TABLE I.
Patient Characteristics.

Characteristic	High Signals in the Cochlea Group	No Signal in the Cochlea Group	*P* Value
No. patients	31	17	
Age (yrs)	50.5 ± 17.7	49.3 ± 14.7	0.9
Sex (female/male)	17/14	6/11	0.19
Number of patients with vertigo	12	3	$P < 0.01$
Ear (right/left)	16/15	8/9	0.76
High signals in vestibule	10	1	$P < 0.01$
Gadolinium enhancement	16	0	$P < 0.01$
Initial audiogram (dB)	80 ± 17	75 ± 19	0.38
Final audiogram (dB)	55 ± 27	31 ± 21	$P < 0.01$
Period to initial visit	9 ± 8	5 ± 4	0.08
Period to MRI	15 ± 12	10 ± 8	0.08

High signal = high signal in the affected inner ear on precontrast three-dimensional fluid-attenuated inversion recovery (3D-FLAIR); High signals in vestibule = high signals in the vestibule on precontrast 3D-FLAIR; Gadolinium enhancement = gadolinium enhancement in the affected inner ear on postcontrast 3D-FLAIR; Period to initial visit = period from the onset of sudden sensorineural hearing loss to the initial visit; Period to MRI = period from the onset of sudden sensorineural hearing loss to magnetic resonance imaging.

the following factors were independently related to a worse hearing prognosis for patients with sudden SNHL: high signals in the affected inner ear on precontrast 3D-FLAIR, the period from the onset of sudden SNHL to the first visit, and the initial audiogram (Table II). The period between the MRI study and the onset of HL in patients with sudden SNHL did not differ significantly between patients with high signals (15 ± 12 d) and those with no signal (10 ± 8 d) (Mann-Whitney *U* test) (Table I).

Hearing improvement (24 ± 19 dB) in patients who showed Gd enhancement on 3D-FLAIR in the affected inner ear was not significantly different from that (36 ± 25 dB) in patients who did not (Mann-Whitney *U* test). On postcontrast 3D-FLAIR, three patients who had no signal on precontrast 3D-FLAIR showed Gd enhancement on 3D-FLAIR in the affected inner ear.

Ten patients showed high signals in both the cochlea and the vestibule on precontrast 3D-FLAIR in the affected ear, and 8 of these 10 patients suffered from vertigo at the onset of sudden SNHL. One patient showed high signals only in the vestibule, without high signals in the cochlea on precontrast 3D-FLAIR in the affected inner ear, and this patient suffered from vertigo at the onset of sudden SNHL. In this patient, 3D-FLAIR was performed 17 days after the onset of sudden SNHL. There was a relationship between the vertigo at the onset of sudden SNHL and high signals in the vestibule of the affected inner ear ($P < .05$; χ^2 test). Furthermore, the final hearing levels of patients with high signals in the vestibule were significantly worse than those of patients who did not have these high signals ($P < .05$; Mann-Whitney *U* test).

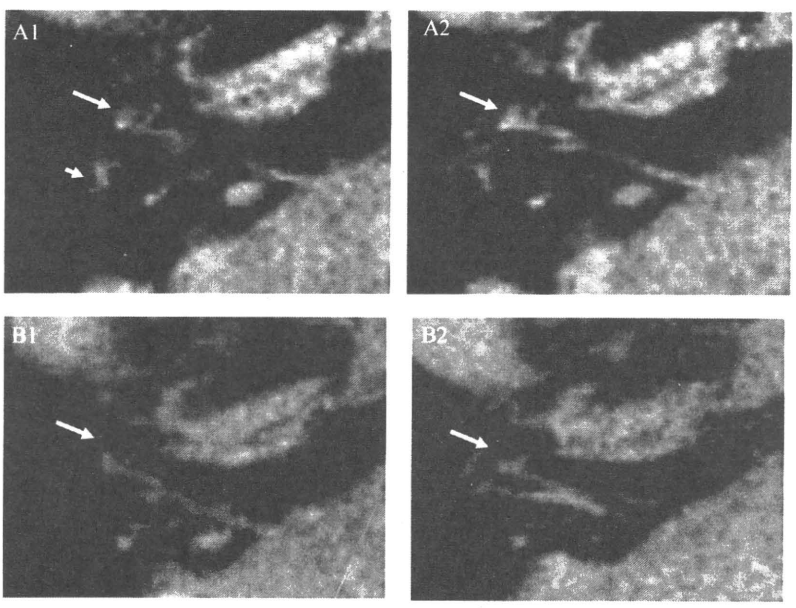

Fig. 1. Axial magnetic resonance image. Right inner ear of a 45-year-old man with vertigo and right sudden sensorineural hearing loss. (A1) First three-dimensional fluid-attenuated inversion recovery (3D-FLAIR) image before enhancement. Bright signals are very high in right cochlea (long arrow) and vestibule (short arrow). (A2) First 3D-FLAIR image after enhancement. Gadolinium enhancement is not observed in right cochlea (long arrow). (B1) Second 3D-FLAIR image before enhancement. No bright signal is visible in right cochlea or vestibule. (B2) Second 3D-FLAIR image after enhancement. No gadolinium enhancement is visible in right cochlea.

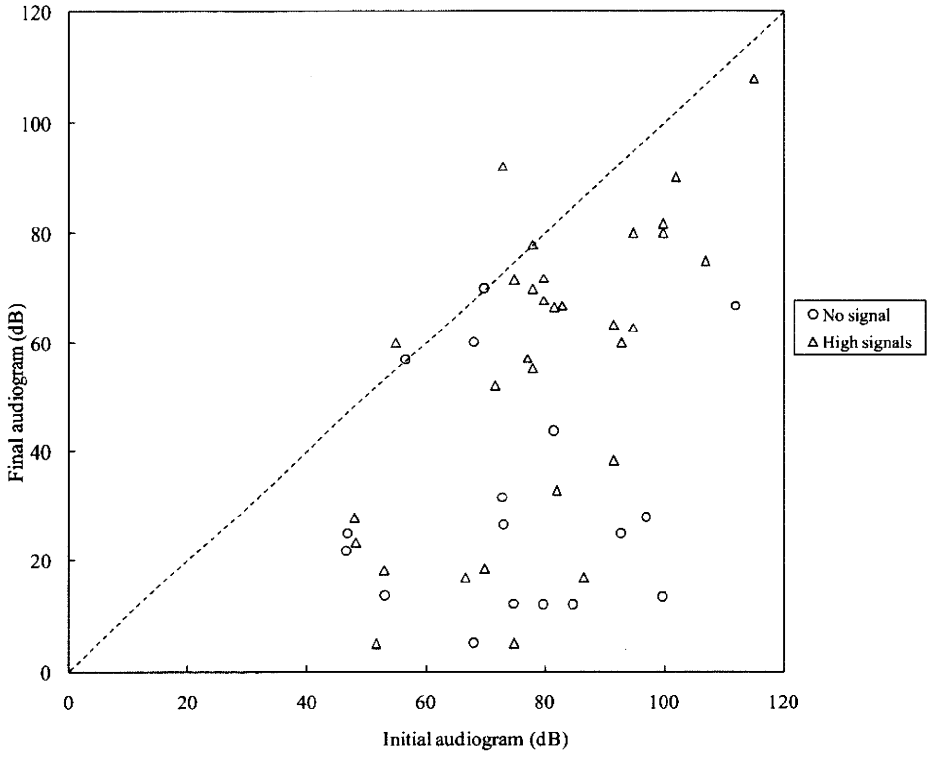

Fig. 2. Hearing improvement in patients with and without high signals in affected inner ear on precontrast three-dimensional fluid-attenuated inversion recovery (3D-FLAIR) magnetic resonance imaging. Spots near transverse dotted line show bad prognoses for sudden sensorineural hearing loss (SNHL). Conversely, spots near bottom line show good prognoses for sudden SNHL. High signals in cochlea in affected ear on precontrast 3D-FLAIR suggest poor hearing prognosis in sudden SNHL.

DISCUSSION

The major finding of this study is that 3D-FLAIR findings are closely related to hearing prognosis in sudden SNHL. This is the first report to demonstrate that high signals in the cochleae of affected ears on precontrast 3D-FLAIR suggest poor hearing prognoses in sudden SNHL. We have previously reported that four of eight patients with sudden SNHL showed high signals in the inner ear on precontrast 3D-FLAIR and that one of these four patients showed Gd enhancement in the affected inner ear on 3D-FLAIR.[4] However, in that study, our data were insufficient to evaluate the relationship between the 3D-FLAIR findings and hearing prognosis. It demonstrated that 3D-FLAIR is a very useful tool with which to predict the prognosis before treatment.

