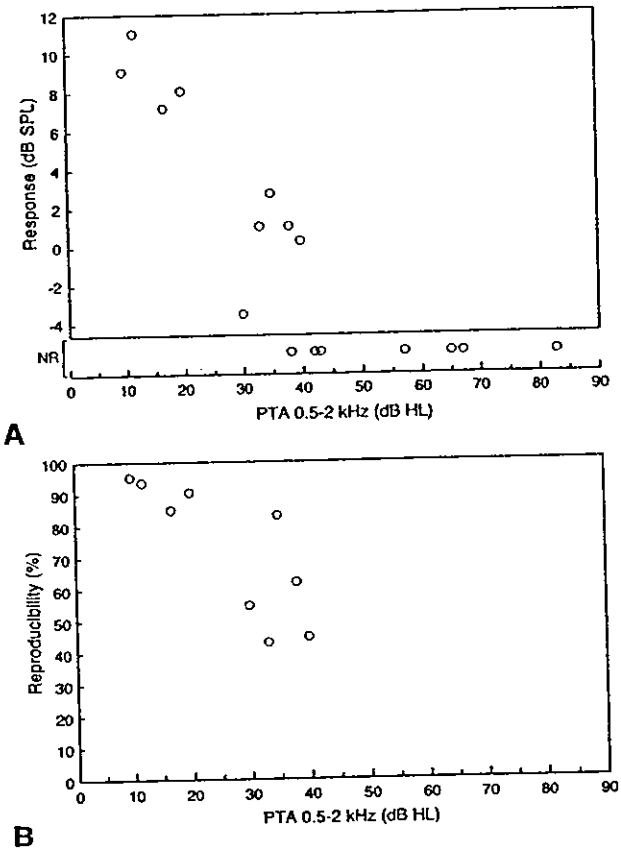

Fig 5. Response (A) and reproducibility (B) of transient evoked otoacoustic emissions as function of mean of pure tone thresholds at 0.5, 1, and 2 kHz ("PTA 0.5-2 kHz") for each ear.

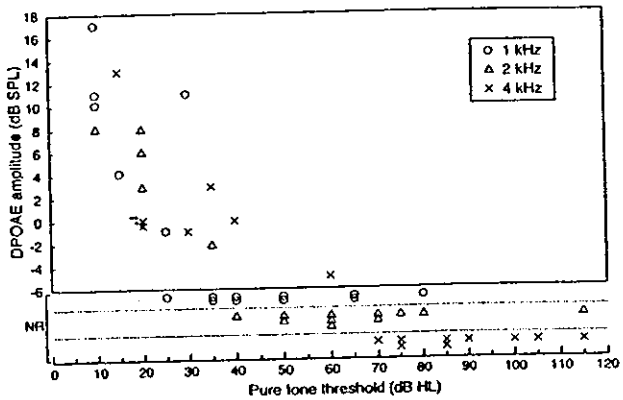

Fig 6. Distortion product otoacoustic emission (DPOAE) amplitude as function of pure tone threshold at DPOAE-tested frequency for each ear. Tests were conducted at 1, 2, and 4 kHz. Symbols between horizontal lines in lower part of Figure (indicated by NR) represent no DPOAE response. Overlapping symbols were moved as indicated in Fig 3.

ily members who were tested.[10] Thus, all of the present subjects who were maternally related members of this family can be considered to carry the A1555G mutation, and all of the present audiological findings can be considered to represent the effects of the A1555G mutation.

A battery of audiological tests conducted in the present study showed a consistent pattern of audiological characteristics, indicating a common pathophysiological mechanism in the development of hearing loss due to the A1555G mutation. Exclusively sloping or sharp sloping audiograms were noted in all subjects with hearing loss except for 1 individual whose hearing loss resulted from long-term noise exposure. In subjects with slight or mild hearing loss according to the PTA 0.5-2 kHz, the pure tone thresholds at 8 kHz were always the most elevated. Even in the subjects who did not report any hearing loss at the time of interview, 11 of the 63 ears (17.5%) exhibited significantly elevated pure tone thresholds at 8 kHz. This frequency was significantly higher than the frequency expected in ears of an otologically normal population. As a result, the relatively frequent occurrence of elevated pure tone thresholds at 8 kHz was considered to be a subclinical audiological feature associated with the mitochondrial A1555G mutation.

These audiogram characteristics have been known in sensory presbycusis, a type of age-related audi-

TABLE 2. CHARACTERISTICS OF AUDITORY BRAIN STEM RESPONSES

	Right Ear					Left Ear				
		Threshold* (dB nHL)		Latency† (ms)			Threshold* (dB nHL)		Latency† (ms)	
Subject	PTA 2-4 kHz (dB HL)	I	V	I	V	PTA 2-4 kHz (dB HL)	I	V	I	V
III-2	55	90	70	1.9	5.9	60		90		7.1
IV-4	80		70		6.4	87.5		70		6.8
IV-11	72.5		80		5.9	67.5	80	70	2.2	6.0
IV-35	92.5	105	100			115		105		
V-7	115					110		105		
V-10	115					115				
V-12	115					115				

PTA 2-4 kHz — average of pure tone thresholds at 2 kHz and 4 kHz.
*Threshold of wave I and wave V.
†Latency of wave I and wave V with click stimulation at 90 dB nHL.

TABLE 3. MITOCHONDRIAL DNA SEQUENCE VARIANTS IN SUBJECTS

Gene Product	*Nucleotide Change*
D-loop	A73G, T152C, A263G, 311insC, T489C
12S rRNA	A750G, A1438G, A1555G
16S rRNA	A2706G, A3145G
NADH dehydrogenase 2	A4715G, A4769G
Cytochrome c oxidase 1	T6632C, A6752G, C7028T, C7196A
Cytochrome c oxidase 2	A8188G
ATP synthase 6	G8584A, A8701G, A8860G, T9090C
Cytochrome c oxidase 3	T9540C
NADH dehydrogenase 3	A10398G, C10400T
NADH dehydrogenase 4	T10873C, G11719A
NADH dehydrogenase 5	C12705T
NADH dehydrogenase 6	C14668T
Cytochrome b	C14766T, T14783C, G15043A, G15301A, A15326G, A15487T, T15784C
D-loop	C16185T, C16186T, C16223T, C16260T, T16298C

tory impairment resulting from the degeneration of sensory hair cells and supporting cells primarily at the basal turn of the cochlea.[20] Several other mitochondrial DNA mutations have been proposed to play roles in age-related dysfunction in organs such as the central nervous system and muscle,[21] and therefore, the A1555G mutation may act analogously to promote auditory dysfunction by a mechanism similar to that of sensory presbycusis.

The speech audiometry results in the present subjects indicated cochlear dysfunction in subjects with slight to severe hearing loss, and these subjects did not exhibit features of retrocochlear dysfunction. The SISI and OAE tests also detected cochlear dysfunction almost simultaneously with or even earlier than the deterioration of pure tone thresholds, indicating that cochlear dysfunction, especially outer hair cell dysfunction, occurred at quite an early stage of hearing loss in the affected subjects. The observed ABR thresholds and latencies also indicated cochlear damage. In agreement with these results, excellent auditory performance with a cochlear implant has been reported in a patient with profound hearing loss due to the A1555G mutation.[22] Given that selective damage to the outer hair cells induces only mild to moderate hearing loss,[23] it would be expected that other cochlear components would thus be damaged in cases of more advanced hearing loss.

