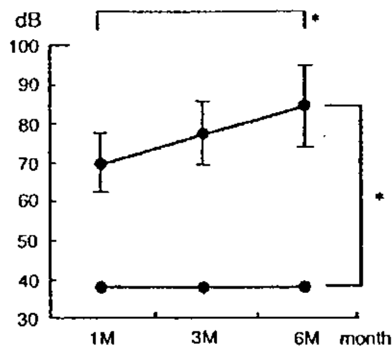

Fig. 2. Time course of ABR thresholds for wild and homozygous type IX collagen knock-out mice using click stimuli. Note the progressive nature of hearing impairment in knock-out mice. *$P < 0.01$ (Bonferroni/Dunn test.).

burst stimuli), and 95.8 dB (SD = 13.5) (32 kHz tone burst stimuli). Differences between the two groups were statistically significant in all frequencies ($P < 0.01$). Most prominent in our data was the progressive hearing loss in knock-out mice with increasing age, in contrast with wild mice, which showed normal hearing levels until 6 months of age (Fig. 2).

3.2. Morphological analysis

The morphological abnormality, which was found to be restricted within the tectorial membrane, is already recognized in the basal turn at the age of 1 month (Fig. 3F). Such abnormality in shape was progressive and found in the middle turn at the age of 3 months (Fig. 3H). Morphometrical analysis showed that significant difference is already noted in the basal turn at 1 month (Fig. 4C) and occurred in the middle turn at 3 months (Fig. 4B). In the apical turn, no significant difference at the light microscopic level was noted until 6 months (Fig. 4A). Electron microscopy confirmed that the tectorial membrane is composed of two different components, radial collagen fibril bundles and striated sheet matrix (Fig. 5A–C). The former fibers were predominantly found in the basal turn, and gradually decreased toward the apical turn (Fig. 5A–C). In knock-out mice, fibers were disorganized and aggregated and fused fiber-like structures were observed in all turns (Fig. 5D–F). Furthermore, many vacant regions were recognized among the disorganized fibers (Fig. 5D–F).

4. Discussion

The present morphological as well as physiological analysis showed that homozygous type IX collagen knock-out mice have progressive hearing loss. The morphological changes are correlated with the progressive hearing loss detected in ABR. The morphological changes in the tectorial membrane at the light microscopic level may be mainly due to disorganization of the collagen fibers detected by electron microscopy. With regard to the composition of the tectorial membrane, electron microscopic study indicated that it is composed of two different components, radial collagen fibril bundles and striated sheet matrix (Goodyear and Richardson, 2002). Previous immuno-electron microscopic findings further indicated that type II and IX collagens are co-

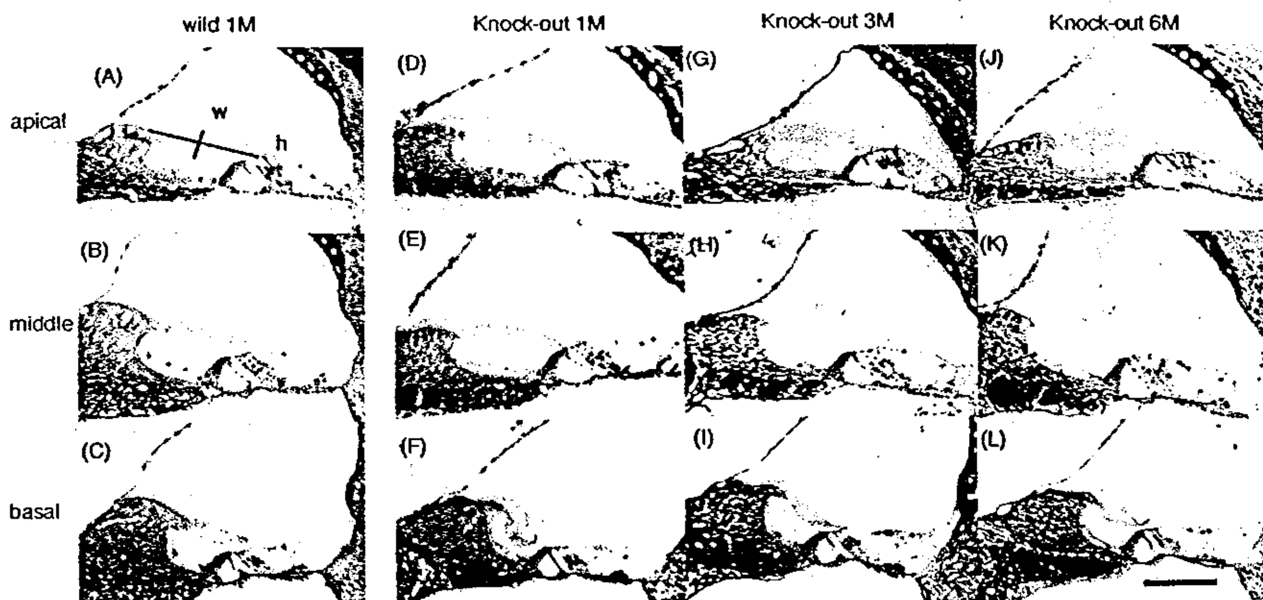

Fig. 3. Light microscopic findings of the cochlea in 1-month-old wild mice (A–C), 1-month- (D–F), 3-month- (G–I), and 6-month- (J–L) old knock-out mice. Note the crooked shaped tectorial membrane in the basal turn in 1-, 3-, and 6-month-old mice, and the middle turn in 3-, and 6-month-old mice. In the wild mouse, tectorial membrane appeared to be normal in shape until 6 months. Height (*h*) was defined as the maximum height measured on the photograph of the tectorial membrane of the cochlea, and the width (*w*) as the maximum length perpendicular to the height (A). Apical turn (A, D, G, J), middle turn (B, E, H, K), basal turn (C, F, I, L). Bar = 100 μm.

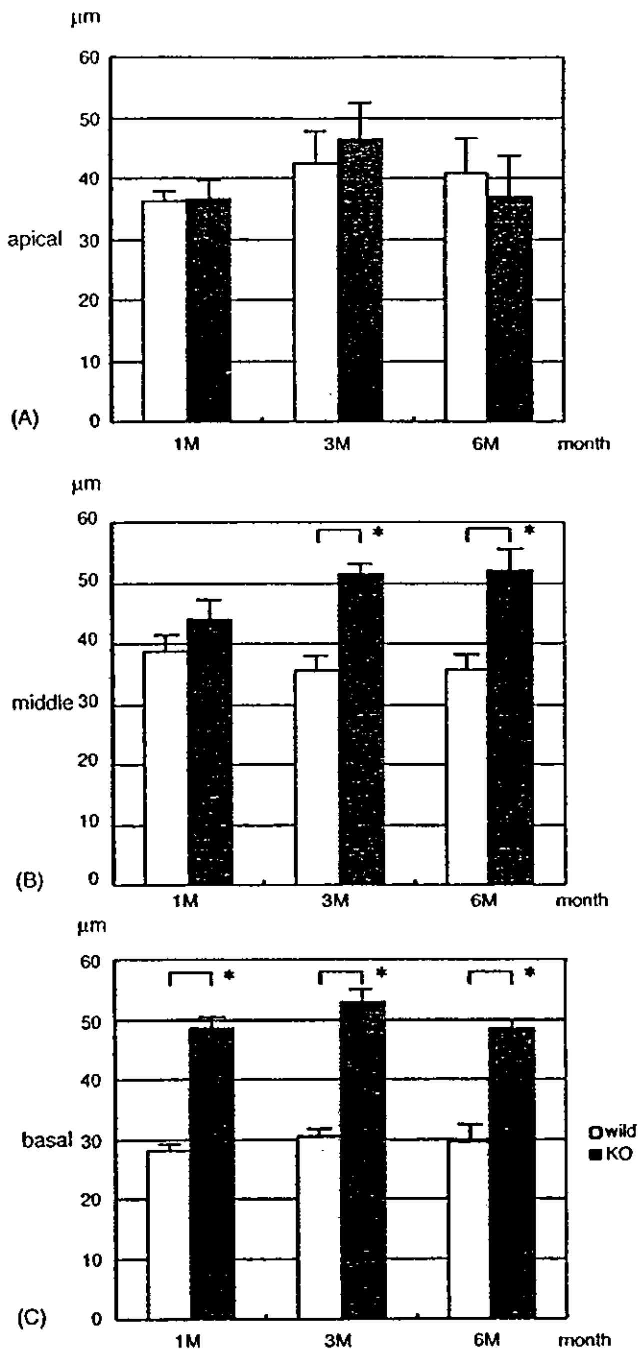

Fig. 4. Width of the tectorial membrane of the apical turn (A), middle turn (B), and basal turn (C) for wild and homozygous type IX collagen knock-out mice. Significant difference is already noted in the basal turn at 1 month and occurred in the middle turn at 3 months. In the apical turn, no significant difference was noted until 6 months. $^{*}P < 0.01$ (Mann–Whitney).

localized in the former collagen fibril bundles. Our recent electron microscopic study indicated that type IX collagen may contribute to the three-dimensional integrated structure of type II collagen molecules, and absence of collagen fibril bundles in homozygous type IX collagen knock-out mice. We also demonstrated that type II collagen is co-localized with type IX collagen in the tectorial membrane, but type II collagen failed to be immunocytochemically detected in the tectorial membrane of homozygous 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 (Asamura et al., 2004).