In this study, we have shown the possibility of identifying poor prognostic factors with 3D-FLAIR. The prognostic factors for sudden SNHL reported previously include age,[13] male sex,[14] the type of audiogram,[13] the level of HL,[14] tinnitus and vertigo,[14] the method of treatment,[13] and the time of treatment initiation.[13] However, the relationship between the hearing prognosis and the imaging findings is not clear. Precontrast high signals on 3D-FLAIR may reflect minor hemorrhage or an increased concentration of protein in the inner ear, which has passed through blood vessels with increased permeability or has originated in disrupted cells in the inner ear. We have shown that precontrast high signals on 3D-FLAIR are independently related to hearing prognosis. Therefore, precontrast high signals on 3D-FLAIR may be a new prognostic factor in sudden SNHL.

Sudden SNHL is caused by a variety of unknown pathophysiologic mechanisms. To develop more effective therapies, it is necessary to understand more precisely the pathology of sudden SNHL. We have also shown an asso-

TABLE II.
Multivariate Regression Model of Impact on Final Audiogram.

Independent Variables	Values	β (Standard Regression Coefficient)	Standard Error	t	*P* Value
Age		0.158	0.19	1.38	0.174
Sex	1 = male, 0 = female	−0.005	6.34	−0.04	0.964
Vertigo	1 = yes, 0 = no	0.091	7.3	0.74	0.464
Period to initial visit		0.312	0.5	2.58	$P < 0.05$
High signals in the cochlea	1 = yes, 0 = no	0.248	7.3	2.07	$P < 0.05$
Initial audiogram		0.416	0.2	3.36	$P < 0.01$

$R^2 = 0.502$, adjusted $R^2 = 0.429$.
Period to initial visit = period from the onset of sudden sensorineural hearing loss to the initial visit; high signals in the cochlea = high signals in the cochlea on precontrast 3D-FLAIR.

ciation between 3D-FLAIR findings and clinical signs, especially those pertaining to vertigo. 3D-FLAIR MRI may be a key to resolving these problems. Therefore, it is possible that 3D-FLAIR MRI will provide made-to-order treatments for patients with sudden SNHL in the future. Further study is necessary to clarify the relationship between MRI findings and the efficiency of several treatments to develop more effective therapies.

CONCLUSIONS

High signals in the affected ear indicate a poor hearing prognosis in patients with sudden SNHL. 3D-FLAIR findings may be one of the prognostic factors in sudden SNHL. We believe that this method contributes to the definition of a prognosis for patients with sudden SNHL.

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The role of mtDNA mutations in the pathogenesis of age-related hearing loss in mice carrying a mutator DNA polymerase γ

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Abstract

Mitochondrial DNA (mtDNA) mutations may contribute to aging and age-related diseases. Previously, we reported that accumulation of mtDNA mutations is associated with age-related hearing loss in mice carrying a mutator allele of the mitochondrial *Polg* DNA polymerase. To elucidate the role of mtDNA mutations in the pathogenesis of age-related hearing loss or presbycusis, we performed large scale gene expression analysis to identify mtDNA mutation-responsive genes and biological process categories associated with mtDNA mutations by comparing the gene expression patterns of cochlear tissues from 9-month-old mitochondrial mutator and control mice. mtDNA mutations were associated with transcriptional alterations consistent with impairment of energy metabolism, induction of apoptosis, cytoskeletal dysfunction, and hearing dysfunction in the cochlea of aged mitochondrial mutator mice. TUNEL staining and caspase-3 immunostaining analysis demonstrated that the levels of apoptotic markers were significantly increased in the cochleae of mitochondrial mutator mice compared to age-matched controls. These observations support a new model of how mtDNA mutations impact cochlear function whereby accumulation of mtDNA mutations lead to mitochondrial dysfunction, an associated impairment of energy metabolism, and the induction of an apoptotic program. The data presented here provide the first global assessment at the molecular level of the pathogenesis of age-related disease in mitochondrial mutator mice and reveal previously unrecognized biological pathways associated with mtDNA mutations.

Keywords: mtDNA mutations; *Polg*; Age-related hearing loss; Presbycusis; Apoptosis; Mitochondrial dysfunction; Aging

1. Introduction

The mitochondrial theory of aging postulates that reactive oxygen species (ROS) generated inside mitochondria damage key mitochondrial components such as mtDNA, membranes, and respiratory chain proteins (Balaban et al., 2005). Such damage accumulates with time, leads to age-related mitochondrial dysfunction, and causes energy depletion, compromising the organism. Mitochondria are the main source of cellular energy, generating most cellular ATP. The mammalian mitochondrial genome consists of 37 genes, encoding 13 proteins of the electron transport oxidative phosphorylation system (Anderson et al., 1981). Since mtDNA plays an essential role in energy metabolism, mtDNA mutations have been hypothesized to contribute to mammalian aging. Indeed, previous studies have shown that mtDNA point mutations accumulate with aging in humans (Michikawa et al., 1999), and that accumulation of mtDNA mutations leads to premature aging in mitochondrial mutator mice, indicating a causal role of mtDNA mutations in mammalian aging (Kujoth et al., 2005; Trifunovic et al., 2004).

Specific mutations in mtDNA have also been implicated in the development of age-related diseases (Corral-Debrinski

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et al., 1992; Dirks et al., 2006; Seidman et al., 2002). Age-related hearing loss, also known as presbycusis, is one of the most common diseases of aging and the number of people affected is rapidly growing as the elderly population increases. However, the pathogenesis of age-related hearing loss remains unclear. The accumulation of mtDNA mutations has been associated with the development of age-related hearing loss (Kujoth et al., 2005). In support of this hypothesis, hearing loss is a common symptom in individuals harboring inherited mtDNA mutations (Chinnery et al., 2000). Several mutations in *Polg* (mitochondrial DNA polymerase gamma) have been identified as a cause of human disorders such as Alpers syndrome and deafness (Mancuso et al., 2004; Nguyen et al., 2005). Specific mtDNA point mutations also contribute to mitochondrial disorders in humans such as mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and myoclonic epilepsy and ragged red fibres (MERRF), the symptoms of which include hearing loss (Chinnery et al., 2000; Fischel-Ghodsian, 2003). Furthermore, more than 100 different deletions of mtDNA have been associated with mitochondrial disorders (Chinnery et al., 2000), and of these, some mtDNA deletions cause Kearns–Sayre syndrome which also involves hearing loss (Chinnery et al., 2000).

We have shown previously that mtDNA mutations play a role in the development of age-related hearing loss in mice carrying a mutation in *Polg* (Kujoth et al., 2005). These mice were created by introducing a two base substitution, which results in a critical residue substitution in the exonuclease domain of *Polg*, impairing its mtDNA proof-reading ability (Kujoth et al., 2005). These animals accumulate mtDNA mutations with aging, and exhibit a striking premature aging phenotype, consisting of osteoporosis, reduced lifespan, and hearing loss (Kujoth et al., 2005; Trifunovic et al., 2004). Thus, these mice represent a useful model for investigating a role of mtDNA mutations in the pathogenesis of age-related hearing loss. In the present study, we performed genome-wide DNA microarray analysis using cochlear tissues from 9-month-old mitochondrial mutator and control mice. We then performed data analysis to identify age-related, mtDNA mutation-responsive genes and biological process categories statistically associated with mtDNA mutation-responsive genes. Quantitative RT-PCR (QRT-PCR), TUNEL staining, immunostaining, histology, and hearing assessment were performed to corroborate the microarray results. Together, these approaches revealed previously unrecognized biological process pathways associated with mtDNA mutations, providing a new model of how mtDNA mutations impact cochlear function.

2. Materials and methods

2.1. Animals

$Polg^{D257A/D257A}$ mice have been previously described and were backcrossed four generations onto the C57BL/6 background (Kujoth et al., 2005). Mice were housed in the Genetics and Biotechnology Center of University of Wisconsin-Madison-approved Animal Care Facility. Experiments were performed in accordance with the protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee (Madison, WI).

2.2. Assessment of hearing

Detailed protocols for ABR measurements have been described (Someya et al., 2007). Briefly, ABRs were measured with a tone burst stimulus (4, 8, and 16 kHz) using an ABR recording system (Intelligent Hearing System, Miami, FL). Mice were anesthetized with a mixture of xylazine hydrochloride (10 mg/kg, i.m.) and ketamine hydrochloride (40 mg/kg, i.m.). We used five mice per group for this study. Following the hearing measurements, the same mice were sacrificed to conduct microarray analysis of cochlear gene expression. Data were analyzed using the two-tailed standard *t*-test. All data were reported as mean ± S.E.M.