The PTA testing confirmed various levels of hearing loss in the present subjects, none of whom had a history of aminoglycoside exposure. To explore possible genetic factors that may have contributed to such phenotypic differences, we sequenced the entire mitochondrial DNA for 8 subjects who presented with various levels of hearing loss. Previously, the coexistence of two mitochondrial mutations, A1555G and G7444A, was identified in Mongolian subjects with hearing loss, and these subjects appeared to present earlier onset and increased severity of hearing loss as compared to patients with the A1555G mutation alone.[24] This finding suggests that an additional new mitochondrial DNA mutation may be responsible for the intrafamilial phenotypic differences in this family. However, our analysis revealed that all 8 subjects had identical mitochondrial DNA sequences, thus indicating that the observed phenotypic differences were not related to any variations in the mitochondrial DNA. In addition, except for the A1555G mutation, no known pathogenic mutations were found in the total mitochondrial DNA sequences; thus, the A1555G mutation is probably the only mitochondrial mutation involved in hearing loss in this family. The degree of hearing loss was similar in the affected subjects within the same sibling group, but varied between the sibling groups. These results suggest that nuclear modifier genes may also be involved in phenotypic differences in the present family, as previously reported in an Arab-Israeli family.[25,26]

In conclusion, our study revealed that various degrees of hearing loss could be caused by an A1555G mutation in the mitochondrial DNA with identical sequences, without any additional pathogenic mutations, even in the absence of aminoglycoside exposure. The affected subjects exhibited audiograms that are characteristic of sensory presbycusis, and also shared common audiological features such as a cochlear origin for all levels of hearing loss and a high degree of vulnerability of outer hair cells. These results further our understanding of the genetic and pathophysiological mechanisms of hearing loss associated with the A1555G mutation, and may aid in the diagnosis and development of new therapies for the treatment of this genetic hearing loss.

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TYPE IX COLLAGEN IS CRUCIAL FOR NORMAL HEARING

K. ASAMURA,[a] S. ABE,[b] Y. IMAMURA,[c] A. ASZODI,[d] N. SUZUKI,[a] S. HASHIMOTO,[a] Y. TAKUMI,[a] T. HAYASHI,[c] R. FÄSSLER,[d] Y. NAKAMURA[b] AND S. USAMI[a]*

[a]*Department of Otorhinolaryngology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan*

[b]*Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*

[c]*Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1, Komaba, Meguro-ku, Tokyo 153-8902, Japan*

[d]*Max Planck-Institut for Biochemistry, Department of Molecular Medicine, 82152 Martinsried, Germany*

Abstract—cDNA microarray analysis indicated that *COL9A1* and *COL9A3* are highly expressed in the human inner ear, suggesting that type IX collagen has a crucial functional role in the inner ear. This study further confirmed, by means of real-time PCR, the presence of collagen type IX genes in the mouse inner ear. Immunocytochemical analysis also revealed that type IX collagen is distributed in the tectorial membrane, where it co-localizes with type II collagen, indicating that type IX collagen may contribute to the three-dimensional integrated structure of type II collagen molecules. Mice with targeted disruption of the *col9a1* gene were shown through assessment by auditory brain stem response to have hearing loss, suggesting an important role of type IX collagen in maintaining normal hearing. At the light microscopic level, the tectorial membrane of knock-out mice was found to be abnormal in shape, and electron microscopy confirmed disturbance of organization of the collagen fibrils. An antibody against type II collagen failed to detect type II collagen in the tectorial membrane of type IX collagen knock-out mice, suggesting that a lack of type IX collagen may affect the three-dimensional structure of type II collagen molecules. These findings indicate that genes encoding each chain of type IX collagen may fulfill an important function associated with the tectorial membrane in the auditory system. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: knock-out mouse, tectorial membrane, type II collagen, hearing impairment.

Collagen in the inner ear is currently of great interest because mutations of several types of collagen have been reported to be responsible for syndromic as well as non-syndromic hearing loss (see Hereditary Hearing Loss Homepage: http://www.uia.ac.be/dnalab/hhh/), including Stickler syndrome (*COL2A1*, *COL11A1*, and *COL11A2*; Richards et al., 1996; Vikkula et al., 1995; Williams et al., 1996), Marshall syndrome (*COL11A1*; Griffith et al., 2000), Alport syndrome (*COL4A3*, *COL4A4*, and *COL4A5*; Barker et al., 1990; Mochizuki et al., 1994), and DFNA13 (*COL11A2*; McGuirt et al., 1999). In the inner ear, these collagens are localized in the tectorial membrane, basement membrane, spiral ligament, and the spiral limbus (Cosgrove et al., 1996; Goodyear and Richardson, 2002; Richardson et al., 1987; Slepecky et al., 1992a,b; Thalmann, 1993).

Genes that are expressed specifically in auditory tissues are likely to be good candidates to screen for genetic alterations in patients with deafness, and several genes associated with deafness have been efficiently identified by way of organ-specific and subtractive approaches (Abe et al., 2003a,b; Verpy et al., 2000; Yasunaga et al., 1999). cDNA microarray analysis indicated that the *COL9A1* and *COL9A3* genes are highly expressed in the human inner ear (Abe et al., 2003a), suggesting that type IX collagen has a crucial functional role in the inner ear.

In the present study, to evaluate the contribution of type IX collagen to hearing function, we performed: 1) real-time PCR experiments to ascertain the expression of the genes for three subunits of collagen type IX in the inner ear, and 2) audiological, immunohistochemical and morphological evaluations of type IX collagen functional knock-out mice.

EXPERIMENTAL PROCEDURES

Extraction of total RNA and reverse transcription

For real-time PCR analysis, inner ears of three mice of the C57B6 strain were removed and then quickly frozen together by liquid nitrogen. These tissues were homogenized, and Trizol (Life Technologies, MD, USA) was used to extract total RNA from the specimen. In order to remove trace amounts of contaminating DNA, treatment with RNase-free DNase I (Takara, Shiga, Japan) was performed. PolyA+ RNA was purified using an Oligotex-dT30 mRNA Purification Kit (Takara) following the manufacturer's instructions. Approximately 0.5 μg of polyA+ RNA was reverse transcribed with oligo (dT) primer and Superscript II reverse transcriptase (Invitrogen, CA, USA).

Real-time PCR analysis

The amplified product was detected by the SYBR Green Intercalater method. The 1 μl of cDNA solution and PCR mixture containing 0.005 U of TaKaRa EX *Taq*TM DNA polymerase, 0.5 μM of specific upstream and downstream primers designed by the NIH primer software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) based on GenBank sequence, were mixed with SYBR Green, and then amplified by a Smart Cycler System (Cepheid, CA, USA). Primers used to amplify each gene were Col9a1-F (5′-CTGGTGTACCTGGGTCTCGT-3′) and Col9a1-R (5′-TTCTCCTTTCGCCCCTTTAT-3′): Col9a2-F (5′-AGTGGGGCTACCAGGTTTCT-3′) and Col9a2R (5′-AACCCCTCCCTCACTGAACT-3′): Col9a3-F (5′-GCAAAGATGGTCGAGATGGT-3′) and Col9a3-R (5′-GTAGCCAGCCACTGTCCATT-3′). As an internal control, β-actin was analyzed with specific primers (Sigma

*Corresponding author. Tel: +81-263-37-2666; fax: +81-263-36-9164.
E-mail address: usami@hsp.md.shinshu-u.ac.jp (S. Usami).
Abbreviations: ABR, auditory brainstem response; Ct, cycle threshold; MED, multiple epiphyseal dysplasia; PBS, phosphate buffer saline.