Some discrepancy exists between light microscopic findings and electron microscopic findings; light microscopic morphology remained intact until 6 months in the apical turn, but electron microscopic findings demonstrated aggregated and fused collagen fiber-like structures, similar to those seen in the basal turn, in the apical turn of 1-month-old mice (Fig. 5D). This could be explained by the regional differences in composition within the tectorial membrane. A previous study indicated that collagen fibrils are not distributed homogeneously in the tectorial membrane, but there is a radial gradient of fibril concentration (Weaver and Schweitzer, 1994). According to that study, the concentration of fibrils in the limbal (inner) zone of the tectorial membrane is greater than that in the marginal (outer) zone in all of the investigated cochlear locations. This gradient is greatest in the basal, high frequency coding region of the cochlea. The present electron microscopic findings supported the predominant distribution of radial collagen fibril bundles in the basal turn (Fig. 5A–C). This could explain why the tectorial membrane in the basal turn is more prone to be crooked.

The all frequency involved, progressive nature of hearing loss found in this study was associated with the predominant and progressive morphological changes in the basal turn (and later middle turn as well). ABR thresholds obtained using tone burst stimuli of 8, 16, 32 kHz corresponded to the region which is located 40.6%, 58.6% and 75.6%, respectively, from the apex of the cochlea (Ou et al., 2000). In this respect, two-thirds of the cochlea is already functionally damaged whereas light microscopically it has a normal appearance. Such disturbance of mechanical property is due to the disorganization of the collagen fibers as described above. At the light microscopic level, the tectorial membrane of the middle turn is not crooked in 1-month-old knock-out mice. However, in the electron microscopic level, the tectorial membrane was already associated with disorganized collagen fibers. These abnormalities in collagen fibrils may result in a change of the elasticity of the tectorial membrane, which may cause moderate hearing loss at 8 kHz.

These data are well correlated with the clinical data of the patients with *COL9A3* mutations who were reported to have a moderate, all frequency involved, progressive, bilateral sensorineural hearing impairment (Asamura et al., 2004). Therefore, the data provided important aspects of progressiveness and frequency unevenness of hearing loss. Homozygous type IX collagen knock-out mice used in this study have been shown to have no detectable abnormalities at birth but to develop non-inflammatory joint diseases including severe joint disease resembling human osteoarthritis (Fassler et al., 1994; Hagg et al., 1997).

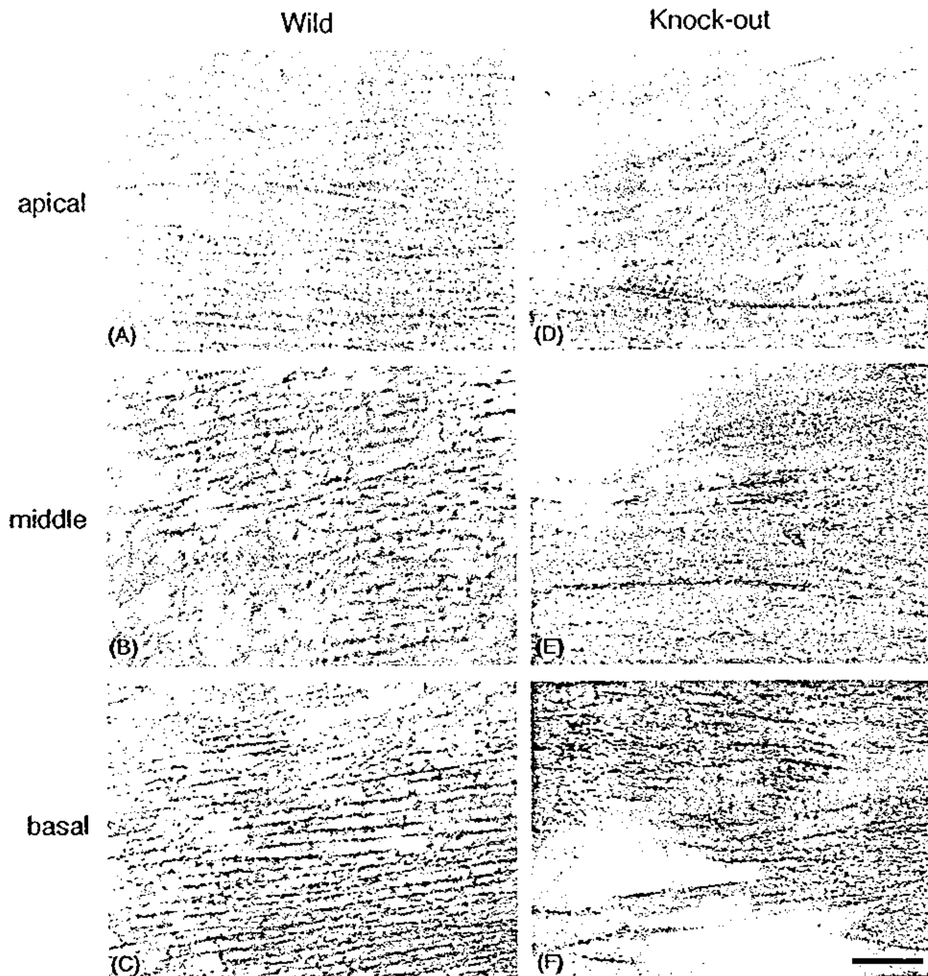

Fig. 5. Electron microscopic findings of the cochlea in 1-month-old wild mice (A–C) and homozygous type IX collagen 1-month-old knock-out mice (D–F) (A, D: apical turn; B, E: middle turn; C, F: basal turn). Tectorial membrane of wild mice shows thick fibers running in well-organized manner (A–C). The thick fibers were predominantly found in the basal turn, and gradually decreased toward the apical turn (A–C). In contrast, tectorial membrane of knock-out mice showed disorganized and aggregated/fused fiber-like structures, and vacant regions are apparent in all turns (D–F). Bar = 200 nm.

5. Conclusion

The type IX collagen knock-out mice analysis revealed progressive hearing loss, and the morphological changes in the tectorial membrane causing it to be abnormal in shape, starting in the basal turn and progressive toward the apical turn. Electron microscopy confirmed the absence of cross-striated patterns in the thick fibers and disorganization of fibrils. These results suggest that type IX collagen may be essential for an intact three-dimensional structure in association with type II collagen or with other molecules. The present study suggests that the mice are also a good animal model for progressive hearing loss caused by mutations in the gene encoding type IX collagen.

Acknowledgements

We thank Dr. Guy van Camp (University of Antwerp) and Dr. Shigeyuki Wakitani (Shinshu University) for their critical comments, and A.C. Apple-Mathews for help in preparing the manuscript. This work was supported by the Ministry of Health and Welfare, Japan (S.U.), and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (S.U.).

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Auris Nasus Larynx xxx (2005) xxx–xxx

AURIS NASUS LARYNX
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Mutation analysis of *COL9A3*, a gene highly expressed in the cochlea, in hearing loss patients

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Received 26 October 2004; received in revised form 6 December 2004; accepted 14 January 2005

Abstract

cDNA microarray analysis indicated that *COL9A3* is one of the highly expressed genes in the cochlea. This suggests that collagen type IX has a crucial functional role in the inner ear and may be a candidate gene for hearing loss. Mutation analysis was carried out to find possible disease-causing mutations in this gene. The direct-sequencing method was applied to the *COL9A3* gene in 159 non-syndromic sensorineural deafness patients and 150 normal controls. Two possible disease-causing mutations were identified: an in-frame deletion of three amino acid residues (G181–P183 del) and a missense mutation (D617E). The patients with the mutations showed a moderate progressive bilateral sensorineural hearing impairment in all frequencies. The present data indicate that mutations of *COL9A3* may cause non-syndromic hearing impairment.
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Keywords: *COL9A3*; Hearing loss; Mutation screening

1. Introduction

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 [1,2]. cDNA microarray analysis indicated that *COL9A3* is one of the highly expressed genes in the inner ear [1], suggesting that collagen type IX has a crucial functional role in the inner ear. Collagen IX is known to be one of the important components together with types II and V collagens in the tectorial membrane of the organ of Corti [3,4]. The tectorial membrane, composed of collagens and non-collagenous glycoproteins, lies over the surface of the organ of Corti and plays a crucial role in the first step of sound transduction. Type IX collagen belongs to the FACIT (fibril-associated collagen with interrupted triplet helices) group of collagens, which is known to bind to the surface of fibril-forming type II collagen [5–7]. The alpha chain of collagen IX contains three triple helical domains (COL1, COL2, and COL3) separated by four non-triple helical (NC1-4) domains [5–7] (Fig. 1). In the present study, we performed screening for mutations in *COL9A3*, a gene reported to be one of those highly expressed in the cochlear tissues.