2.3. Assessment of histology

Detailed protocols for tissue processing have been described (Sakamoto et al., 2004). Briefly, 4% paraformaldehyde-fixed and paraffin-embedded specimens were sliced into 4 μm sections, mounted on silane-coated slides, stained with Haematoxylin and Eosin (HE), and observed under a light microscope (Leica Microsystems, Bannockburn, IL). The Rosenthal's canal was divided into three regions: apical, middle and basal (Keithley et al., 2004) and the apical and basal turns were used for evaluation of cochlear histology. We used five mice for each group ($n = 5$) that were different mice than those used in the microarray analysis. Five modiolar sections obtained in every fifth section from one unilateral cochlea were evaluated per mouse. The same animals were used for spiral ganglion cell counting, TUNEL staining, and immunostaining.

Spiral ganglion cells (SGCs) were counted in the apical turn of the cochlear sections in the field of 0.3 μm × 0.225 μm as seen using a 40× objective by direct observation. SGCs were identified by the presence of a nucleus. The SGC density was calculated as the number of SGCs per 1 mm^2. Five sections of the unilateral apical turn were evaluated in one cochlea per mouse. Data were analyzed using the two-tailed standard *t*-test. All data were reported as mean ± S.E.M.

2.4. RNA sample preparations

Detailed protocols for gene expression profiling analysis using Affymetrix microarrays by Affymetrix algorithms have been described (Affymetrix, 2004; Lee et al., 1999). Briefly, we pooled two cochleae from each mouse for one sample because the amount of mRNA from one cochlea of one mouse was insufficient for a single GeneChip (Affymetrix, Santa

Clara, CA). We hybridized each sample to a single GeneChip and used five samples per group.

2.5. Statistical analysis

Signals in each image were scaled to a whole chip target intensity of 1500 using GeneChip® Operating Software (GCOS) 1.3 in order to minimize an overall variability in hybridization intensities. A gene probe set was considered "expressed" if it displayed a "present" call in at least one GeneChip based on the Affymetrix "present/absent call" algorithms. All genes considered "not expressed" were eliminated from our analysis. Further analysis and comparisons between the two groups were also done using GCOS 1.3 and Significance Analysis of Microarray (SAM) (Tusher et al., 2001). To identify mtDNA-responsive genes, each 9-month-old WT sample ($n=5$) was compared to each 9-month-old D257A sample ($n=5$), generating a total of 25 pairwise comparisons. The change in gene expression was considered significant if the fold change was >1.0 based on the Affymetrix algorithms (Affymetrix, 2004), the P-value was <0.02 using the two-tailed standard t-test, and FDR was <10% based on the SAM algorithms (Tusher et al., 2001). We then used Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Dennis et al., 2003) and Expression Analysis Systematic Explorer (EASE) (Hosack et al., 2003) (http://apps1.niaid.nih.gov/david/) to assign identified genes to "Gene Ontology (GO): Biological Process" categories of Gene Ontology Consortium (www.geneontology.org) and used EASE to determine the total number of identified genes that were assigned to each biological process category, the total number of genes on the array in each biological process category, and to identify "GO: Biological Process" categories statistically associated with mtDNA mutation-responsive genes by performing Fisher Exact tests (Fisher Exact score < 0.05). Five genes were manually assigned to "perception of sound" category using the gene list of "Hereditary Hearing Impairment in Mice" by The Jackson Laboratory (http://www.jax.org/hmr/master_table.html). The complete set of microarray data has been submitted to the GEO (Gene Expression Omnibus) repository (http://www.ncbi.nlm.nih.gov/geo/) with GEO Accession number GSE4866. For further details of the methods (MIAME checklist), see Supplementary information (Supplementary note).

2.6. Quantitative RT-PCR

Detailed protocols for QRT-PCR analysis have been described (Someya et al., in press). Detection of mRNA was carried out with the TaqMan EZ RT-PCR kit using the Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Oligonucleotide primers and MGB fluorescent probes (TaqMan Gene Expression Assays) were purchased from Applied Biosystems. Each sample used for QRT-PCR analysis consisted of the two cochleae pooled from one mouse. We used four mice per group ($n=4$) for this study. mRNA samples used for QRT-PCR analysis were from different mice than those used for microarray analysis which had been exhausted for that purpose. All data were reported as mean ± S.E.M.

2.7. TUNEL staining

Detailed protocols for TUNEL staining have been described (Someya et al., 2007). Briefly, TUNEL staining for apoptotic nuclei was performed using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, stained sections were viewed by light microscopy and spiral ganglions in the apical turns were evaluated for TUNEL positivity. We used five mice for each group ($n=5$) that were different mice than those used in the microarray analysis, but the same as those used for spiral ganglion cell counting and immunostaining. Data were evaluated using the two-tailed standard t-test. All data were reported as mean ± S.E.M.

2.8. Immunostaining

Detailed protocols for immunohistochemical staining have been described (Someya et al., 2007). Immunohistochemical staining for caspase-3 was performed using the Anti-Active Caspase-3 pAb (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, positively-stained cells were observed in sections of the cochleae by direct observation using a light microscopy. Spiral ganglions in the apical turns were evaluated for caspase-3 positivity. We used five mice for each group ($n=5$) that were different mice than those used in the microarray analysis, but the same as those used for spiral ganglion cell counting and TUNEL staining. Data were evaluated using the two-tailed standard t-test. All data were reported as mean ± S.E.M.

3. Results

3.1. Assessment of hearing and histology

The C57BL/6 strain possesses mutations in the Cdh23 gene which result in onset of age-related hearing loss and cochlear degeneration beginning at 8–10 months (Keithley et al., 2004; Noben-Trauth et al., 2003). Because the background of our mice is C57BL/6 and both *Polg*$^{D257A/D257A}$ (D257A) and *Polg*$^{+/+}$ wild-type (WT) mice possess the mutations, we first evaluated hearing function of 8–9-month-old WT mice and confirmed that they did not show any age-related hearing loss at the frequencies examined under the housing conditions of our animal facility. We then chose to study 9-month-old WT mice and age-matched D257A mice, an age at which WT mice do not have hearing loss due to the *Cdh23* mutations.

To determine whether D257A mice display age-related hearing loss, we measured auditory brainstem response (ABR) thresholds in 9-month-old WT and D257A mice. Nine-month-old WT mice displayed normal hearing. However, the age-matched D257A mice displayed significant age-related hearing loss ($P < 0.05$, $n = 5$) (Fig. 1). To confirm whether D257A mice display age-related cochlear degeneration, we performed histological examination of the cochleae from 2- and 9-month-old WT and D257A mice ($n = 5$). Examination of the histology of the apical cochlear region confirmed that two of the five cochleae from 9-month-old D257A mice displayed severe loss of SGCs (Fig. 2J and L). Consistent with our previous studies, all the cochleae from 9-month-old D257A mice displayed severe loss of SGCs in the basal turn (Fig. 3G) (Kujoth et al., 2005). In contrast, all the cochleae from 2-month-old WT and D257A mice dis-

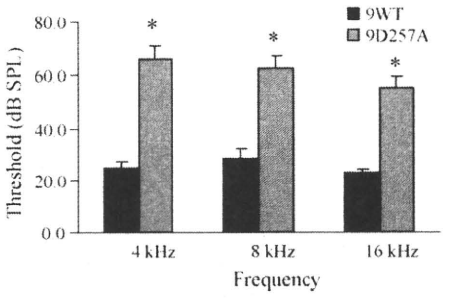

Fig. 1. Age-related hearing loss. Means of ABR thresholds (dB SPL, decibels sound pressure level) for 9-month-old WT and D257A mice at 4, 8, and 16 kHz. The mean ABR thresholds of 9-month-old D257A mice were significantly elevated compared to the age-matched WT mice ($^{*}P < 0.05$, $n = 5$), showing age-related hearing loss. Error bars represent S.E.M.