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Genosys, Hokkaido, Japan). Product specificity was confirmed by: i) melting curve analysis; ii) agarose gel electrophoresis.

Animals

Type IX collagen knock-out mice were generated by targeted disruption of the *Col9a1* gene as described previously (Fässler et al., 1994). In mice with an inactivated *Col9a1* gene, Col9a2 and Col9a3 polypeptides were demonstrated not to be expressed, although the mRNAs for both Col9a subunits are normally transcribed (Hagg et al., 1997). Therefore, *Col9a1* null mice are thought to be collagen IX functional knock-out mice (Hagg et al., 1997). Type IX collagen knock-out mice develop non-inflammatory joint diseases (Fässler et al., 1994; Hagg et al., 1997). These mice show no detectable abnormalities at birth but develop a severe joint disease resembling human osteoarthritis (Fässler et al., 1994; Hagg et al., 1997).

Auditory testing

Five wild type (12 weeks old), and 5 heterozygous (12 weeks old), and 5 homozygous (12 weeks old) collagen IX functional knock-out mice were evaluated by auditory brainstem response (ABR). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) by i.p. injection and ABR measurements were performed in a quiet room. Click stimuli were generated by a square pulse of 0.1 ms duration and specified amplitude using a digital signal-processing platform (Tucker-Davis Technologies, FL, USA), and analyzed with PowerLab systems (ADInstruments, NSW, Australia). For recording, stainless steel needle electrodes were placed at the vertex and ventrolateral to the left and right ears. Acoustic stimuli evoked by clicks were delivered to the animals through a tweeter modified with a coupler for insertion into the external canal. ABR waveforms were recorded in 5–10 dB intervals down from a maximum amplitude of 85 dB until no waveform could be visualized.

Morphological analysis

For morphological studies, cochlear tissues were fixed with 2.5% glutaraldehyde (in 0.1 M phosphate buffer saline: PBS; pH 7.4) and then treated with OsO_4 (in 0.1 M PBS, pH 7.4), dehydrated in graded ethanols and propylene oxide, and embedded in Durcupan (Fluka, Buchs, Switzerland). For light microscopy, semithin sections were stained with Toluidine Blue. For electron microscopy, ultrathin sections were post-stained with uranyl acetate and lead citrate, and were viewed on a JEOL-1200CX TEM.

Immunohistochemical analysis

The animals were anesthetized and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.25 was injected through the tympanic membrane prior to systemic transcardial perfusion with the same fixative. The temporal bones were removed immediately after perfusion, and post-fixed with 4% paraformaldehyde at 4 °C for 3 h. Then the tissues were cryoprotected in 10% and 30% sucrose (24 h each). Serial cryostat sections (15 μm thick) were cut and placed on silane-coated slides. For double staining of type II and type IX collagen, the specimens were incubated as follows: (1) the blocking process was used to prevent non-specific immunoreactivity; non-specific binding sites were saturated with normal horse serum (Vector Laboratories, CA, USA) at room temperature for 30 min; (2) a mixture of mouse monoclonal anti-type II collagen antibody (Neomarkers, CA, USA) and rabbit polyclonal antibody to mouse type IX collagen (LSL, Tokyo, Japan) diluted appropriately with PBS containing 0.3% Triton X-100, overnight at room temperature; (3) a mixture of secondary antibodies, biotinylated anti-mouse IgG (H+L) (Vector Laboratories) and goat F $(ab')_2$ anti-rabbit IgG (H+L) TRITC (Leinco Technologies, MO, USA), 2 h at room temperature; (4) streptavidin–fluorescein (Amersham Biosciences, NJ, USA), 2 h at room temperature. Imaging was performed using the Leica TCS SP2 AOBS Spectral Confocal Scanning System (Leica, Wetzlar, Germany).

All studies were carried out in accordance with Shinshu University School of Medicine and National Institutes of Health guidelines for the ethical treatment of experimental animals. The number of mice used and their suffering were minimized in the present study.

RESULTS

Real-time PCR analysis

The expression of the type IX collagen genes, *Col9a1*, *Col9a2*, *Col9a3*, was confirmed along with expression of β-actin in mice inner ears using reverse transcription and subsequent real-time PCR analysis. PCR of all samples generated a smooth, S-shaped growth function of fluorescence, also consistent with a single PCR product (Fig. 1A). Cycle threshold (Ct) was determined as the first cycle in which there is a significant increase in fluorescence above the background fluorescence. Cts of *col9a1*, *col9a2*, *col9a3*, were 26.28, 26.46, and 23.95 respectively. Theoretically, the amounts of *col9a1*, *col9a2*, *col9a3* were given as $1:2^{26.28-26.46}:2^{26.28-23.95}$ (1:0.88:5.03). After PCR, amplification was verified by gel electrophoresis. Each band was detected at the appropriate band size (Fig. 1B).

Audiological evaluation

ABRs were recorded from type IX collagen knock-out mice and controls at 12 weeks. Although wild-type animals maintained normal hearing (35±0 dB) the homozygous knock-out mice showed hearing loss (62±6.7 dB; mean±S.D.; Fig. 2) and their average decrease in hearing threshold was 27± 6.7 dB. The average ABR threshold was significantly different ($P<0.005$). Heterozygous knock-out mice showed no detectable hearing loss when compared with the wild-type animals (data not shown).

Morphological analysis

The morphologic inner-ear abnormality found at the light microscopic level was in the tectorial membrane, which appeared to be enlarged and with obvious crooked deformation in homozygous knock-out mice (Fig. 3B, D).

At the electron microscopic level, in the wild-type animals, the tectorial membrane contains two distinct components as previously described (Goodyear and Richardson, 2002; Hasko and Richardson, 1988). The radial collagen fibril bundles, which have a cross-striated pattern, coursed in a parallel and well-organized manner among a laminated, striated-sheet matrix (Fig. 3E). Contrarily, in the tectorial membrane in homozygous knock-out mice, striated-sheet matrix was loose and only aggregated and fused fibers which did not show a cross-striated pattern, coursed among the striated-sheet matrix (Fig. 3F). There were no detectable differences in other regions, such as the spiral ligament (Fig. 3G, H).

Heterozygous knock-out mice did not show any detectable morphological changes either at light or electron microscopic observation (data not shown).