2. Materials and methods

2.1. Subjects

We screened 147 Japanese and 12 Korean probands with non-syndromic sensorineural hearing impairment. The composition of the subjects was as follows: 95 subjects from autosomal dominant or mitochondrial families (two or more generations affected); 15 subjects from autosomal recessive families (normal hearing parents and two or more

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doi:10.1016/j.anl.2005.01.011

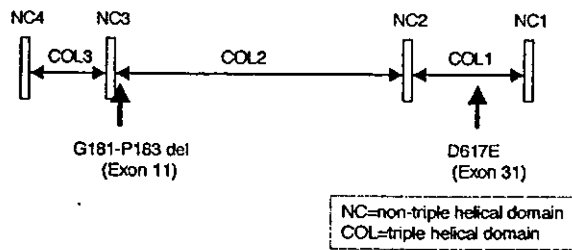

Fig. 1. Scheme showing gene structure of *COL9A3*, which contains three triple helical domains (COL1, COL2, and COL3), separated by four non-triple helical (NC1-4) domains. Arrows indicate the mutations that have been found in this study.

affected siblings); 49 subjects with sporadic deafness (also compatible with recessive inheritance or non-genetic hearing loss). None of the patients had any other associated neurological signs. The control group consisted of 150 unrelated Japanese individuals with normal hearing evaluated by auditory testing. All subjects gave prior informed consent for participation in the project and the study was approved by the Ethical Committee of Shinshu University.

2.2. Mutation analysis

All 32 exons and flanking intronic sequences except for exon 1 of the *COL9A3* gene [8] were amplified by polymerase chain reaction PCR. Primers were designed to flank all of the exon–intron boundaries through use of the Primer3 web-based server (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Each genomic DNA sample (40 ng) was amplified, using Ex-Taq polymerase (Takara), for 5 min at 95 °C, followed by 37 three-step cycles of 95 °C for 30 s, 53–63 °C for 30 s, and 72 °C for 1.5 min, with a final extension at 72 °C for 10 min, ending with a holding period at 4 °C in a Perkin-Elmer thermal cycler. The PCR products varied in size at about 230–760 bp, and they were treated with 0.1 μl exonuclease I (Amersham) and 1 μl shrimp alkaline phosphatase (Amersham) by incubation at 37 °C for 30 min, and inactivation at 80 °C for 15 min. After the products were purified, we performed standard cycle-sequencing reactions with ABI Big Dye terminators in an ABI 377 autosequencer (Applied Biosystems).

Table 1
Nucleotide changes detected by mutation analysis of the *COL9A3* gene

CHANGE	EXON	AMINO ACID	ALLELE FREQUENCY/CHROMOSOME	
			In Patients	In Control Subjects
308 G>A	5	R103Q	6/318	4/300
541-549 9bp del	11	G181-P183 del	2/318	0/300
1304 C>A	25	A435E	6/318	4/300
1649 C>T	30	P550L	2/318	0/300
1740 T>C	30	P580P	79/318	45/300
1851 C>A	31	D617E	2/318	0/300
2044 C>A	32	R2044R	16/318	Not done

3. Results

Direct DNA-sequencing identified two possible disease-causing mutations as well as five polymorphisms (Table 1). One mutation was a homozygous nine-base-pair (in-frame) deletion at position 541–549 in exon 11, removing a Gly–Pro–Hypro triplet in the 5′-end of the COL2 domain (Fig. 2). This mutation was detected in a 38-year-old female, who was affected by a moderate progressive bilateral sensorineural hearing impairment in all frequencies. Her parents were consanguineous, but not available for testing. Anamnestically, the onset of hearing impairment was at about the age of 5 and gradually progressed. She had a history of vertigo at the age of 35, but had no abnormal findings in a temporal bone CT scan. Aside from hearing loss, she was phenotypically normal. Consanguinity is a strong indication of true homozygosity and it is unlikely that the paired allele is a different large deletion leading to hemizygosity of the nine-base-pair deletion. Even if it were, the argument that this person's hearing loss is due to a defect in both *COL9A3* alleles would remain strong.

There is weaker evidence of possible dominant inheritance. This was seen in two families with a missense mutation, D617E (1851 C > A) in exon 31 (Fig. 3), localized in the COL1 domain. It was found in two (one Japanese and one Korean) independent autosomal dominant families (Fig. 3). The affected individuals with this missense mutation had moderate bilateral all frequency involved progressive sensorineural hearing loss unassociated with other symptoms. The age at onset of hearing impairment was 30 and 41 years, respectively. The probands did not have mutations in any of the other genes known to cause deafness at an appreciable frequency (*GJB2*, *SLC26A4*, or the 1555A > G mutation in the mitochondrial 12S rRNA) (data not shown).

4. Discussion

The present mutation analysis in the *COL9A3* gene detected two possible disease-causing mutations; an in-frame deletion of three amino acid residues (G181–P183 del) in the COL2 domain and a missense mutation (D617E)

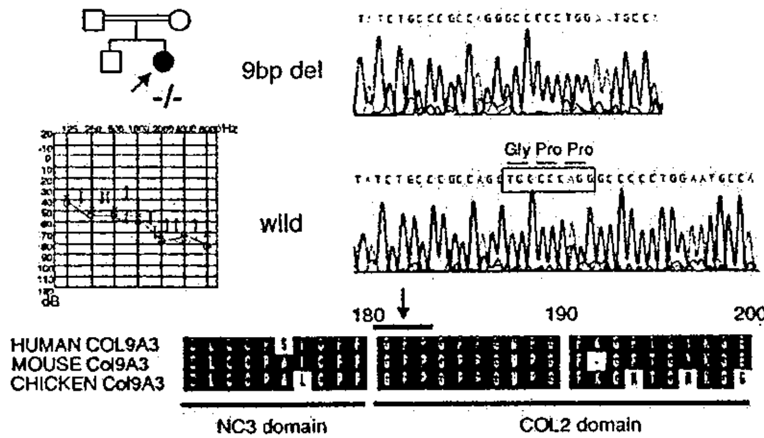

Fig. 2. Electrophoregram, pedigree, and audiogram of the affected family with the G181–P183 del mutation. Electrophoregram showing the mutation in the *COL9A3* gene. Corresponding normal sequence is shown in the lower panel. Alignment of deduced amino acid sequences of the *COL9A3* genes of human, mouse, and chicken. The conserved amino acids are shown with black background and the non-conserved amino acids are shown with white background. Dot shows gap in the alignment. Arrow indicates mutation point found in the present study, showing conservation between human, mouse, and chicken.

in the COL1 domain (Fig. 1). Both mutations were found in domains, where mutations had never been reported [9–11]. The deletion in the COL2 domain appears to be recessive. The missense mutation in the COL1 domain appeared dominant. Each family was too small and only a limited number of DNA samples was available. The recessive mutation is likely a pathologic rather than a rare or functionally neutral change because: (1) it was not found in any of the controls or SNP databases (http://snp.ims.u-tokyo.ac.jp/), (2) it involved a deletion of amino acid residues that are all conserved in the mouse and the chicken, and (3) the pattern of audiogram (all frequency involved), severity, and progressiveness is reminiscent of the type IX collagen knock-out mouse [12,13] (Fig. 2).

The dominant mutation (D617E) is also a candidate for pathology because: (1) it was not found in any of the controls or SNP databases (http://snp.ims.u-tokyo.ac.jp/), (2) it occurred at a highly conserved amino acid residue, and (3) the hearing loss is moderate and progressive, similar to the type IX collagen knock-out mouse [12,13] (Fig. 3). It is not unusual to see different mutations in the same gene having different expressions. *MYO7A* mutations, for example, are seen in both dominant and recessive families [14,15]. The association of splice mutations in exon 2 with dominantly

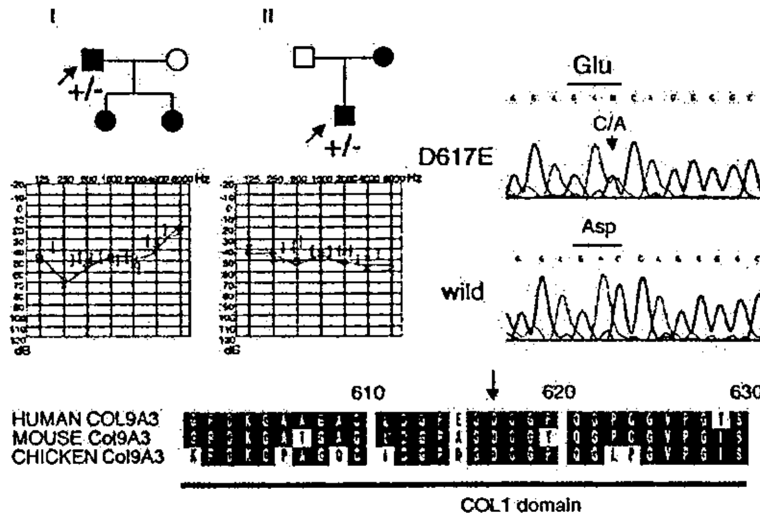

Fig. 3. Electrophoregrams, pedigrees, and audiograms of the affected families with the D617E mutation. Electrophoregram showing the mutation in the *COL9A3* gene. Corresponding normal sequence is shown in the lower panel. Alignment of deduced amino acid sequences of the *COL9A3* genes of human, mouse, and chicken. The conserved amino acids are shown with black background and the non-conserved amino acids are shown with white background. Arrow indicates mutation point found in the present study, showing conservation between human, mouse, and chicken.

inherited multiple epiphyseal dysplasia (MED; MIM132400) [9–11] clearly demonstrate that *COL9A3* mutations can present with dominant inheritance.

