such general rules can be drawn with regard to genotype–phenotype correlation, determination of these correlations will facilitate the prediction of the course of hearing and help in making decisions regarding treatment/intervention.

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