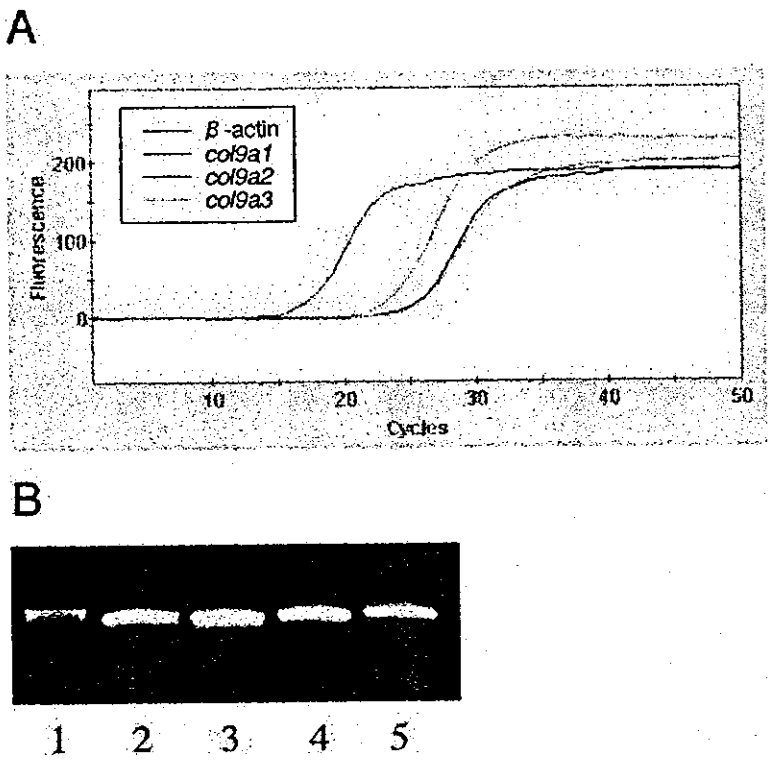

Fig. 1. (A) Cybergreen fluorescence observed across PCR amplification cycles of mice inner ear samples for type IX collagen and β-actin. (B) Agarose gel electrophoresis of *Col9a1*, *Col9a2*, *Col9a3* and β-actin after real-time PCR. A single band of the expected size is observed. The lanes are: (1) DNA standard (331 bp); (2) *Col9a1* (275 bp); (3) *Col9a2* (245 bp); (4) *Col9a3* (283 bp); (5) β-actin (392 bp).

Immunohistochemical analysis

Type II collagen-immunoreactivity was found in the tectorial membrane, the spiral limbus and the spiral ligament in wild-type animals (Fig. 4A). Type IX collagen-immunoreactivity was predominantly distributed in the tectorial membrane. Weak immunoreactivity was also found in the spiral limbus and the spiral ligament (Fig. 4C). In contrast, there was no type IX collagen-immunoreactivity in the cochlea of knock-out mice (Fig. 4D). Type II collagen-immunoreactivity was absent from the tectorial membrane, whereas immunoreactivity was stable in the limbus and spiral ligament as in wild-type animals (Fig. 4B). Heterozygous knock-out mice showed the same immunocytochemical findings as in wild-type animals (data not shown).

DISCUSSION

Several collagens, including II, IV, V, IX and XI, have been reported to be distributed in the cochlea, and are thought to have important roles in hearing function (Cosgrove et al., 1996; Goodyear and Richardson, 2002; Richardson et al., 1987; Slepecky et al., 1992a,b; Thalmann, 1993). These collagens have been considered as important extracellular matrix components in the inner ear. The tectorial membrane, composed of collagens and non-collagenous glycoproteins and lying over the surface of the organ of Corti, plays a crucial role in the first step of sound transduction. Type IX collagen was known to be one of the important components, together with type II and V collagens, in the tectorial membrane of the organ of Corti (Slepecky et al., 1992a,b). Type IX collagen belongs to the fibril-associated collagen with interrupted triplet helices group of collagens, which is known to bind to the surface of fibril-forming type II collagen (Eyre et al., 2002; van der Rest and Mayne, 1988; Wu et al., 1992). Electron microscopic studies revealed that the tectorial membrane contains two different components; radial collagen fibril bundles and a laminated, striated-sheet matrix (Goodyear and Richardson, 2002; Hasko and Richardson, 1988).

The present study revealed the existence and the important functional roles of type IX collagen in the cochlea: 1) gene expression of three α chains of type IX collagen in mice cochlea, 2) mild to moderate hearing impairment assessed by ABR in type IX collagen knock-out mice, and 3) altered architecture of the tectorial membrane in type IX collagen knock-out mice.

Reverse transcription and subsequent real-time PCR analysis ascertained gene expression of three α chains of type IX collagen, *col9a1*, *col9a2*, *col9a3*, in mice inner ears. This supports the finding that these genes are highly expressed in the cochlea (Abe et al., 2003a).

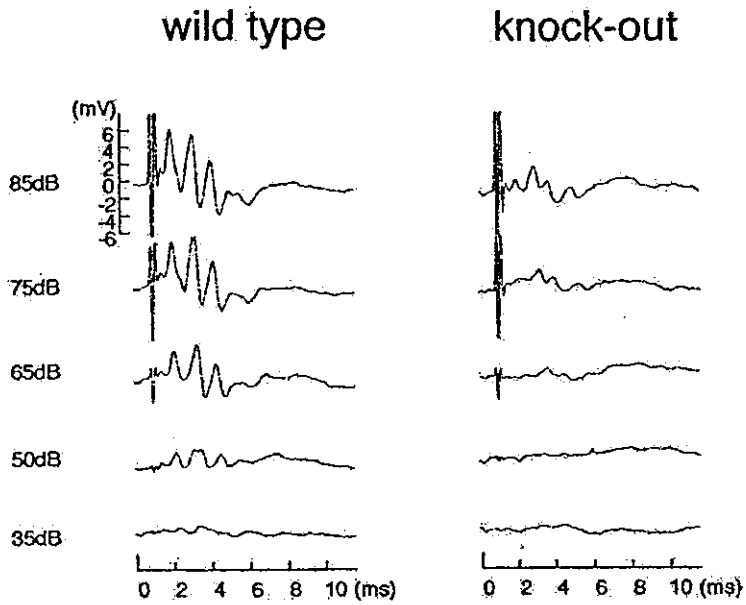

Fig. 2. ABR responses of wild-type mice and knock-out mice at the age of 12 weeks. Although wild-type animals maintained normal hearing (35±0 dB), the homozygous knock-out mice showed hearing loss (62±6.7 dB; mean±S.D.). The average ABR threshold was significantly different ($P<0.005$).

At light microscopic level, the tectorial membrane was found to be deformed in shape and detached from the organ of Corti (Fig. 3B, D). The lower surface of the tectorial membrane is in contact with the stereocilia of outer hair cells and with Hensen cells at the lateral edge of the organ of Corti (Kimura, 1966; Lim, 1972). Therefore, the tectorial membrane is supposed to be functionally connected not only to the input of mechanoelectrical transducers in hair-cell stereocilia, but also to the output of electromechanical transducers in the outer hair cell membrane. By changing the arrangement of the collagen fibrils in the tectorial membrane from a parallel and close pattern to a disorganized, random and widely separated pattern with altered architecture, the electrical and chemical properties of the tectorial membrane may be varied.