Five neutral variants, which did not fulfill the above criteria, were also found in this study (Table 1). Among them, we could not conclude whether P550L, which was localized in the NC2 domain and was detected in two independent families, is a pathologic mutation. P550L occurred at conserved amino acid residues and was not found in any of the control samples, but was not associated with the hearing loss within the family.

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*) [16–18], Alport syndrome (*COL4A3*, *COL4A4*, and *COL4A5*) [19,20], and DFNA13 (*COL11A2*) [21]. These collagens are localized in the tectorial membrane, basement membrane, spiral ligament, and the spiral limbus [3,4,22–24].

Collagen IX was known to be one of the important components, together with types II and V collagens, in the tectorial membrane of the organ of Corti [3,4]. Recently, we have demonstrated that the type IX collagen knock-out mice showed progressive hearing loss, and are associated with the abnormal shape of the tectorial membrane, in which organization of the collagen fibrils were disturbed [12,13]. These data obtained in knock-out mice are well correlated with the clinical data of the patients with *COL9A3* mutations, who were reported here to have a moderate, all frequency involved, progressive, bilateral sensorineural hearing impairment. Such characteristic audiological features closely resemble those found in patients with deafness caused by mutations in the gene encoding the collagens in the tectorial membrane (DFNA13, caused by *COL11A2* mutations and Stickler syndrome, caused by *COL2A1*, *COL11A1*, and *COL11A2* mutations), although some were reported to be non-progressive [25,26].

According to Eyre et al. [6], COL1 and COL2 domains are crucial to maintain the correct linear distance between type II collagen molecules. The mutations in COL1 and COL2 domains found in this study are predicted to affect the three-dimensional structure of the triple-helix domain of the collagen protein and may lead to abnormal integrity of collagen fibers in the tectorial membrane.

COL9A3 is known to be responsible for MED [9–11]. *COL9A3* gene mutations responsible for MED are clustered in the acceptor splice site of intron 2, resulting in the skipping of exon 3 of the *COL9A3* gene [9–11]. Whether non-syndromic hearing loss found in the present study is a continuum phenotype caused by *COL9A3* requires further discussion, because: (1) the present patients did not have short stature or early-onset osteoarthritis, characteristic features of MED and (2) hearing loss has not been reported in MED patients. However, it is noted that mutations causing MED have only been found in the COL3 domain, whereas mutations causing non-syndromic hearing loss were found in the COL1 and COL2 domains. Therefore, the phenotypic consequence is possibly positionally dependent, as postulated in *COL11A2* [21]. It is also possible that two diseases that have been classified into different categories are both part of a continuous spectrum of disease caused by *COL9A3* with overlapping phenotypes, because of their relative mild phenotypes and delayed onset.

Acknowledgements

We thank all the subjects that participated in the present project. We also thank Dr. Hee-Nam Kim (Yonsei University College of Medicine) and Dr. Tetsuo Watanabe (Oita Medical School) for sending samples of their patients, Dr. Guy van Camp (University of Antwerp), Dr. William J. Kimberling (Boys Town National Research Hospital), and Dr. Shigeyuki Wakitani (Shinshu University) for their critical comments, and A. C. Apple-Mathews for help in preparing the manuscript. This work was supported by a Health Sciences Research Grant (Research on Eye and Ear Science, Immunology, Allergy and Organ Transplantation) from the Ministry of Health and Welfare of Japan (S.U.), the Acute Profound Deafness Research Committee of the Ministry of Health and Welfare of Japan (S.U.), and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (S.U.).

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Auris Nasus Larynx xxx (2005) xxx–xxx

AURIS NASUS LARYNX
INTERNATIONAL JOURNAL OF ORL & HNS

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Genetic features, clinical phenotypes, and prevalence of sensorineural hearing loss associated with the 961delT mitochondrial mutation

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Received 26 October 2004; received in revised form 6 December 2004; accepted 14 January 2005

Abstract

To examine the frequency of the 961delT mitochondrial point mutation, considered to be associated with aminoglycoside-induced hearing loss, restriction fragment length polymorphism (RFLP) analysis was performed in (1) 334 unrelated sensorineural hearing loss (SNHL) patients and (2) 56 patients with aminoglycoside antibiotic injection history. Approximately 2% of the SNHL patients had the 961delT mutation, raising the possibility of a relatively high prevalence of this mutation among hearing impaired populations. However, the following findings cast doubt on whether this mutation is truly associated with hearing loss: (1) a similar frequency found in the control subjects, (2) hearing loss that was not segregated within the families, (3) rates of heteroplasmy and aging that were not correlated with the severity of hearing loss, and (4) a low prevalence among the aminoglycoside-induced hearing loss patients (1/56 = 1.8%). The present analysis did not agree with the concept that the 961delT mutation causes aminoglycoside-induced hearing loss.
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Keywords: Mitochondria; 961delT; Hearing impairment; Aminoglycoside antibiotics

1. Introduction

Mitochondrial mutations have been demonstrated to be responsible for syndromic as well as non-syndromic hearing impairments (see Hereditary Hearing Loss Homepage [http://dnalab-www.uia.ac.be/dnalab/hhh/]).

One mitochondrial gene in particular, *12S rRNA*, has received much attention in relation to aminoglycoside-induced hearing loss. To date, three mutations in this gene, 1555A > G, 1494C > T, and 961delT, have been reported as possibly associated with aminoglycoside-induced hearing loss [1–6]. A series of reports have provided evidence that patients with the 1555A > G mutation have susceptibility to aminoglycosides, and subsequent hearing loss even after exposure to small dosages [1,3,4]. The 1555A > G mutation was also demonstrated to cause hearing loss even without aminoglycoside injection, and consequently may be related to inner ear susceptibility. It may be therefore, that the mutation manifests itself as a diminished ability to repair cochlear damage from a variety of causes, including noise, aging, etc. [1,3,4]. Recently, our wide screening revealed a high frequency of the 1555A > G mutation among hearing impaired populations [7].

The 1494C > T mutation has recently been reported in a large Chinese family with maternally transmitted aminoglycoside-induced and non-syndromic deafness [6]. Clinical features indicated that the injection of aminoglycoside antibiotics induced or worsened deafness. In vitro experiments indicated significant average increase in doubling time in lymphoblastoid cell lines derived from the individuals with the 1494C > T mutation when compared to four control cell lines.

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doi:10.1016/j.anl.2005.01.010

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In contrast to the 1555A > G and 1494C > T mutations, the status of 961delT is rather ambiguous. This mutation was first reported in one out of 35 sporadic patients with a history of aminoglycoside exposure [2]. Two later reports described both a family and a single patient with aminoglycoside-induced hearing loss with this mutation [5,8]. However, little is known about the genetic features, clinical phenotypes, and frequency of this mutation among neither the hearing impaired nor the general populations. In the present study, details of the 961delT mitochondrial mutation in the hearing impaired population in Japan was evaluated.

2. Materials and methods

The frequency of the 961delT mutation was examined using restriction fragment length polymorphism (RFLP) analysis in two groups of Japanese. Group 1 consisted of 334 unrelated sensorineural hearing loss (SNHL) patients and group 2 consisted of 56 patients who had a history of aminoglycoside injection. Two control groups were employed: (1) 176 subjects who had no hearing loss as evaluated with pure tone audiometry, (2) 366 individuals from the general population from several regions in Japan. All participants in the project had provided written informed consent.

All patients had also been examined for mutations in the *GJB2* gene [MIM 121011] and the 1555A > G mutation in the mitochondrial *12S rRNA* gene [MIM 561000], both described as common causes of deafness [7,9,10].

The 334 subjects of group 1 had a mean age of 21.9 years (range: 0–78) and had visited outpatient clinics due to SNHL. Pure-tone audiograms were performed for 300 patients. Hearing of the 34 patients under 3y.o. was evaluated by auditory brainstem response (ABR) or conditioned orientation reflex audiometry (COR). Eight subjects had a history of aminoglycoside injection. There were no other associated neurological signs. The mean age of onset was 12.8 years (range: 0–76). One hundred and seventy-four subjects had a family history with one or more other family members having hearing loss (80 compatible with autosomal dominant or mitochondrial, 94 with autosomal recessive). Severity was classified by using a pure-tone average over 500, 1000, 2000 and 4000 Hz in the better hearing ear. The mean hearing level of the 300 subjects was 59.2 dB, 39% of the subjects had mild hearing loss (up to 49 dB), 22.0% had moderate (50–69 dB), 18.4% severe (70–89 dB) and 20.6% profound (90 and over dB).