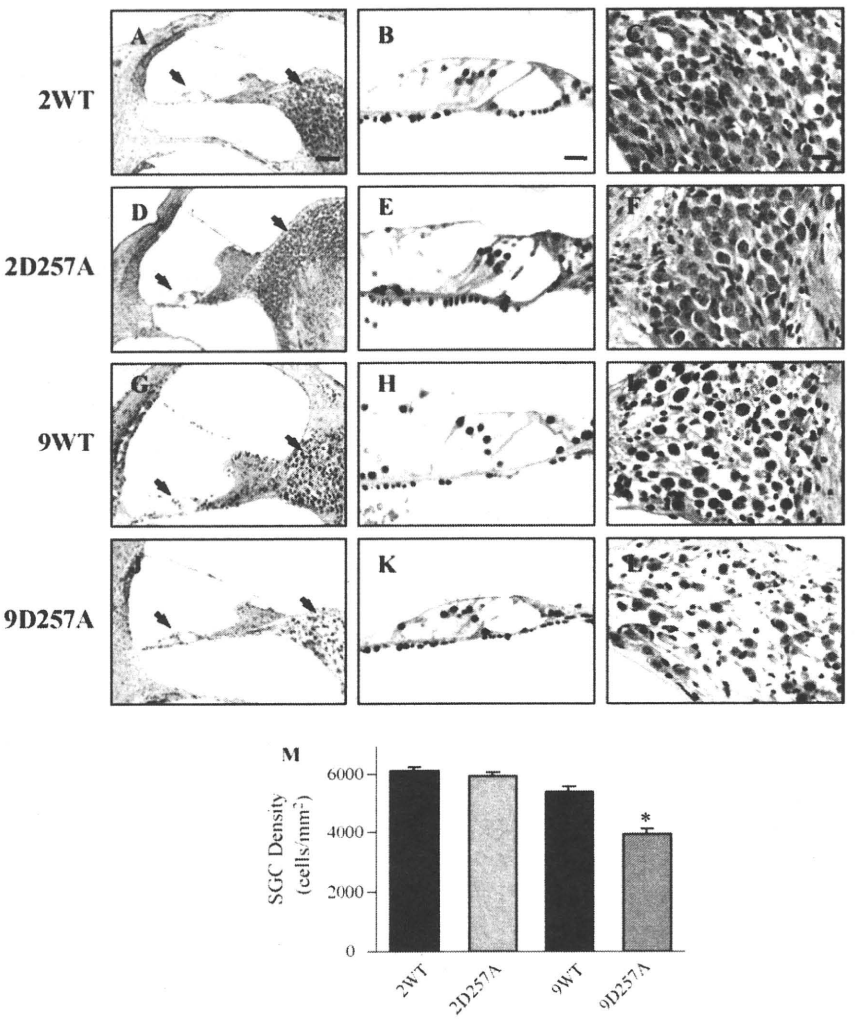

Fig. 2. Age-related cochlear degeneration. Cochlear apical turn from 2-month-old WT (A–C) and D257A (D–F), and 9-month-old WT (G–I) and D257A (J–L) mice. Arrows indicate hair cells and spiral ganglion cells (SGCs). The cochleae from 2-month-old WT and D257A, and 9-month-old WT mice displayed no or only a few loss of the hair cells and spiral SGCs, whereas the cochleae from 9-month-old D257A mice displayed severe loss of SGCs. The mean SGC density of D257A mice was significantly lower than that of age-matched WT (M) ($^{*}P < 0.05$, $n = 5$). Error bars represent S.E.M. Scale bar = 100 μm (left panel). Scale bar = 25 μm (middle and right panel).

played no or only a few loss of SGCs (Fig. 2A, C, D, and F). The five cochleae from 9-month-old WT mice also displayed no clear loss of SGCs (Fig. 2G and I). Decreased SGC density is one of the hallmarks of presbycusis in mammals (Keithley et al., 2004). The mean SGC density of 9-month-old D257A mice was significantly lower than that of age-matched WT (Fig. 2M) ($P < 0.05$, $n = 5$). There was no significant difference in the SGC density between 2-month-old WT, age-matched D257A, and 9-month-old WT mice (Fig. 2M). Hair cell loss is another hallmark of presbycusis (Francis et al., 2003). Examination of the histology of the basal cochlear region confirmed that two of the five cochleae from 9-month-old D257A mice displayed severe loss of outer hail cells (OHCs) and inner hair cells (IHCs) (Fig. 3G and H), whereas all the cochleae from 2-month-old WT and D257A and 9-month-old WT mice displayed no or only a few loss of the OHCs and IHCs (Fig. 3A–F). All the cochleae from 2- and 9-month-old WT and D257A mice also displayed no or only a few loss of the OHCs and IHCs in the apical cochlear region (Fig. 2B, E, H, and K). Together, these observations confirm that D257A mice display age-related hearing loss and cochlear degenerations by 9 months of age.

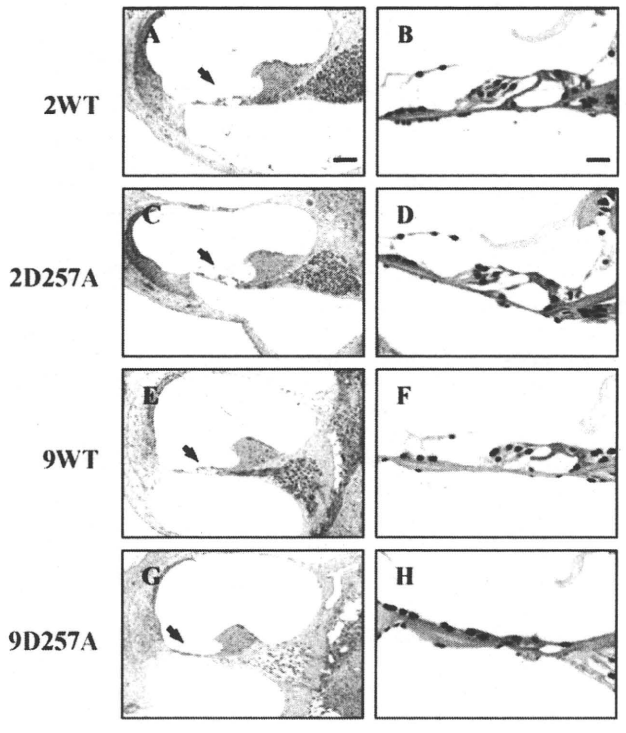

Fig. 3. Age-related hair cell loss. Cochlear basal turn from 2-month-old WT (A and B) and D257A (C and D), and 9-month-old WT (E and F) and D257A (G and H) mice. Arrows indicate hair cells. The cochleae from 9-month-old D257A mice displayed severe loss of the out hair cells (OHCs) and inner hair cells (IHCs), whereas the cochleae from 2-month-old WT and D257A, and 9-month-old WT mice displayed no or only a few loss of the OHCs and IHCs. Scale bar = 100 μm (left panel). Scale bar = 25 μm (right panel).

3.2. Overview of microarray and statistical analysis

To identify mtDNA mutation-responsive genes and associated biological process categories, we conducted genome-wide gene expression analysis using RNA samples isolated from cochlear tissues of 9-month-old WT and D257A mice ($n = 5$). We selected cochlear tissue for our investigation because the cochlea is primarily composed of long-lived, high oxygen-consuming postmitotic cells (Pickles, 2004), a feature shared with other critical postmitotic tissues such as heart and brain which are sites of multiple, major age-related diseases and show accumulation of mtDNA mutations (Corral-Debrinski et al., 1992). We used the Affymetrix mouse genome 430 2.0 array, which contains 45,037 gene probe sets representing more than 34,000 mouse genes and ESTs. We then performed statistical analysis using the Affymetrix algorithms (Affymetrix, 2004), SAM algorithms (Tusher et al., 2001), and our own data analysis criteria. We found that 1289 gene probe sets were significantly up-regulated, and 662 gene probe sets were significantly down-regulated in cochlear tissues from 9-month-old D257A mice. The gene probe sets were further assigned to "GO: Biological Process" categories of the Gene Ontology Consortium using DAVID (Dennis et al., 2003), resulting in classification of 466 up-regulated and 378 down-regulated genes. Gene probe sets were considered "genes" if they had been assigned a "gene symbol" annotation by DAVID. We then used EASE (Hosack et al., 2003) to determine the number of identified genes and the total number of genes on the array in each biological process category, and to test the statistical significance of coregulation (overrepresentation) of identified genes within each biological process category. For this analysis, we used the Fisher Exact test ($P < 0.05$), whose score represents the probability that an overrepresentation of a certain biological process category occurs by random chance (Hosack et al., 2003). A summary of the Gene Ontology Biological Process categories statistically associated with mtDNA-responsive genes is shown in Table 1. A list of selected genes altered in expression is shown in Table 2. For all genes identified, see Supplementary information (Table 3).

3.3. mtDNA mutations result in up-regulation of genes involved in apoptosis and stress response

EASE analysis revealed that two apoptotic process categories, "regulation of apoptosis" and "anti-apoptosis", were significantly associated with the mtDNA mutation-responsive genes (Fisher Exact score < 0.05), and 15 genes within these categories were significantly up-regulated in the cochleae ($P < 0.02$, false discover rate (FDR) < 10%) (Table 1). Of the 15 genes identified, 10 genes were involved in regulation of apoptosis, including phosphatase and tensin homolog (*Pten*), BCL2-like 11 (apoptosis facilitator) (*Bim*), baculoviral IAP repeat-containing 6 (*Birc6*), and beta-amyloid binding protein precursor (*Bbp*) (Table 2).