The absence of cross-striated patterns in the radial collagen fibril bundles and aggregated/fused fibrils found at the electron microscopic level in the homozygous knock-out mice (Fig. 3F) suggests that type IX collagen may be essential for intact three dimensional structure in association with type II collagen or with other molecules. These ultrastructural changes in structural integrity may cause the crooked shape of the tectorial membrane in the homozygous knock-out mice. Such changes in the structure were also supported by the fact that antibody against type II collagen was unable to detect type II collagen in type IX collagen knock-out mice. In contrast to such electron microscopic findings in the tectorial membrane where type II and type IX collagens are co-localized, collagen fibrils in the spiral ligament remained unchanged in appearance (Fig. 3H). This could be explained by the fact that type IX collagen is present in the spiral limbus and the spiral ligament, but is less abundant. These electron microscopic findings are supported by the subsequent immunocytochemical results. The present immunoreactivities of type II and IX collagens were in line with the previous reports (Fig. 4A, C; Goodyear and Richardson, 2002; Slepecky et al., 1992b). Type II collagen is distributed in the tectorial membrane as well as the spiral limbus and the spiral ligament (Fig. 4B, E). Type IX collagen is predominantly found in the tectorial membrane and found to be co-localized with type II collagen (Fig. 4C, E). In type IX collagen knock-out mice, an antibody against type II collagen was able to detect type II collagen in the limbus and spiral ligament where type IX collagen is less abundant (Fig. 4B, F). However, interestingly, the antibody failed to detect type II collagen in the tectorial membrane where type IX collagen is abundantly distributed, suggesting that a lack of type IX collagen may affect the three-dimensional structure of type II collagen molecules (Fig. 4B, F). In the cartilage, where type II and IX collagens are also known to be co-localized, type II collagen-immunoreactivity of knock-out mice was not affected (Hagg et al., 1997) as shown in the tectorial membrane in this study. In the case of the tectorial membrane in knock-out mice, aggregated/fused collagen fibril-like structures are found among a laminated, striated-sheet matrix (Fig. 3F). Such morphological change has not been found in the cartilage. Therefore, the difference in staining pattern of type II collagen could be explained by a unique structure of the complex fiber arrangements in the tectorial membrane. The antibody may not detect such an abnormal three dimensional structure. It is understandable that tectorial membrane must have a very fine and sensitive structure as the first step for transduction of sounds. Taken

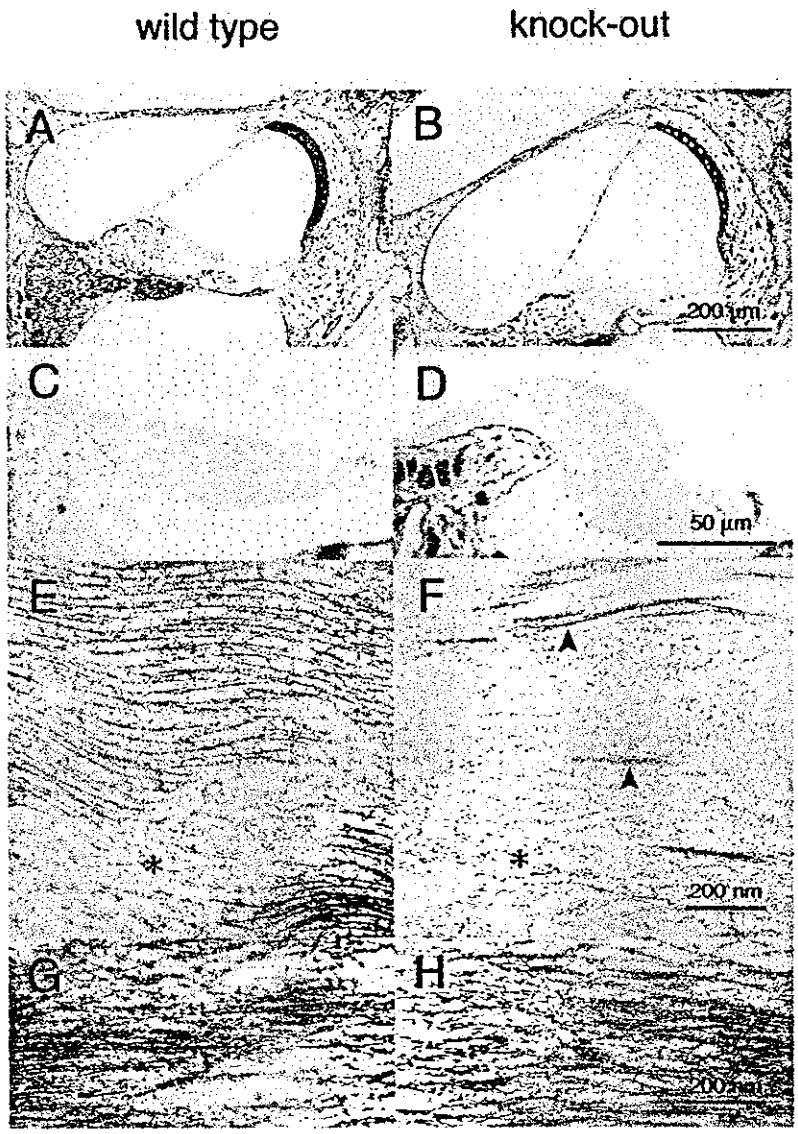

Fig. 3. Morphological characteristics found in wild-type (A, C, E, G) and homozygous knock-out mice (B, D, F, H). (A–D) Light microscopic features of wild-type animals (A, C) and knock-out mice (B, D). Cochlea of wild-type controls showed a normal appearance of the organ of Corti with compact and well-organized tectorial membrane (A, C). In contrast, knock-out mice showed enlarged and crooked tectorial membranes, but normal organ of Corti. Scale bar=200 μm (A, B) and 50 μm (C, D). (E–H) The ultrastructural features of the tectorial membrane (E, F) and spiral ligament (G, H). The radial collagen fibril bundles which have a cross-striated pattern coursed in a parallel and well-organized manner among a laminated, striated-sheet matrix (Fig. 3E). In the tectorial membrane in homozygous knock-out mice, striated-sheet matrix is loose and only aggregated and fused fibers (arrowheads) without cross-striated pattern coursed among the striated-sheet matrix (Fig. 3F). In the spiral ligament, collagen fibrils are parallel in both wild-type (G) and knock-out (H) mice, and the structure is much the same. Asterisks (*) show the striated-sheet matrix. Scale bar=200 nm.

together, these immunocytochemical and electron microscopic observations suggest type IX collagen plays an important role in maintaining the mechanical stability of the tectorial membrane in association with type II collagen.

Targeted deletion of the genes encoding the collagenous and non-collagenous molecules of tectorial membrane resulted in various malformations of the tectorial membrane and subsequent hearing impairment in animals. *Col11a2*-mutant mice were shown to have a crooked shaped tectorial membrane and hearing impairment (McGuirt et al., 1999). Electron microscopy revealed disorganized and widely spaced collagen fibrils (McGuirt et al., 1999). These morphological abnormalities resemble those found in the tectorial membrane of the *Col9a1*-mutant mice in this study. In *Tecta*-mutant mice, the tectorial membrane is completely detached from the hair cells of the organ of Corti (Legan et al., 2000).