The 56 patients of Group 2 had a mean age of 44.6 years (range: 2–92) and known history of aminoglycoside injection of varying dosage and duration. The mean hearing level of these patients was 72.4 dB, consisting of 16.0% mild, 17.9% moderate, 14.3% severe, and 51.8% profound hearing loss.

Restriction fragment length polymorphism (RFLP) analysis was used to identify the 961delT mutation. MtDNA fragments were amplified using the primer pair, sense 5′-AGCATCAAGCACGCAGCAAT-3′ (bases 754–773) and anti-sense 5′-GGATATGAAGCACCGCCA-3′ (bases 1191–1174). After amplification, expected sizes (438 bp) of PCR products were confirmed on 2% agarose gel and digested with restriction enzyme MnlI (New England Biolabs, Beverly, MA, USA) at 37 °C overnight. As seen in Fig. 1, normal controls had three fragments, while in mutated DNA fragments, loss of the *MnlI* site, caused by the 961delT mutation, resulted in two fragments. The 961delT mutation was confirmed by direct sequencing using an ABI sequencer 377XL (Perkin Elmer Inc., Boston, MA, USA).

Heteroplasmy rate of the 961delT mutation was examined using a fluorescence bio-imaging analyzer FMBIOII® (Hitachi Software Engineering, Tokyo, Japan). For quantitative control, plasmids containing wild-type and 961delT mutation were prepared as follows.

The primers for detecting 961delT with the specific sites of restriction enzymes were sense *Bam*HI-bases 754–773 5′-CCCGGATCCAGCATCAAGCACGCAGCAAT-3′ and anti-sense bases 1191–1174-*Hin*dIII 5′-GGGAAGCTT GGATATGAAGCACCGCCA-3′. Prepared PCR products were inserted into pUC-18 vector (Takara, Tokyo, Japan). The PCR products and vector were digested with *Bam*HI and *Hin*dIII. Ligation reactants were transformed into *Escherichia coli* DH5α. Positive colonies were incubated in Luria–Bertani (LB) liquid medium containing ampicilin. QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA) was used for purification of plasmid DNA according to the manufacturer's protocol.

Patients' DNA samples and mixed plasmid (wild/mutant; 100%, 50%, 25%, 10%, 5%, 0%) were amplified using the sense primer with Rhodamine, sense 5′-Rho-AGCATCAAGCACGCAGCAAT-3′ and anti-sense primer (same as above). Digested samples were separated by 6.0% acrylamide gel electrophoresis and visualized by a fluorescence image analyzer. These images were quantitated by Image Analysis Ver.2.0 (Hitachi Software Engineering, Tokyo, Japan) (Fig. 2). All analyses were performed in duplicate.

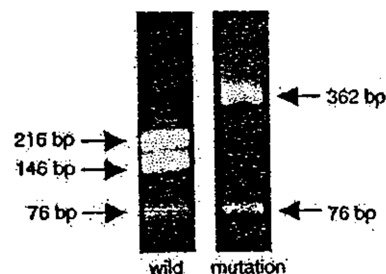

Fig. 1. Restriction patterns of the patients with 961delT mutation (right) and wild type (left). The restriction enzyme divides normal PCR products into three fragments, while in mutated DNA there are two fragments, due to loss of the *MnlI* site, caused by the 961delT mutation.

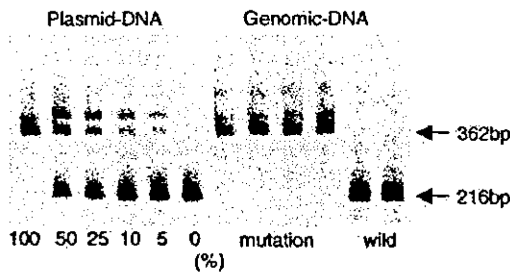

Fig. 2. Digested samples visualized by a fluorescence image analyzer. The left six lanes are mixed plasmid (ratio of mutant: 100%, 50%, 25%, 10%, 5%, 0%, respectively), and the right six lanes are the patients with the 961delT mutation (four lanes) and wild type (two lanes).

3. Results

3.1. Frequency

The 961delT mutation was recognized in 7/334 (2.1%) in the unrelated hearing impaired patients (group 1) and 1/56 (1.8%) in the patients with aminoglycoside injection history (group 2). This mutation was also detected in 2/176 (1.1%) in the subjects without hearing loss (control 1) and 8/366 (2.2%) in the general population (control 2). There were no statistically significant differences between the groups (Mann–Whitney *U*-test).

3.2. Heteroplasmy rate

As illustrated in Fig. 1, digested samples visualized on an agarose gel represented a homoplasmic pattern. The precise rate of heteroplasmy assessed by a fluorescent image analyzer was from 97% to 100%.

3.3. Segregation

Available pedigrees of the patients associated with the 961delT mutation are shown in Fig. 3. One patient was found in a dominant family, three patients were found in recessive families, and one patient was found in a simplex family. Pedigrees indicated that hearing loss phenotype is not associated with the 961delT mutation.

3.4. Hearing assessment

The mean hearing level of the patients with the 961delT mutation was 34.7 dB, 66.7% of the subjects had mild hearing loss (up to 49 dB), 22.2% had moderate (50–69 dB), 5.6% severe (70–89 dB) and one patient had profound (90 and over dB) loss. Overlapping audiograms collected from the subjects who had the 961delT mutation are shown in Fig. 4. There is a wide variety of hearing impairment from normal to high frequency predominant and mild to severe.

3.5. Other involved genes

All patients with the 961delT mutation had been examined for mutations in the *GJB2* gene and the 1555A > G mutation in the mitochondrial *12S rRNA* gene. Among the 334 unrelated hearing loss subjects, 13 patients (3.9%) had mutations in the *GJB2* gene and nine patients (2.7%) had the 1555A > G mutation. Among the 56 patients with known aminoglycoside antibiotic injection, 11 patients (19.6%) had the 1555A > G mutation. These frequencies together with the 961delT mutation are summarized in Table 1. None of the patients had both the 961delT and the1555A > G mutations, but one patient was associated with homozygous 235delC and the 961delT mutations and showed profound hearing loss (asterisks in Fig. 4).

4. Discussion

4.1. Frequency

Approximately 2% of patients with SNHL possessed the 961delT mutation, suggesting the possibility that it may have an important contribution to the etiology of hearing loss patients in Japan. However, a similar frequency was also found in the control populations, raising the question of whether the 961delT mutation is a sole cause of hearing loss or not. In this study, we used two different control groups: control 1 consisting of subjects in whom pure tone audiometry confirmed the absence of hearing loss, and control 2 who were randomly selected from the general population in Japan. Although the mutation frequency (1.1%) in control 1 seemed to be somewhat lower than that in SNHL patients (2.1%), statistical assessment did not show any significant difference ($p = 0.509$; Mann–Whitney *U*-test). Moreover, a similar frequency was found in the general population (2.2%), indicating that the 961delT mutation is commonly found in the general population.

4.2. Relationship to aminoglycoside ototoxicity

In this study, only one out of the 56 patients (1.8%) with a history of aminoglycoside injection had the 961delT mutation, therefore the significance of this mutation in relation to aminoglycoside-induced hearing loss was not evident. This is in contrast to the 1555A > G mutation, which was present in only 2.7% (9/334) in group 1, but in 19.6% (11/56) in group 2. This high frequency of the 1555A > G mutation among patients with a history of aminoglycoside injection is compatible with our previous report [10], and further confirmed the high genetic susceptibility of this mutation to aminoglycoside ototoxicity.

4.3. Segregation

We were unable to find any families similar to that reported by Casano et al., in which hearing loss and the