Table 1
List of "GO: Biological Process" categories of genes significantly associated with mtDNA mutation-responsive genes in the aging cochlea

Biological process categories	N	TN	Fisher
Up-regulated (466 classified genes)			
Ribonucleoprotein complex	23	242	0.000
Ribosome	14	147	0.003
RNA binding	21	285	0.005
Protein binding	71	1401	0.020
Anti-apoptosis	5	41	0.023
Regulation of apoptosis	10	123	0.025
Intracellular transport	25	423	0.031
Down-regulated (378 classified genes)			
Cytoskeleton	39	440	0.000
Extracellular matrix	25	237	0.000
Collagen	8	32	0.000
Structural molecule activity	32	456	0.000
Cell motility	15	158	0.000
Calcium ion binding	28	421	0.000
Myosin	6	33	0.001
Cell adhesion	22	425	0.017
Carbohydrate metabolism	13	239	0.044

N: the number of identified genes in the category; FC: fold change; TN: the total number of genes in the category represented on the GeneChip; Fisher: Fisher Exact test score.

Bim is a member of the proapoptotic Bcl2 family and a well-described component of mitochondrial apoptosis pathway, leading to cytochrome *c* release (Cheng et al., 2001; Putcha et al., 2001). *Birc6* is also involved in mitochondrial pathways of apoptosis (Ren et al., 2005). Phosphatase and tensin homolog is a tumor suppressor phosphatase that regulates Akt kinase activity and may promote apoptotic death of hippocampal neurons (Gary and Mattson, 2002). The EASE analysis also revealed that 13 genes in the "programmed cell death" category were up-regulated, including serum/glucocorticoid regulated kinase (*Sgk*), programmed cell death 6 interacting protein (*Pdcd6ip*), and tumor necrosis factor receptor superfamily, member 1a (*Tnfrsf1a*) ($P < 0.02$, FDR < 10%, Fisher Exact score = 0.21) (Table 2). *Sgk* is a member of a serine/threonine kinase family and is involved in the early pathogenesis of Parkinson's disease (Schoenebeck et al., 2005). The protein encoded by *Pdcd6ip* regulates neuronal death involving caspase pathways (Trioulier et al., 2004). *Tnfrsf1a* is a member of a TNFR superfamily and is involved in activation of Bid, a Bcl-2 family member and inducer of mitochondrial pathways of apoptosis (Yin, 2000). QRT-PCR analysis was conducted for *Pten* and *Bim*, to validate the microarray results for expression of these genes. The QRT-PCR results were in agreement with the microarray findings that expression of these proapoptotic genes were increased in the cochlea of D257A mice (Fig. 4).

Proteolysis also plays a key role in cell death (Hengartner, 2000). The EASE analysis revealed that 14 genes involved in the "proteolysis and peptidolysis" category were found to be up-regulated, including a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 4 (*Adamts4*), a disintegrin and metalloproteinase domain 15

Table 2
List of selected genes significantly associated with mtDNA mutations

Gene ID	FC	*P*-value	FDR	Symbol
Regulation of apoptosis (10)				
BB831420	1.69	0.000	2.9	Gsk3b
AA214868	2.07	0.000	3.2	Pten
BM120925	1.66	0.000	4.0	Bim
NM_007566	1.37	0.001	4.1	Birc6
AF201288	1.11	0.003	6.2	Tsc22d3
BB768208	1.25	0.004	6.6	Sgk3
AB012278	1.35	0.006	7.5	Cebpb
NM_026810	1.23	0.009	8.9	Mlh1
AK011828	1.66	0.010	8.9	Hip1
AF353993	1.32	0.013	9.7	Bbp
Programmed cell death (13)				
NM_011361	1.56	0.000	3.8	Sgk
BC026823	1.39	0.002	5.6	Pdcd6ip
L26349	1.26	0.003	5.9	Tnfrsf1a
Proteolysis and peptidolysis (14)				
BB443585	2.37	0.002	5.4	Adamts4
AK008695	1.63	0.004	6.2	2210010C04Rik
NM_022022	1.08	0.005	6.6	Ube4b
AK020649	1.45	0.005	6.9	Adam 15
BB131619	1.62	0.006	7.5	Lnx1
NM_007800	2.27	0.007	8.2	Ctsg
AK013777	2.42	0.008	8.2	Ptpn21
NM_008585	1.55	0.008	8.2	Mep1a
NM_133216	1.28	0.011	8.9	Xpnpep1
BC013654	1.56	0.011	8.9	Trfr2
Response to stress (15)				
AF047377	1.42	0.002	5.0	Hspb1
BF466143	2.23	0.003	5.9	Wasl
BM234360	1.54	0.003	5.9	Fn1
L77884	2.62	0.003	5.9	Lyst
NM_011558	2.27	0.004	6.6	Tcrg-V4
BB298208	3.39	0.005	6.6	Chek1
BB075261	1.48	0.005	6.9	Fcgr1
NM_016928	1.64	0.006	7.5	Tlr5
NM_011728	1.46	0.007	8.2	Xpa
NM_010824	2.01	0.011	8.9	Mpo
Carbohydrate metabolism (13)				
BQ176370	−1.47	0.00	2.9	9230112O05Rik
NM_008063	−1.29	0.000	4.0	Slc37a4
NM_008832	−1.83	0.001	5.0	Phka1
AW208566	−4.83	0.001	5.0	Lyzs
BG073164	−1.42	0.002	6.2	Pgm2l1
NM_007933	−5.77	0.003	6.9	Eno3
BI685536	−1.35	0.004	7.5	B4galt6
NM_008062	−1.34	0.004	7.5	G6pdx
AK007262	−1.57	0.004	8.2	1700124B08Rik
NM_011079	−3.91	0.004	8.2	Phkg
Mitochondrion (13)				
BC008518	−1.16	0.000	3.8	Nnt
NM_007702	−2.69	0.000	4.0	Cidea
AB022340	−4.11	0.001	4.5	Acsm3
AV278574	−1.42	0.001	5.4	Aldh6a1
NM_007472	−1.65	0.002	6.2	Aqp1
NM_080575	−1.57	0.003	6.9	Acas2l
NM_026331	−1.89	0.004	7.5	Mscp
NM_022032	−1.49	0.004	7.5	Perp
AK017491	−1.47	0.004	8.2	Mipep
BG866501	−1.38	0.005	8.9	Hadhb

Table 2 (*Continued*)

Gene ID	FC	*P*-value	FDR	Symbol
Cytoskeleton (39)				
BG094386	−1.27	0.000	2.9	Dag1
BQ032637	−1.49	0.000	3.8	Jak1
BC021484	−1.70	0.000	3.8	Sspn
AK003186	−1.98	0.000	4.0	Tpm2
AF237628	−38.21	0.000	4.0	Lmod2
AK003182	−7.87	0.000	4.0	Myl1
AJ002522	−8.77	0.001	5.0	My h1
NM_010861	−3.49	0.002	6.2	Myl2
BC008538	−136.05	0.003	6.2	Myh2
NM_016754	−6.87	0.005	8.2	Mylpf
Extracellular matrix (25)				
AW550625	−1.91	0.000	2.9	Col3a1
BF227507	−2.95	0.000	2.9	Col1a2
NM_008610	−1.74	0.000	3.8	Mmp2
BC019502	−1.71	0.000	3.8	Bgn
AV229424	−1.58	0.000	4.0	Col5a2
BF158638	−1.35	0.001	5.4	Col4a1
NM_007732	−14.01	0.002	5.6	Col1 7a 1
AF011450	−1.30	0.003	6.6	Col15a1
AF064749	−1.33	0.003	6.6	Col6a3
U08020	−3.18	0.004	7.5	Col1a1
Cell adhesion (22)				
BE199556	−1.43	0.000	3.8	Pard3
AF038562	−5.52	0.000	3.8	Lgals7
NM_009675	−2.53	0.000	3.8	Aoc3
BI110565	−2.01	0.000	4.0	Postn
BB776961	−1.33	0.001	4.5	Cdh13
NM_019759	−2.14	0.002	5.6	Dpt
BB534670	−2.31	0.003	6.9	Cd36
NM_11582	−2.92	0.004	6.9	Thbs4
BQ175493	−1.56	0.004	7.5	Itga8
NM_016685	−2.35	0.007	9.7	Comp
Perception of sound (6)				
BI143915	−1.69	0.001	4.3	Ntn1*
BQ176524	−1.72	0.001	5.4	Bsn*
U08020	−3.18	0.004	7.5	Col1a1*
BQ175493	−1.56	0.004	7.5	Itga8*
NM_010081	−6.85	0.006	8.9	Dst*
NM_013624	−1.52	0.007	9.7	Otog
Ion transport (12)				
BG791642	−1.73	0.001	4.4	Abcc9
BE945884	−2.61	0.001	5.0	Gabra1
BQ175666	−1.55	0.001	5.4	Gabrb3
NM_013540	−2.04	0.004	6.9	Gria2
BB515151	−1.39	0.005	8.2	Tcn2
BM119753	−1.51	0.006	9.7	Kcna6
AF089751	−1.49	0.006	9.7	P2rx4
BB236747	−1.48	0.006	9.7	Clic5
BI650739	−1.33	0.007	9.7	Ubl4
NM_08557	−1.57	0.007	9.7	Fxyd3

Gene ID: representative public gene ID; FC: fold change; *P*-value: *P*-values for the comparison of 9-month-old WT vs. 9-month-old D257A group; FDR: false discovery rate; symbol: gene symbol.