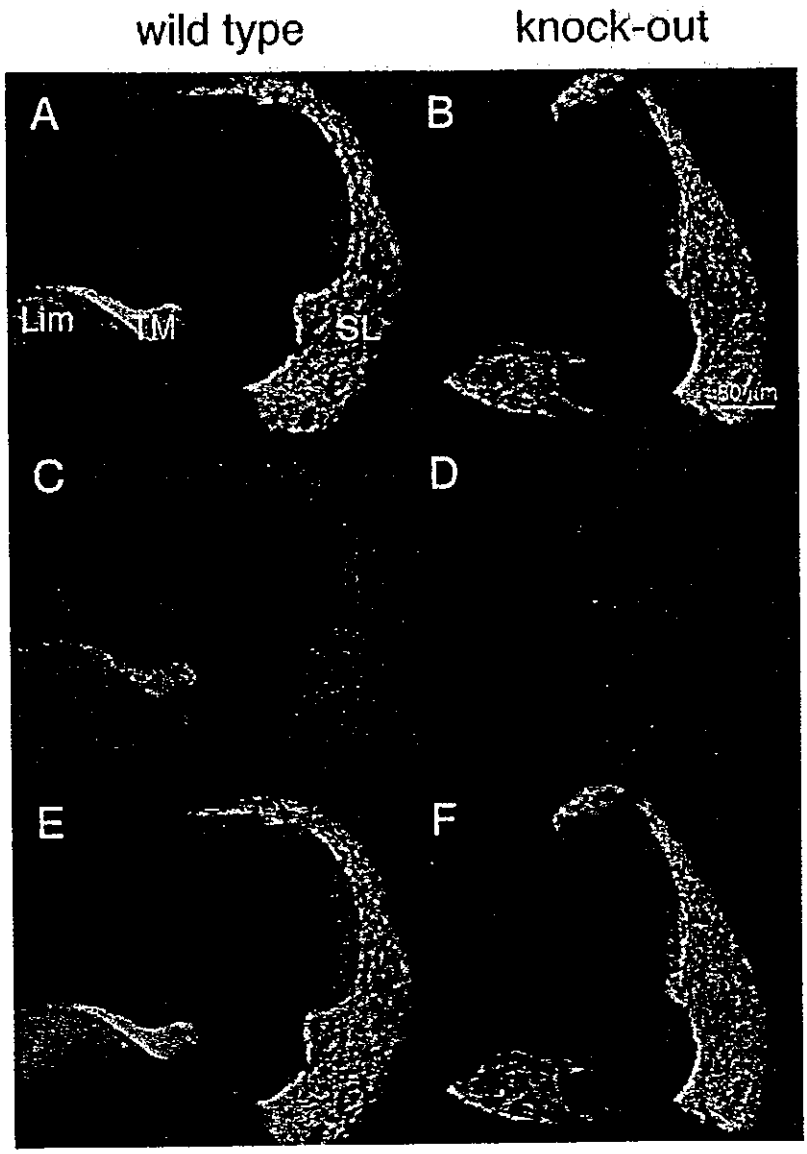

Fig. 4. Immunohistochemistry of cochlear tissue from 12-week-old wild-type controls (A, C, E) and knock-out (B, D, F) mice. Double staining with monoclonal antibodies to type II collagen and with polyclonal antibodies to type IX collagen. Antibody recognized type II collagen in the tectorial membrane, the spiral limbus, and the spiral ligament in wild-type animals (green) (A), but the antibody failed to recognize type II collagen in the tectorial membrane of knock-out mice (B). Type IX collagen is predominantly found in the tectorial membrane and found to be co-localized with type II collagen (red) (C). There was no immunoreactivity in all areas of the knock-out mice (D). Double immunostaining showed these two molecules are co-localized (yellow) in the tectorial membrane (E). Lim, spiral limbus; SL, spiral ligament; TM, tectorial membrane. Scale bar=80 μm.

In the tectorial membrane in the *Otog*-mutant mice, large zones lacking normal fibrillar structures and containing fiber-rod structures have been shown (Simmler et al., 2000). The characteristic features in these mutant mice are that abnormal findings are restricted within the tectorial membrane and the architecture of the organ of Corti is otherwise normal.

In humans, it has already been known that mutations in collagen genes, including *COL2A1*, *COL11A1* and *COL11A2*, encoding collagenous components of the tectorial membrane, cause hearing impairment. Mutations in *COL2A1*, *COL11A1*, and *COL11A2* cause Stickler syndrome, which is known as an autosomal dominant disorder characterized by vitreo-retinal anomalies, joint laxity, palatal clefting, facial dysmorphism, and hearing loss (Richards et al., 1996; Vikkula et al., 1995; Williams et al., 1996). Recent reports expanded the phenotypes caused by *COL11A1* and *COL11A2*. Marshall syndrome, which has a very similar phenotype to that of the Stickler syndrome, was reported to

be associated with splicing mutations in *COL11A1* (Griffith et al., 2000). *COL11A2*, the responsible gene for Stickler syndrome also causes DFNA13 non-syndromic hearing impairment (McGuirt et al., 1999). *TECTA*, a gene which encodes non-collagenous molecules specific to the tectorial membrane, was demonstrated to be the responsible gene for DFNA8/12 and DFNB21 (Mustapha et al., 1999; Verhoeven et al., 1998).

These reports suggested that mutations in genes encoding collagen type IX are possible candidates for causing human deafness. *COL9A1*, *COL9A2* and *COL9A3* genes are known to be responsible for multiple epiphyseal dysplasia (MED; MIM132400; Bonnemann et al., 2000; Briggs and Chapman, 2002; Czarny-Ratajczak et al., 2001; Paassilta et al., 1999). Although hearing loss has not been reported in MED patients, it would be very interesting to perform detailed audiometric testing in MED patients or perform screening to find possible mutations in the non-syndromic deafness patients.

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ORIGINAL ARTICLE

Tomohiro Oguchi · Akihiro Ohtsuka
Shigenari Hashimoto · Aki Oshima · Satoko Abe
Yumiko Kobayashi · Kyoko Nagai · Tatsuo Matsunaga
Satoshi Iwasaki · Takashi Nakagawa · Shin-ichi Usami

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

T. Oguchi · A. Ohtsuka · S. Hashimoto · A. Oshima
S. Usami (✉)
Department of Otorhinolaryngology,
Shinshu University School of Medicine,
3-1-1 Asahi, Matsumoto 390-8621, Japan
E-mail: usami@hsp.md.shinshu-u.ac.jp
Tel.: +81-263-372666
Fax: +81-263-369164

S. Abe
Abe ENT Clinic, 2-2-5 Nishi-magome, Ota-ku,
Tokyo 143-0026, Japan

Y. Kobayashi
Department of Otorhinolaryngology,
Iwate Medical University, 19-1 Uchimaru,
Morioka, Iwate 020-8505, Japan

K. Nagai
Department of Otorhinolaryngology,
Gunma University School of Medicine,
4-2 Aramaki-machi, Maebashi, Gunma 371-8510, Japan

T. Matsunaga
Department of Otolaryngology/Laboratory of Auditory Disorders,
National Tokyo Medical Center, National Institute of Sensory
Organs, 2-5-1 Higashigaoka, Meguro-ku,
Tokyo 152-8902, Japan

S. Iwasaki
Department of Otorhinolaryngology, Hamamatsu University
School of Medicine, 1-20-1 Handayama,
Hamamatsu 431-3192, Japan

T. Nakagawa
Department of Otorhinolaryngology, Graduate School of Medical
Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku,
Fukuoka 812-8582, Japan