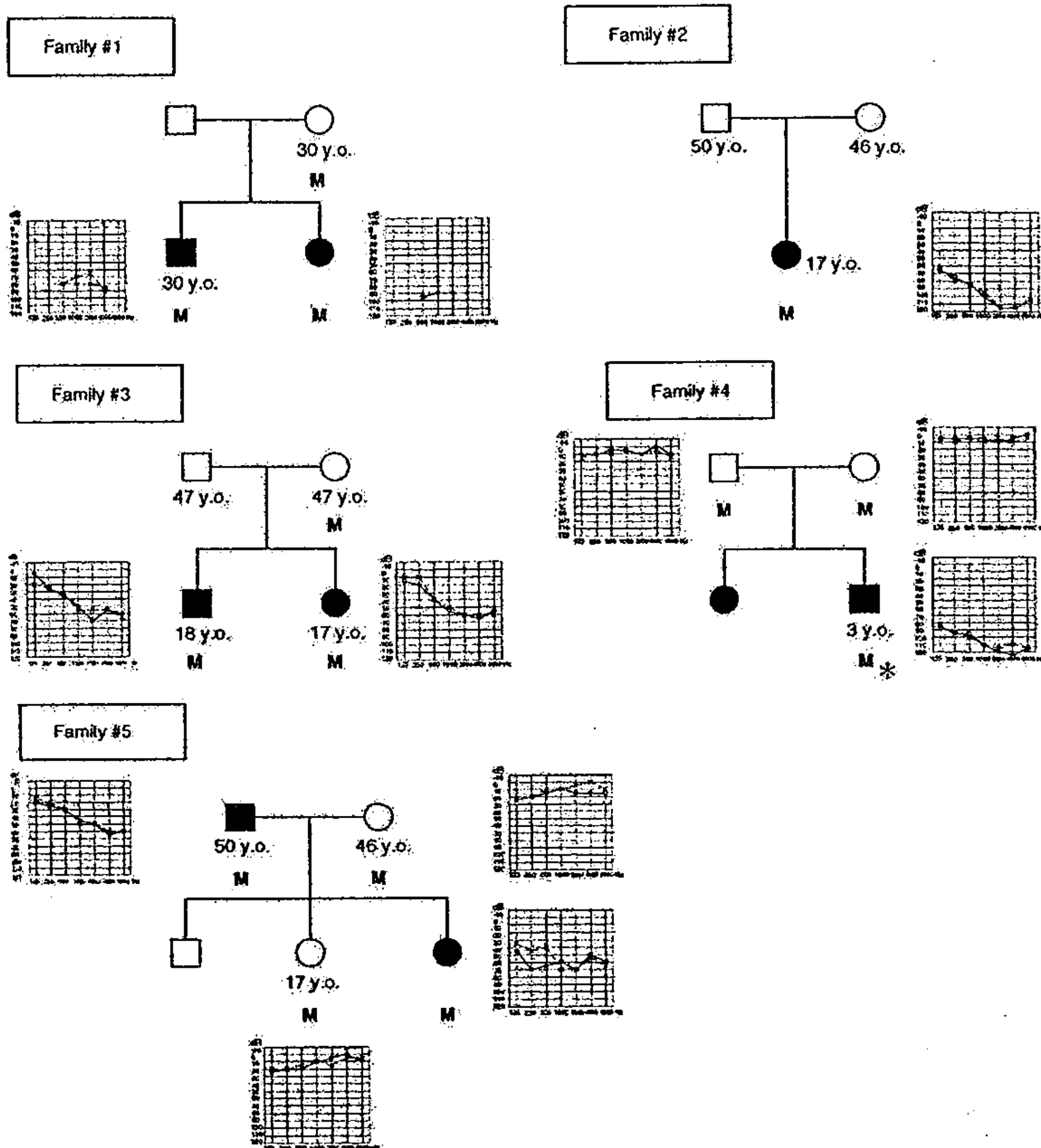

Fig. 3. Pedigrees with individual subjects' audiograms. "M" indicates patients associated with the 961delT mutation. Note that even though the 961delT mutation is inherited through the mother, hearing loss is not. One patient (asterisk) of family #4 was homozygous for the 235delC mutation in the *GJB2* gene.

mutation were co-segregated [5]. As shown in Fig. 3, hearing loss phenotype was not associated with the 961delT mutation, indicating that hearing loss may possibly occur independently of the 961delT mutation.

4.4. Clinical phenotypes

Concerning phenotypes caused by the 961delT mutation, two previous reports described moderate to severe high frequency-involved SNHL [5,8]. In contrast, in this study, overlapping audiograms collected from the subjects with the 961delT mutation showed a wide variety of hearing impairment from normal to high frequency involved mild to severe hearing loss (Fig. 4). It is unlikely from the present results that the 961delT mutation solely causes hearing loss. It may be that the rate of aging and/or heteroplasmy contributes to the severity of hearing loss. To assess this possibility, overlapping audiograms were divided into four age groups: 0–19, 20–39, 40–59 and over 59. However, the severity of hearing loss was independent of age (data not

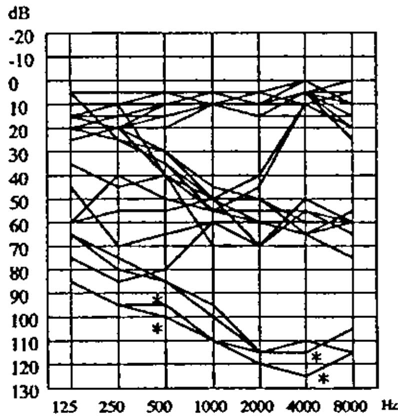

Fig. 4. Overlapping audiograms from subjects with the 961delT mutation. There is a wide variety of hearing impairment from normal to mild to severe hearing loss. One patient was associated with homozygous 235delC mutations (asterisks).

shown). With regard to the rate of heteroplasmy, all the samples were between 97% and 100%, and there were no large variations that could have caused such a variety of hearing levels.

4.5. Other involved genes

One patient with the 961delT mutation was also associated with homozygous 235delC mutations. The patient showed profound hearing loss (asterisks in Fig. 4), in agreement with reports of the *GJB2* mutations causing moderate to severe hearing loss [11]. Recent genotype/phenotype correlation study in the Japanese revealed that most of the patients with the 235delC mutation showed comparatively more severe hearing loss [12].

In conclusion, the present detailed genetic as well as clinical analysis using a relatively large cohort did not agree with the concept that the 961delT mutation causes aminoglycoside-induced hearing loss. Such discrepancy with conclusions from previous studies may have occurred because of the difference in applied techniques and control evaluation. The first report by Bacino et al. used SSCP and heteroduplex methods [2], which are more indirect and insensitive approaches, to identify the 961delT mutation. Therefore, they may have passed over a subtle base change including the 961delT mutation as also suggested by Tang et al. [13]. The following two case reports lacked control evaluation [5,8] as well as segregation analysis [8], which must be performed to prove that a mutation is disease causing. Recently, three nucleotide changes, 961T > G, 956–960insC, and 961insC, in the vicinity of the nucleotide 961 were reported [13,14]. These data suggested that this region may be more variable, and therefore a careful assessment will be needed to discuss whether these changes are associated with disease or not.

Acknowledgments

We thank all the subjects that participated in the present project. We would also like to thank Ms. Yukari Nakamura, Ms. Naoko Anaguchi, and Ms. Yumi Saito for technical assistance, Ms. A.C. Apple-Mathews for help in preparing the manuscript, and Dr. Yoshimitsu Fukushima for providing normal control samples. This study was supported by a Health Sciences Research Grant (Research on Eye and Ear Science, Immunology, Allergy and Organ Transplantation) from the Ministry of Health and Welfare of Japan and by the Acute Profound Deafness Research Committee of the Ministry of Health and Welfare of Japan. To our great sorrow, our co-author Dr. Satoshi Horai passed away suddenly during the preparation of this paper. We are grateful for his contributions to our project and pray for the repose of his soul.

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Table 1
Frequencies of the 961delT and 1555A > G mutations in patients and controls

Subjects	n	961delT	1555A>G
Group 1 (SNHL patients without selection bias)	334	7 (2.1%)	9 (2.7%)
Group 2 (SNHL patients with history of aminoglycoside injection)	56	1 (1.8%)	11 (19.6%)
Control 1 (subjects with no hearing loss)	176	2 (1.1%)	
Control 2 (general population)	366	8 (2.2%)	

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REPRINTED FROM *ANNALS OF OTOLOGY, RHINOLOGY & LARYNGOLOGY*
February 2005 Volume 114 Number 2
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AUDIOLOGICAL FEATURES AND MITOCHONDRIAL DNA SEQUENCE IN A LARGE FAMILY CARRYING MITOCHONDRIAL A1555G MUTATION WITHOUT USE OF AMINOGLYCOSIDE

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To elucidate the pathophysiological and genetic mechanisms of hearing loss associated with the homoplasmic mitochondrial A1555G mutation in the absence of aminoglycoside exposure, we conducted audiological and genetic analyses on 67 maternally related members of a large Japanese family carrying this mutation. A consistent pattern was evident in the audiograms, with features of sensory presbycusis, cochlear origin at all levels of hearing loss, and a high degree of vulnerability of outer hair cells. That the degree of hearing loss was similar in affected subjects within the same sibling group but differed between sibling groups suggests the involvement of nuclear modifier genes. Total mitochondrial DNA sequences were completely identical among subjects with various levels of hearing loss, and lacked additional pathogenic mutations. For the diagnosis of sensorineural hearing loss, the mitochondrial A1555G mutation should be considered when these features are present even in the absence of aminoglycoside exposure.

KEY WORDS — cochlea, hereditary hearing loss, mitochondria, nonsyndromic hearing loss.