(metargidin) (*Adam15*), and cathepsin G (*Ctsg*) ($P < 0.02$, FDR < 10%, Fisher Exact score = 0.88) (Table 2). The proteins encoded by *Adamts4* and *Adam15* are involved in proteolysis of neurocan, aggrecan, and versican, members of a proteoglycan family, and components of the extracellular matrix in various tissues (Gao et al., 2002). Cathepsins are cysteine proteases that are released from lysosomes and play a key role in apoptotic cell death (Chwieralski et al., 2006). Up-regulation of these proteolysis genes provides further evidence of increased transcriptional apoptotic activity in the aging cochlea (Table 2).

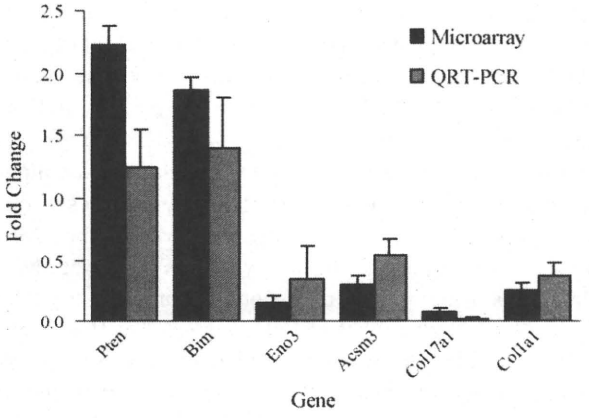

Fig. 4. QRT-PCR validation of microarray data. The data represent the fold-change in gene expression of 9-month-old D257A cochlea compared to age-matched WT based on average signal intensities of microarray hybridizations ($n = 4$) and gene expression levels determined by quantitative RT-PCR ($n = 4$). A consistent agreement between fold-change determined by the two methods was observed. Error bars represent SEM. Fold change: 9-month-old WT to age-matched D257A ratio. QRT-PCR: quantitative RT-PCR.

Stress responses may trigger apoptotic cell death (Mattson, 2000). The EASE analysis revealed that 15 genes involved in the "response to stress" category were up-regulated, including heat shock protein 1 (*Hspb1*) and myeloperoxidase (*Mpo*) ($P < 0.02$, FDR < 10%, Fisher Exact score = 0.65) (Table 2). The generation of ROS plays an important role in immune-mediated defense against infection, tumor cells, and cell damage (Aratani et al., 1999). Myeloperoxidase produces hypochlorous acid from hydrogen peroxide and chloride, and plays a key role in microbial killing or immune-mediated cell destruction (Aratani et al., 1999). In the mammalian brain, small heat shock proteins are induced in response to physiological stress such as tissue injury (Murashov et al., 1998). *Hspb1* is a member of the small heat shock protein family and has been characterized as a molecular chaperone required for protection of motor neurons under conditions of stress (Murashov et al., 1998). Together, these results provide evidence that mtDNA mutations result in significant up-regulation of genes involved in multiple apoptotic and stress pathways in the cochlea of aged mitochondrial mutator mice.

3.4. mtDNA mutations result in down-regulation of genes involved in energy metabolism

The EASE analysis revealed that one of the key energy metabolism categories, "carbohydrate metabolism", was significantly suppressed in D257A mice (Fisher Exact

score < 0.05), and that 13 genes in this category were significantly down-regulated in the cochlea ($P < 0.02$, FDR < 10%) (Table 1). These include phosphorylase kinase alpha 1 (*Phka1*), phosphoglucomutase 2-like 1 (*Pgm2l1*), and phosphorylase kinase gamma 1 (*Phkg1*) (Table 2). The glycogen phosphorylase kinases such as phosphorylase kinase alpha 1 and phosphorylase kinase gamma 1 are key enzymes involved in glycogen metabolism and regulate glycogen breakdown by activating glycogen phosphorylase (Wuyts et al., 2005). *Pgm2l1* is a member of a Phosphoglucomutase gene family involved in glycolysis and gluconeogenesis (Levin et al., 1999). Phosphoglucomutases regulate the interconversion of glucose 1-phosphate and glucose 6-phosphate in the glycolysis pathway (Levin et al., 1999). The EASE analysis also revealed that 13 genes in the "mitochondrion" category were down-regulated, including aquaporin 1 (*Aqp1*), PERP, TP53 apoptosis effector (*Perp*), and hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit (*Hadhb*) ($P < 0.02$, FDR < 10%, Fisher Exact score = 0.79) (Table 2). Aquaporins are membrane proteins that regulate water transport. Specific members of this water channel family have been shown to be expressed in the mitochondria of mammalian brain (Amiry-Moghaddam et al., 2005). Aquaporin 1, a member of the water channel family is localized in the stria vascularis of rat cochlea and is thought to play a key role in ion transport of the auditory system (Sawada et al., 2003). The protein encoded by *Hadhb* is a subunit of a multienzyme complex or mitochondrial functional protein that is involved in mitochondrial β-oxidation (Orii et al., 1997). QRT-PCR analysis was conducted for *Eno3* and *Acsm3* from these energy metabolism categories (Table 2), to validate the microarray results for expression of these genes. A consistent agreement between the two methods was observed (Fig. 4). Together, these observations provide evidence that mtDNA mutations result in down-regulation of genes involved in energy metabolism in the cochlea of aged mitochondrial mutator mice, consistent with mitochondrial dysfunction and associated impairment of energy metabolism.

3.5. mtDNA mutations result in down-regulation of genes involved in cytoskeletal regulation

The EASE analysis revealed that six cytoskeletal regulation categories, including "cytoskeleton", "extracellular matrix", "collagen", "structural molecule activity", "myosin", and "cell adhesion", were significantly suppressed in D257A mice (Fisher Exact score < 0.05), and that 132 genes in these categories were significantly down-regulated as a result of age-related accumulation of mtDNA mutations ($P < 0.02$, FDR < 10%) (Table 1). Of the 132 genes identified, 39 genes were associated with the "cytoskeleton" category, including 5 myosin encoding genes: *Myl1*, *Myh1*, *Myl2*, *Myh2*, and *Mylpf* (Table 2). Myosins are a family of actin-based molecular motor proteins and play an important role in the auditory system (Gillespie, 2002). For example, the protein encoded by *Myo7a* is localized along the actin-rich stereocilia of hair cells in the cochlea (Gillespie, 2002; Wolfrum et al., 1998). Further, specific mutations in *myo7a*, *myo15*, and *myo6* are associated with deafness (Kros et al., 2002; Melchionda et al., 2001). Twenty-five genes in the "extracellular matrix" category were significantly down-regulated, including eight collagen encoding genes: *Col3a1*, *Col1a2*, *Col5a2*, *Col4a1*, *Col17a1*, *Col15a1*, and *Col6a3* (Table 2). Type IX collagen is thought to play a key structural role in the tectorial membrane of the organ of Corti in cochlea (Suzuki et al., 2005). Specific mutations in *Col1a1*, *Col4a3*, *Col4a4*, *Col4a5*, and *Col11a2* cause conductive hearing loss in humans (Chen et al., 2005; Gratton et al., 2005; Iliadou et al., 2006). The EASE analysis also revealed that 22 genes in the "cell adhesion" category were significantly down-regulated, including cadherin 13 (*Cdh13*) (Table 2), a member of a cadherin family that mediates cell–cell adhesion or cell-extracellular matrix interactions (Zheng et al., 2005). Specific mutations in *Cdh23* and *Pcdh15* cause hearing loss in mice (Noben-Trauth et al., 2003; Zheng et al., 2005). QRT-PCR analysis was conducted for *Col17a1*, to validate the microarray results for expression of these genes. A consistent agreement between the two methods was observed (Fig. 4). These results provide evidence that mtDNA mutations result in down-regulation of genes involved in cytoskeletal regulation in the cochlea of aged mitochondrial mutator mice, consistent with cytoskeletal dysfunction.