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

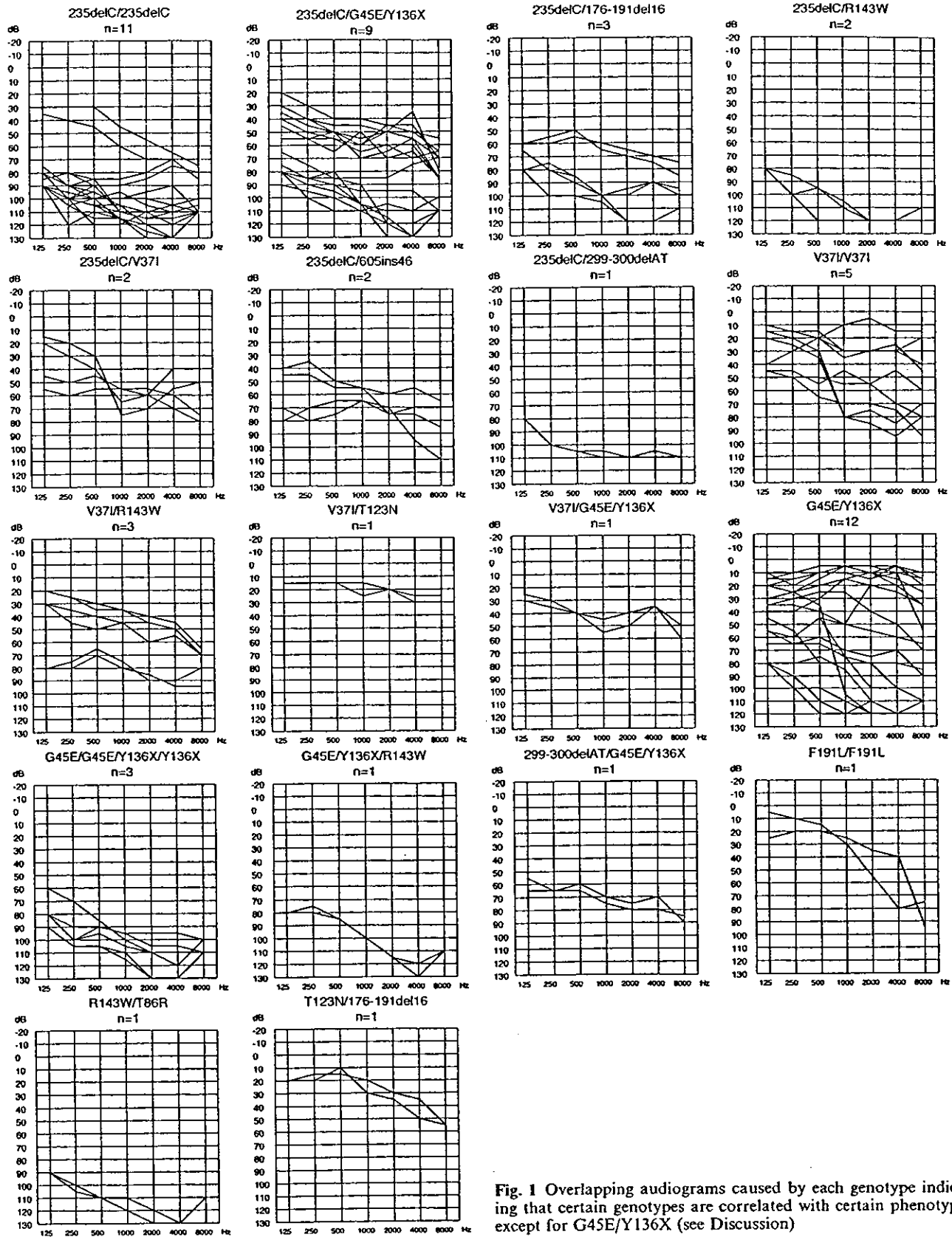

Fig. 1 Overlapping audiograms caused by each genotype indicating that certain genotypes are correlated with certain phenotypes, except for G45E/Y136X (see Discussion)

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

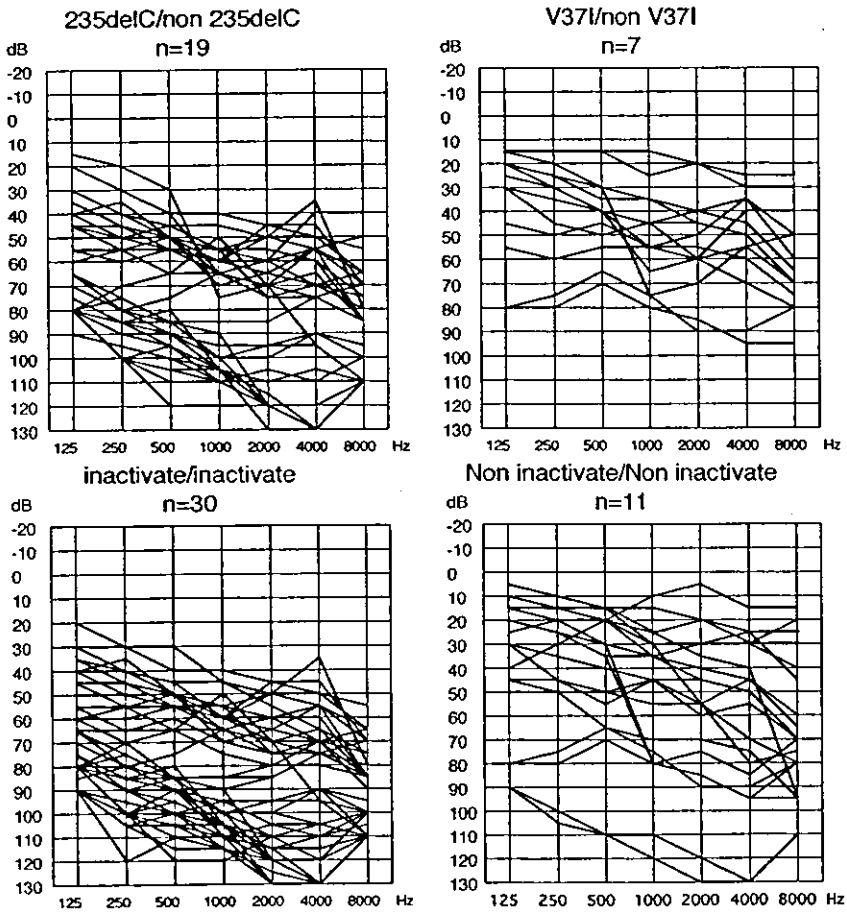

Fig. 2 Overlapping audiograms caused by 235delC/non 235delC, V37I/non V37I, inactivating mutation/ inactivating mutation, and noninactivating mutation/ noninactivating mutation. Note that patients associated with 235delC show relatively severer hearing loss whereas V37I-involved patients show a relatively 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