INTRODUCTION

Various mitochondrial DNA mutations have been reported to cause hearing loss, either on their own or in association with other clinical symptoms such as neuromuscular disorders and diabetes.[1] The homoplasmic A1555G mutation in the mitochondrial 12S ribosomal RNA gene has been the first mitochondrial DNA mutation to be associated with nonsyndromic sensorineural hearing loss.[2] The A1555G mutation was initially identified primarily in subjects with hearing loss following aminoglycoside exposure. Indeed, it has been reported that the increased binding affinity of ribosomal RNA to aminoglycosides as a result of the mutation constitutes the pathogenetic mechanism underlying ototoxic susceptibility.[3] Subsequently, this mutation was also found in subjects who developed hearing loss in the absence of aminoglycoside exposure.[4-8] In these cases, the clinical phenotype ranged from profound congenital hearing loss to moderate progressive hearing loss of later onset to only slight hearing loss. Although these phenotypic differences may be the result of additional mutations in the mitochondrial or nuclear DNA, or of unknown environmental factors, the exact mechanism has not been determined. Furthermore, the pathophysiological mechanism of hearing loss due to the A1555G mutation in the absence of aminoglycoside exposure has not been defined, because there are no reports on temporal bone histopathology in patients with this mutation, and the audiological evaluation of patients has been limited to pure tone audiometry (PTA) in most previous studies. Only one study carried out detailed audiological evaluations, but most subjects exhibited profound hearing loss.[9] Thus, such detailed audiological evaluations of subjects with various levels of hearing loss, especially those with mild or moderate hearing loss, remain to be performed to uncover the pathophysiological mechanism underlying the development of hearing loss.

We previously identified a large Japanese family in which the A1555G mutation is prevalent. None of the family members were previously exposed to aminoglycosides, and the prevalence of hearing loss in maternally related members was much higher than that in the general population.[10] To further elucidate the pathophysiological and genetic mechanisms of

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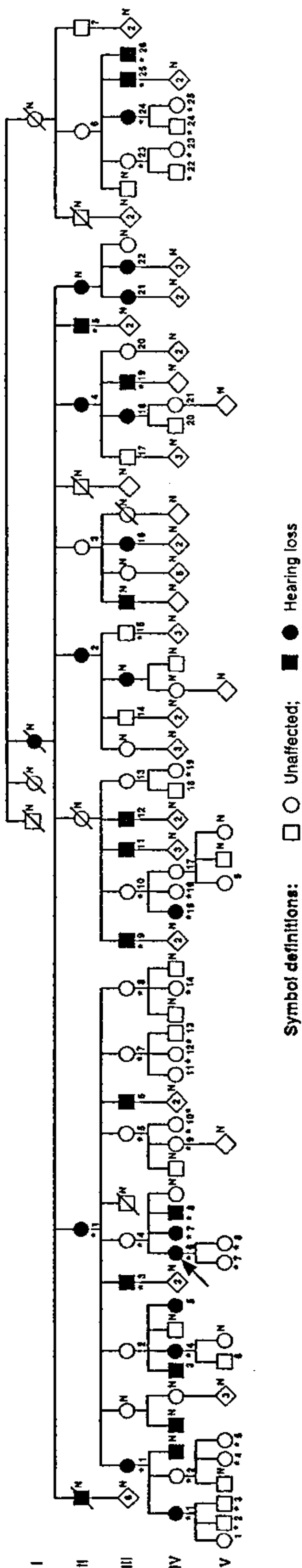

Fig 1. Part of pedigree shows intrafamilial relationship of subjects. Generations are indicated on left in roman numerals, and numbers under symbols represent identification numbers. Family members who were not subjects of this study are indicated by "N" to upper right of symbol. Subjects who reported hearing loss by interview are indicated by solid symbols. Asterisks indicate subjects who were previously tested for A1555G mutation. Arrow indicates proband of family.

the hearing loss due to this mutation, we conducted a battery of audiological tests and sequenced the entire mitochondrial DNA in maternally related members of this family.

MATERIALS AND METHODS

Subjects. The subjects were 67 maternally related members (23 male, 44 female) of a large Japanese family with the homoplasmic mitochondrial A1555G mutation (Fig 1). During interviews prior to PTA testing, 26 of the 67 subjects reported a hearing loss. The original family included 124 maternally related members in 6 generations. The medical histories, clinical phenotypes, and genetic features of these members have been reported previously.[10] In 123 maternally related members whose information about hearing was reliably obtained by interviews, 33 members (penetrance, 26.8%) were considered to have a hearing disability and handicap. The inheritance pattern was maternal and not paternal in this family. Apart from hearing loss, no other significant defects related to mitochondrial mutations were noted in this family. None of the family members had a history of aminoglycoside exposure. All 41 maternally related members who were tested for the A1555G mutation exhibited the mutation in a homoplasmic form. All 41 of these subjects participated in the present study.

Evaluation of Auditory Function. After otoscopic examination, PTA testing was conducted on all subjects. An AA75 audiometer (Rion, Tokyo, Japan) was used in a soundproof room for most subjects. For some subjects, PTA testing was conducted with an AA72B audiometer (Rion) and circumaural earphones in quiet rooms in which background noise was lower than 40 dB sound pressure level (SPL; as measured with an NA29 sound level meter; Rion) with A-weighting. Both air-conducted and bone-conducted thresholds were measured. Subjects who exhibited a pure tone threshold of 30 dB hearing level (HL) or worse at any frequency were given further detailed audiological tests when possible (Table 1). A speech recognition test was conducted with the 67-S monosyllable list (Japan Audiological Society, Tokyo) in 19 subjects. The performance-intensity function was made separately for right and left ears in each subject, and both the maximum speech recognition score and the rollover index were determined.[11] The short increment sensitivity index (SISI) test was performed to examine cochlear dysfunction at 1 or 2 frequencies in 14 subjects. The level of sound stimulation was set at 20 dB above the level of the pure tone threshold at the tested frequencies. Transient evoked otoacoustic emissions (TEOAEs) and distortion product otoacoustic emissions (DPOAEs) were examined to evaluate outer hair cell function with

TABLE 1. SUBJECTS OF DETAILED AUDIOLOGICAL TESTS

Test	*Subjects*
Speech recognition test	II-1, II-5, III-1, III-3, III-9, III-15, III-19, III-21, III-22, III-24, III-25, III-26, IV-1, IV-4, IV-5, IV-6, IV-7, IV-8, IV-15
Short increment sensitivity index test	III-1, III-3, III-9, III-15, III-19, III-22, III-24, III-25, III-26, IV-2, IV-5, IV-7, IV-8, IV-15
Transient evoked otoacoustic emissions and distortion product otoacoustic emissions	II-1, III-3, III-9, III-15, III-19, III-25, III-26, IV-15
Auditory brain stem response	II-1, III-3, III-9, III-26, IV-4, IV-6, IV-8

the ILO292 Otoacoustic Emission Systems (Otodynamics, Hatfield, England) in 8 subjects. For TEOAE analysis, a nonlinear click stimulus train was used at 80 dB SPL, and the number of responses to be averaged was set at 260. The DPOAE measurement was performed at 3 points per octave across the F2 stimulus frequency range of 1,000 Hz to 6,000 Hz with an F2-F1 ratio of 1.221 and at F1 and F2 levels of 70 dB SPL. Each DPOAE result was evaluated with a DP audiogram. The auditory brain stem response (ABR) was evaluated to locate the site of the lesion in the auditory pathway with the Neuropack Σ5504 (Nihon Kohden, Tokyo) in 7 subjects. Alternating click stimulation was presented monaurally at a rate of 10/s through an earphone while the contralateral ear was masked with white noise. The responses were recorded with vertex-earlobe electrodes. A total of 1,000 sweeps were added for each measurement. Thresholds of wave I and wave V were determined, and the latencies of wave I and wave V were measured with the click stimulation presented at 90 dB normal hearing level (nHL).

Total Mitochondrial DNA Sequencing. Total mitochondrial DNA was sequenced for 8 subjects with various degrees of hearing loss. The 8 subjects consisted of the proband (IV-6), her daughter (V-7), her mother (III-4), her grandmother (II-1), and 4 siblings (III-23, III-24, III-25, III-26). Genomic DNA was isolated from peripheral leukocytes of the subjects by conventional methods. As in a previous study,[12] to avoid nuclear pseudogene amplification, we applied the long polymerase chain reaction–based sequencing method. With 96 primer sets designed for sequencing, we sequenced the polymerase chain reaction products using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, California). Each reaction product was then analyzed with an ABI 3700 automated sequencer (PE Applied Biosystems) according to the manufacturer's protocol. The sequence data were compared with those in MITOMAP (http://www.mitomap.org),[13] as well as those from 200 unrelated Japanese without hearing loss.

The study protocol was approved by the Ethics Committee of the National Tokyo Medical Center, and the study was conducted according to the principles of the Declaration of Helsinki. Informed consent was obtained from all individuals who participated in the study.

RESULTS

Pure Tone Audiometry. The results of PTA testing in all of the subjects are summarized in Fig 2. Hearing loss was categorized with respect to the mean air-conducted pure tone thresholds at 0.5, 1, and 2 kHz ("PTA 0.5-2 kHz"),[11] by which 59% of the subjects were classified as having normal hearing (≤15 dB HL), 14% had slight hearing loss (16 to 25 dB HL), 9% had mild hearing loss (26 to 40 dB HL), 4% had moderate hearing loss (41 to 55 dB HL), 5% had moderately severe hearing loss (56 to 70 dB HL), 3% had severe hearing loss (71 to 90 dB HL), and 5% had profound hearing loss (>90 dB HL). The PTAs were symmetric in the right and left ears in the majority of the subjects, in that 56 subjects exhibited the same category of hearing loss on both sides. The remaining 11 subjects showed somewhat asymmetric hearing loss, but the categories differed by only 1 level. All subjects with hearing loss exhibited sloping or sharp sloping audiograms except for 1 subject (III-1) who had a history of noise exposure. This subject's audiogram was typical of noise-induced hearing loss (ie, increased bone-conducted thresholds at 4 kHz). The degree of hearing impairment was similar in affected subjects within the same sibling group, but differed between sibling groups.