3.6. mtDNA mutations result in down-regulation of genes associated with hearing function

Gene expression profiling revealed that six genes involved in the "perception of sound" category were down-regulated, including procollagen, type I, alpha 1 (*Col1a1*) and otogelin (*Otog*) ($P < 0.02$, FDR < 10%) (Table 2). Otosclerosis, a bony dystrophy of the otic capsule caused by abnormal resorption and redeposition of bony tissue, is one of the common causes of hearing loss (Rodriguez et al., 2004). Hearing loss in this disease most commonly results from the fixation of the stapedial footplate to the oval window, which prevents ossicular vibration in response to sound (Rodriguez et al., 2004). Specific mutations in *Col1a1* have been associated with this disorder (Rodriguez et al., 2004). The Otog gene encodes otogelin, an *N*-glycosylated protein, which is thought to play a key role in the deflection of the stereocilia bundles in the hair cells of the cochlea (Simmler et al., 2000). Specific mutations in this gene also cause deafness (Simmler et al., 2000).

In the cochlea, ion transport is needed to maintain proper ionic balance essential for hearing function (Jespersen et al., 2005). The EASE analysis revealed that 12 genes in the "ion transport" category were down-regulated, including gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 1 (*Gabra1*), glutamate receptor, ionotropic, AMPA2 (alpha 2) (*Gria2*), and potassium voltage-gated channel, shaker-related, subfamily, member 6 (*Kcna6*) ($P < 0.02$, FDR < 10%, Fisher Exact score = 0.79) (Table 2). The protein encoded by

Gabra1 is a receptor subunit for gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter in mammalian brain (Ma et al., 2005; Rosmond et al., 2002). *Gria2* encodes a glutamate receptor subunit that is involved in glutamatergic transmission (Mead and Stephens, 2003). QRT-PCR analysis was conducted for *Col1a1*, to validate the microarray results for expression of these genes. A consistent agreement between the two methods was observed (Fig. 4). These observations were consistent with the ABR findings, and together provide evidence that mtDNA mutations result in down-regulation of genes involved in hearing function in the cochlea of aged mitochondrial mutator mice.

3.7. TUNEL staining and immunostaining

Apoptosis results in nuclear DNA fragmentation (Rich et al., 2000). To confirm whether levels of apoptosis are increased in the cochleae of aged D257A mice, we performed the TUNEL staining to examine cochleae from 2- and 9-month-old WT and D257A mice. We selected spiral ganglion cells in the apical region for evaluation of TUNEL staining because spiral ganglion cells were severely degenerated in the basal region of 9-month-old D257A mice. The mean percentage of TUNEL-positive cells in 9-month-old D257A mice (4.1%) was significantly elevated compared to those of 2-month-old WT (0.8%) and D257A (1.0%), and 9-month-old WT mice (1.5%) ($P<0.05$, $n=5$) (Fig. 5A–E).

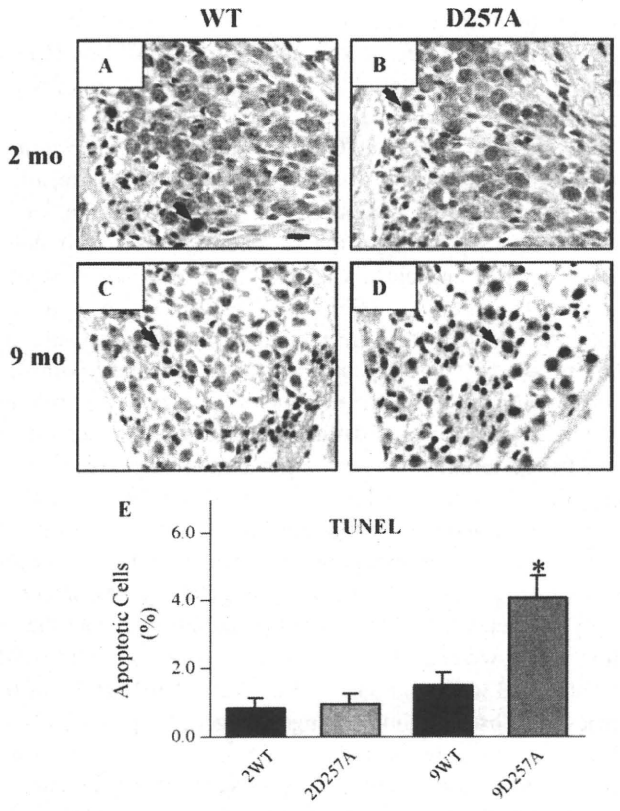

Fig. 5. Apoptosis detection by TUNEL staining. Detection and quantification of DNA fragmentation in the cochlear apical turn from 2- and 9-month-old WT and D257A (A–D) mice. Arrows indicate TUNEL-positive staining cells. The cochleae from 9-month-old D257A mice displayed significantly more TUNEL-positive cells relative to those of age-matched WT (E) ($^*P<0.05$, $n=5$). Error bars represent S.E.M. Scale bar = 25 μm.

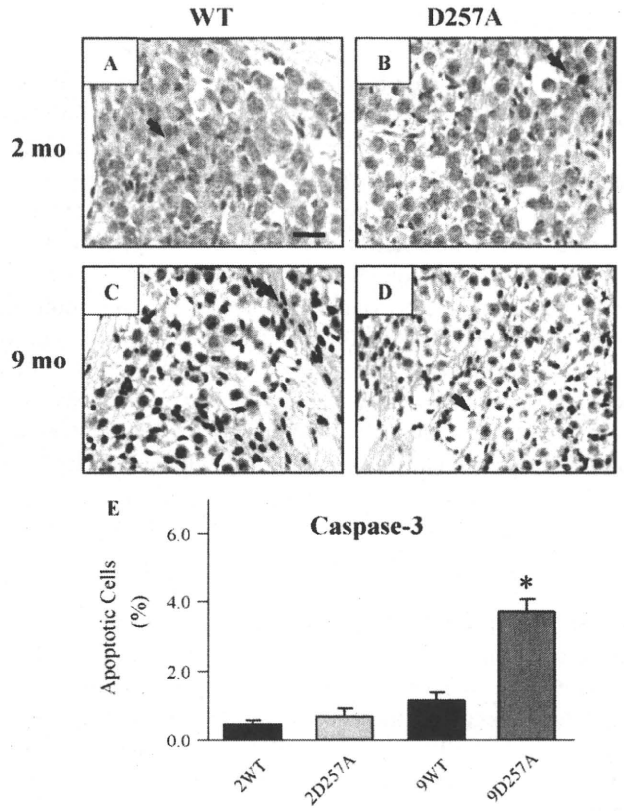

Fig. 6. Apoptosis detection by active caspase-3 staining. Detection and quantification of cleaved caspase-3 in the cochlear apical turn from 2- and 9-month-old WT and D257A (A–D) mice by immunostaining. Arrows indicate cleaved caspase-3-positive staining cells. The cochleae from 9-month-old D257A mice displayed significantly more cleaved caspase-3-positive cells relative to those of age-matched WT (E) ($^*P<0.05$, $n=5$). Error bars represent S.E.M. Scale bar = 25 μm.

An additional indicator of apoptotic cell death is the activation of caspases (Hengartner, 2000; Yuan and Yankner, 2000). To examine whether levels of activated caspase-3 are increased in the cochleae of aged D257A mice, we examined cochleae of 2- and 9-month-old WT and D257A mice by immunostaining using an antibody for cleaved caspase-3. We also selected spiral ganglion cells in the apical region for this study. The mean percentage of cleaved caspase-3-positive cells in 9-month-old D257A mice (3.7%) was significantly elevated compared to that seen in 2-month-old WT (0.5%) and D257A (0.7%), and 9-month-old WT mice (1.2%) ($P<0.05$, $n=5$) (Fig. 6A–E). These results were consistent with the microarray findings, and together provide evidence that mtDNA mutations result in induction of apoptosis in the cochlea of aged mitochondrial mutator mice.