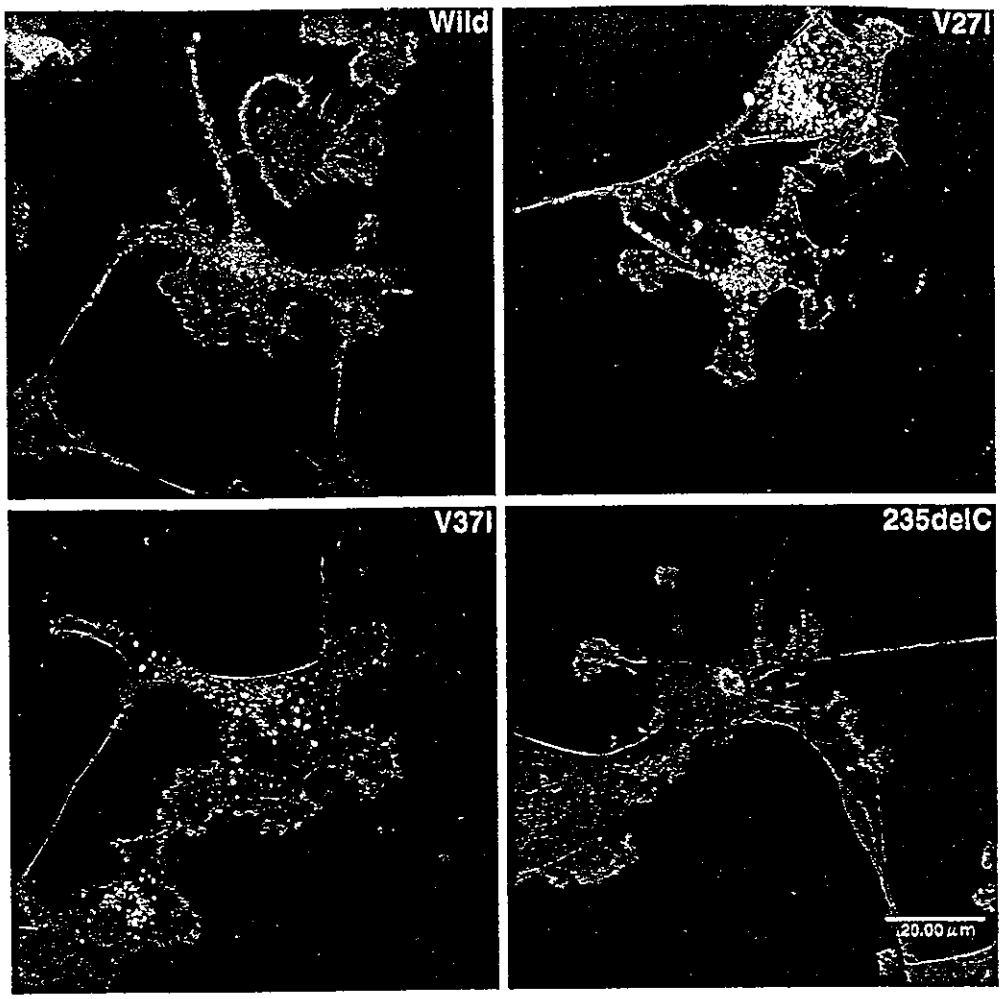

Fig. 3 Protein expression in transfected COS-7 cells. COS-7 cells transfected with GFP-Cx26 wt, GFP-Cx26 V27I, and GFP-Cx26 V37I, which were associated with normal-mild phenotypes, showed a characteristic puncta along the membrane. In contrast, only perinuclear staining was seen in GFP-Cx26 235delC. *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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Deafness Due to A1555G Mitochondrial Mutation Without Use of Aminoglycoside

Tatsuo Matsunaga, MD, PhD; Hiroshi Kumanomido, MD; Masae Shiroma, PhD; Akihiro Ohtsuka, MD; Kenji Asamura, MD; Shin-ichi Usami, MD, PhD

Objectives/Hypothesis: **The objective was to clarify the characteristics of deafness associated with the A1555G mutation within mitochondrial 12S ribosomal RNA gene in the absence of aminoglycoside exposure.** ***Study Design:*** **Clinical and genetic studies in family members with the A1555G mitochondrial mutation were performed.** ***Methods:*** **The subjects were 123 maternally related members of a large Japanese family with the A1555G mutation. All subjects had no previous history of exposure to aminoglycosides. Hearing disability and handicap, tinnitus, and medical histories were analyzed by interviews in all of the subjects, genetic testing was performed in 41 subjects, and pure-tone audiometry was conducted in 26 subjects with hearing disability and handicap.** ***Results:*** **The A1555G mutation was detected in a homoplasmic form (meaning that all the mitochondrial DNA carries the mutation) in all 41 subjects who were screened. The risk for developing postlingual hearing loss was likely to be much higher in the present subjects than in the general population. Both the severity and age at onset of the phenotype were similar in affected subjects within the same sibling group. Pure-tone averages were significantly worse in subjects who developed hearing loss before age 10 years than in those who developed hearing loss later.** ***Conclusion:*** **The present study demonstrated that the prevalence of deafness in individuals with the A1555G mitochondrial mutation was likely to be high even in the absence of aminoglycoside exposure and clearly showed the association of severe to profound hearing loss with the onset of hearing loss before age 10 years.**

Key Words: **Hereditary hearing loss, nonsyndromic, cochlea, mitochondria.**
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INTRODUCTION

The A1555G mutation (i.e., A to G substitution at position 1555) in the mitochondrial 12S ribosomal RNA (rRNA) gene contributes to nonsyndromic sensorineural hearing loss and is prevalent in Japanese[1] and other populations.[2] This mutation is homoplasmic, indicating that all the mitochondrial DNA in different cells and tissues of the subject harbors the mutation. The A1555G mutation was first identified in family members who exhibited aminoglycoside-induced deafness,[3,4] and this ototoxic susceptibility has its basis in the increased binding affinity of aminoglycosides for A1555G rRNA.[5] In addition, this mutation may also induce hearing loss in the absence of aminoglycosides.[3,6–12] The expressivity of the A1555G mutation varies even within the same family and is not strictly associated with aminoglycoside exposure. Prevalence, onset, and severity of the A1555G mutation–associated hearing loss in the absence of aminoglycoside exposure have not been characterized. Furthermore, what modulates the expressivity of this mutation is not clear.

We identified a large Japanese family in which the A1555G mutation was prevalent. All the family members were not previously exposed to aminoglycosides. We conducted genetic tests, interviews, and pure-tone audiometry in maternally related members of this family and elucidated the prevalence, characteristics, and intrafamilial patterns of the auditory dysfunction associated with the A1555G mutation in the absence of aminoglycoside exposure.

PATIENTS AND METHODS

Subjects

Subjects were maternally related members of a large Japanese family in which hearing loss was common in a pattern that was compatible with maternal inheritance (Fig. 1). Because of the inheritance pattern, mutations in the mitochondrial DNA was suspected and the following genetic analysis in the proband (V-10) revealed homoplasmic A1555G mutation in the mitochondrial 12S rRNA gene. There were 124 maternally related family members in all (57 male and 67 female family members) comprising

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From the Department of Otolaryngology (T.M.), National Tokyo Medical Center, Tokyo, Japan; the Department of Speech-Language Pathology and Audiology (T.M., H.K., M.S.), School of Health Science, International University of Health and Welfare, Tochigi, Japan; and the Department of Otorhinolaryngology (A.O., K.A., S-I.U.), Shinshu University School of Medicine, Matsumoto, Japan.

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Send Correspondence to Tatsuo Matsunaga, MD, PhD, Department of Otolaryngology, National Tokyo Medical Center, Higashigaoka 2-5-1, Meguro-ku, Tokyo 152-8902, Japan. E-mail: tmatsuna@ntmc.hosp.go.jp