In 41 subjects who did not report any hearing loss at the time of interview, normal hearing was detected in both ears by PTA over 0.5, 1, and 2 kHz in 32 subjects, slight or mild hearing loss in one or both ears in 8 subjects, and slight hearing loss due to otitis media in 1 subject. The age of the 8 subjects (II-3, II-6, II-7, III-2, III-15, III-17, III-20, III-23) with slight or mild hearing loss ranged from 42 to 80 years. Considering the ages and the degree of hearing loss in these 8 subjects, the lack of reported hearing loss was considered to be reasonable in these subjects. In these 41 subjects, the results of PTA at 8 kHz were analyzed in order to find out whether any subclini-

cal changes existed in their hearing, because the pure tone thresholds at 8 kHz were most prominently affected in the subjects with hearing loss. Thus, subjects whose ages ranged between 18 and 70 years and whose ears were free of otitis media were eligible for this analysis. Sixty-three ears of 32 subjects met this criteria, and 11 of the 63 ears (17.5%) exhibited significantly elevated pure tone thresholds at 8 kHz (>95th percentile) in comparison to the normal range for their respective ages and sexes.[14] A statistical analysis performed with the binomial test on which the threshold probability of the target population is .05 revealed the frequency of the elevated pure tone thresholds at 8 kHz (17.5%) to be significantly higher than the frequency expected in the ears of the otologically normal population ($p < .0001$).

Speech Recognition Test. The relationship between the maximum speech recognition score and PTA 0.5-2 kHz is shown for each ear (Fig 3). The score ranged from 100% in ears with normal hearing to 0% in ears with profound hearing loss. None of the subjects exhibited a disproportionately poor maximum speech recognition score in relation to the magnitude of pure tone thresholds. In 15 of 38 tested ears, the maximum speech recognition score was >50%, and the rollover index of the performance-intensity function could be reliably determined in these 15 ears. Significant amounts of rollover are pathological and are associated with retrocochlear hearing loss. That the rollover index was <40% in all of the 15 ears suggests that retrocochlear dysfunction did not contribute significantly to hearing loss.

SISI Test. The SISI score and the pure tone threshold at the respective frequencies in each ear are shown in Fig 4. We regarded SISI scores of 70% or higher as positive for cochlear dysfunction, while those between 30% and 70% were regarded as semipositive, and those of 30% or lower as negative.[11] The SISI scores were mostly negative at frequencies for which the pure tone threshold was lower than 30 dB HL. In contrast, the SISI scores were predominantly positive at frequencies for which the pure tone threshold was 30 dB HL or higher. A few subjects exhibited

Fig 2. Pattern of pure tone thresholds for all tested frequencies in each subject. Subjects are listed in order of generation and identification number (ID) as designated in Fig 1. Age of each subject is indicated by dot at corresponding division of age scale classified at top. Thin horizontal lines divide different sibling groups, and thick horizontal lines divide generations. Air conduction pure tone thresholds of right and left ears are indicated by following symbols: white square, ≤30 dB HL; dot in white square, 31 to 60 dB HL; gray square, 61 to 90 dB HL; black square, ≥91 dB HL; blank, not tested. Bone conduction pure tone thresholds are shown instead of air conduction thresholds in 2 subjects (IV-18 and V-8) who had otitis media at time of test.

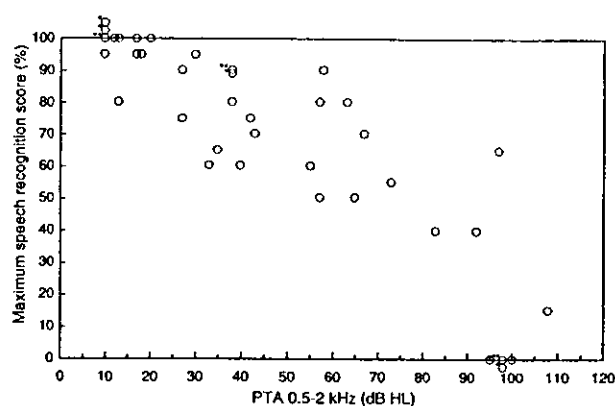

Fig 3. Maximum speech recognition score as function of mean of pure tone thresholds at 0.5, 1, and 2 kHz ("PTA 0.5-2 kHz") for each ear. For ease of visualization, overlapping symbols were moved from original position (indicated by double dots) to neighboring positions (indicated by dot).

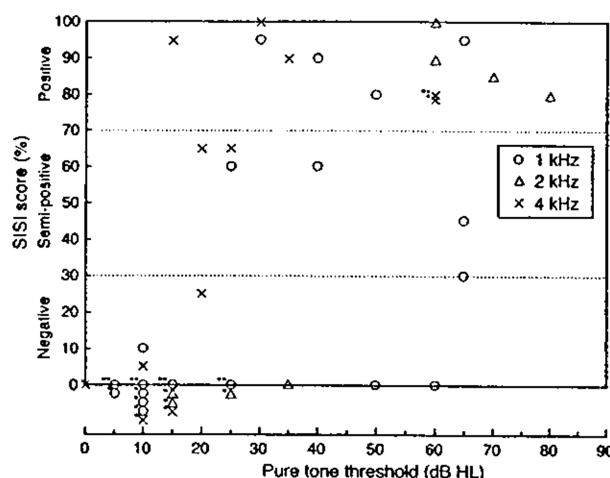

Fig 4. Short increment sensitivity index (SISI) score as function of pure tone threshold at SISI-tested frequency for each ear. Tests were conducted at 1, 2, and 4 kHz. Overlapping symbols were moved as indicated in Fig 3.

semipositive or negative SISI scores despite elevated pure tone thresholds (mostly at 1 kHz). Such occurrences have been noted in previous studies reporting that SISI scores are occasionally semipositive or negative at low frequencies (including 1 kHz) even in ears with cochlear dysfunction.[15]

TEOAE. The TEOAE results were evaluated by the response of the spectral amplitude against noise across a broad frequency range (Fig 5A), as well as by the reproducibility of the time waveform (Fig 5B). The data were plotted against the PTA 0.5-2 kHz in each ear. The response and reproducibility were lower in ears with a PTA 0.5-2 kHz higher than 20 dB HL than in ears with a PTA 0.5-2 kHz of 20 dB HL or lower. No TEOAEs were detected in any of the 6 ears with a PTA 0.5-2 kHz higher than 40 dB HL.

DPOAE. DPOAEs with amplitudes higher than 2 standard deviations above the noise level were considered as positive responses, and DPOAE amplitudes tested at 1, 2, and 4 kHz were compared with the pure tone thresholds measured at the corresponding frequency in each ear (Fig 6). The DPOAE amplitudes were reduced in ears with pure tone thresholds of 20 dB HL or higher at the corresponding DPOAE-tested frequency, and the DPOAE was mostly absent in ears with the pure tone thresholds of 40 dB HL or higher.

ABR. The thresholds of wave I and wave V were determined with the click stimulation, and the latencies of these two waves at 90 dB nHL were measured. The thresholds were then compared with the mean of the air-conducted pure tone thresholds at 2 and 4 kHz ("PTA 2-4 kHz"; Table 2). This frequency range is known to produce the largest ABR components in the cochlea.[16] The relationships of wave I and wave V thresholds and PTA 2-4 kHz were consistent with cochlear dysfunction; ie, the wave V thresholds were almost equal to the PTA 2-4 kHz, and wave I thresholds were higher than wave V thresholds.[17] The wave V latency was within the range predicted by the PTA 2-4 kHz based on the relationship in ears with the corresponding degree of cochlear hearing loss[18] in all but 3 ears (left ear of III-2 and both ears of IV-4) that exhibited relatively long wave V latencies, indicating mild retrocochlear involvement. These 2 subjects were 87 and 62 years old, respectively, and both presented with mild cerebrovascular disease.

Total Mitochondrial DNA Sequence. The mitochondrial DNA sequences were identical in all 8 subjects examined. These subjects exhibited 40 base substitutions relative to the human mitochondrial DNA sequence in MITOMAP, including the A1555G mutation (Table 3). The 39 base substitutions excluding the A1555G mutation were previously reported as polymorphisms in MITOMAP or found in normal Japanese controls — a finding indicating that these substitutions were not related to the observed hearing loss.

DISCUSSION

In our previous study,[10] the proband of the present family exhibited the mitochondrial A1555G mutation in a homoplasmic pattern; ie, all of the mitochondrial genomes in different cells and tissues of the proband harbor the mutation. Because mitochondrial DNA exhibits exclusively maternal inheritance,[19] all of the maternally related members of this family were assumed to carry the A1555G mutation in a homoplasmic form, and this presumption was substantiated by genetic tests that revealed the mutation in a homoplasmic pattern in all 41 maternally related fam-