4. Discussion

The data presented here provide the first comprehensive gene expression analysis of a tissue in aged mitochondrial mutator mice and reveal previously unreported pathways associated with age-related hearing loss that may provide insights into pathogenic mechanisms. It has been postulated that mitochondrial function declines with aging partly due to oxidative damage to mtDNA, and that the resulting mitochondrial dysfunction leads to impairment of energy metabolism. Indeed, mitochondrial ATP content and production has been shown to decline with aging in the rat muscle by ~50% (Dirks et al., 2006). Mitochondrial ATP production also declines with advancing aging in human skeletal muscle (Short et al., 2005). Furthermore, mitochondrial mutator mice display a decline in respiratory enzyme activities and mitochondrial ATP production rates with aging in the heart, suggesting a role of mtDNA mutations as a cause of energy metabolism impairment (Trifunovic et al., 2004). In the present study, we observed transcriptional evidence for a decline in carbohydrate metabolism and mitochondrial function in the cochlea of aged mitochondrial mutator mice, suggesting that accumulation of mtDNA mutations leads to energy metabolism impairment. We have shown previously that the frequency of mtDNA mutations in the cytochrome *b* gene region from 5-month-old D257A mice was ~3–8 times that in the age-matched WT mice for heart, liver, and duodenum (Kujoth et al., 2005). An independent group has also reported that the frequency of somatic mtDNA point mutations in the cytochrome *b* gene region from 8-week-old mitochondrial mutator mice was approximately ~5 times higher than that of the age-matched wild-type mice for brain, and that further increases at 25 weeks were not significant (Trifunovic et al., 2004). These reports suggest that although a substantial load of mtDNA mutations may be present by 2 months of age in the cochlea of D257A mice, it takes several more months to develop age-related hearing loss in these animals. We did not measure the frequency of mtDNA mutations in the cochlear tissue of D257A mice, but similar to all other tissues examined in this model, it is likely that these mice have a substantial burden of mtDNA mutations in this tissue. With aging, this may lead to mitochondrial dysfunction, resulting in impairment of energy metabolism.

A progressive decline in mitochondrial function and associated energy depletion in aging muscle have been associated with sarcopenia, an age-related loss of muscle mass and strength (Dirks et al., 2006). It has been postulated that apoptosis induced by mitochondrial dysfunction and associated impairment of energy metabolism in aged muscle tissue may contribute to the development of sarcopenia (Dirks et al., 2006). Indeed, impairment of respiratory function is associated with induction of apoptosis (Dirks et al., 2006; Kujoth et al., 2005). We have previously reported that aged mitochondrial mutator mice display an increase in the levels of apoptotic markers in liver, testes, thymus, intestine, and muscle (Kujoth et al., 2005). Furthermore, Parkinson's disease, one of the well-known age-related neuronal diseases, has also been associated with apoptotic death of neurons and mitochondrial mutations (Mattson, 2000). Interestingly, caloric restriction, known to retard aging and age-related diseases, has shown to suppress apoptotic cell death in the mouse cochlea and prevents age-related hearing loss (Someya et al., 2007), implicating apoptosis in the development of age-related hearing loss. In the present study, we observed up-regulation of over 50 genes associated with apoptotic pathways and a marked increase in the levels of apoptotic markers in the aging cochlea. Because D257A mice have a substantial burden of mtDNA mutations in post-mitotic tissues, it is possible that these mtDNA mutations lead to mitochondrial dysfunction and associated impairment of energy metabolism, resulting in activation of apoptosis. Together, our observations and these cited reports suggest that apoptosis may play a major role in the pathogenesis of age-related hearing loss in mammals, and that mtDNA mutations is likely to induce apoptotic cell death in the mammalian cochlea.

Structural proteins such as myosins, collagens, and cadherins play a key role in maintaining the structural integrity and motor activity of cochlear cells and mediating the cell-to-cell interaction essential for auditory function (Gillespie, 2002; Suzuki et al., 2005; Zheng et al., 2005). Indeed, defects or mutations in a number of myosin, collagen, and cadherin genes have been associated with hearing loss (Chen et al., 2005; Gratton et al., 2005; Iliadou et al., 2006; Kros et al., 2002; Melchionda et al., 2001; Noben-Trauth et al., 2003; Zheng et al., 2005). Of these mutations, *Cdh23* mutations have been linked to age-related hearing loss in mice and humans (Noben-Trauth et al., 2003). Cadherin 23 is specifically expressed in the stereocillia of the hair cells in the cochlea and plays a key role in perception of sound (Noben-Trauth et al., 2003). The C57BL/6 strain, a mouse homozygous for the *Cdh23* mutation, has increased susceptibility to early onset of presbycusis (Zheng and Johnson, 2001), indicating that this modifier gene plays a key role in its pathogenesis. It is possible that mtDNA mutations may lead to accelerating cochlear degeneration due to the Cdh23 mutations in the C57BL/6 background. Furthermore, it is likely that several other modifier genes contribute to presbycusis and our microarray observations raise the possibility that these unknown modifiers might include genes involved in cytoskeletal regulation identified herein. We note that a dramatic down-regulation of the expression of Myh2 (fold change = −136.0), Lmod2 (−38.2), and Col17a1 (−14.0) genes was observed, implicating these genes as potential candidates for modifier or marker genes of age-related hearing loss. We also observed severe loss of spiral ganglion neurons and down-regulation of over one hundred genes involved in cytoskeletal regulation in D257A mice. Because impaired energy metabolism can lead to apoptosis, it is possible that loss of critical cells such as spiral ganglion neurons due to apoptosis may cause damage to the structural proteins of cochlea, resulting in cytoskeletal dysfunction. Together, our

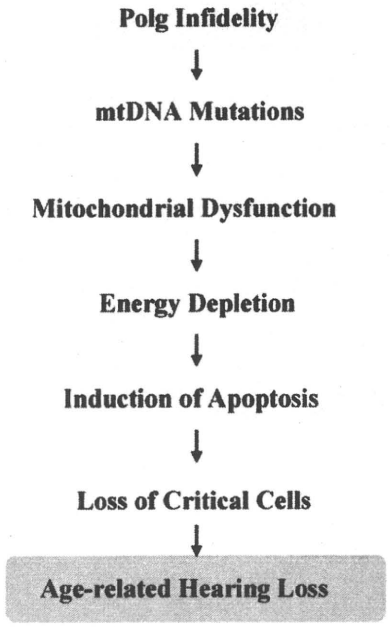

Fig. 7. Model of how mtDNA mutations impact cochlear function. In this model, a *Polg* mutation causes accumulation of mtDNA mutations, leading to mitochondrial dysfunction and associated impairment of energy metabolism. The energy depletion in turn induces apoptosis, leading to loss of critical cochlear cells such as hair cells and spiral ganglion neurons. These processes eventually cause loss of cochlear function, resulting in age-related hearing loss.

observations suggest that cytoskeletal dysfunction may be a key feature of age-related hearing loss.

Our findings suggest a previously unreported model of how mtDNA mutations impact cochlear function (Fig. 7). In this model, *Polg* infidelity causes accumulation of mtDNA mutations, leading to mitochondrial dysfunction and associated impairment of energy metabolism. The energy depletion in turn induces apoptosis, leading to loss of critical cochlear cells such as hair cells and spiral ganglion neurons. These processes eventually cause loss of cochlear function, resulting in age-related hearing loss. The relevance of this model to human presbycusis, mitochondrial diseases, and aging in general is worthy of further exploration.

Conflict of interest

The authors declare no actual or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary information associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2007.01.014.

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REVIEW ARTICLE

The responsible genes in Japanese deafness patients and clinical application using Invader assay

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Abstract

Discovery of deafness genes has progressed but clinical application lags because of the genetic heterogeneity. To establish clinical application strategy, we reviewed the frequency and spectrum of mutations found in Japanese hearing loss patients and compared them to those in populations of European ancestry. Screening revealed that in Japanese, mutations in *GJB2*, *SLC26A4*, and *CDH23*, and the mitochondrial 12S rRNA are the major causes of hearing loss. Also, mutations in *KCNQ4*, *TECTA*, *COCH*, *WFS1*, *CRYM*, *COL9A3*, and *KIAA1199* were found in independent autosomal dominant families. Interestingly, spectrums of *GJB2*, *SLC26A4*, and *CDH23* mutations in Japanese were quite different from those in Europeans. Simultaneous screening of multiple deafness mutations based on the mutation spectrum of a corresponding population using an Invader panel revealed that approximately 30% of subjects could be diagnosed. This assay will enable us to detect deafness mutations in an efficient and practical manner in the clinical platform. We conclude that specific racial populations may have unique deafness gene epidemiologies; therefore, ethnic background should be considered when genetic testing is performed. Simultaneous examination of multiple mutations based on a population's spectrum may be appropriate and effective for detecting deafness genes, facilitating precise clinical diagnosis, appropriate counseling, and proper management.

Keywords: *GJB2, SLC26A4, CDH23, mitochondria, founder effect, hot spot, hearing impairment, genetic testing*

Discovery of deafness genes and clinical application

From several etiological studies, at least 50% of childhood hearing loss has been estimated to be of genetic etiology, with the other 50% due to environmental causes, including newborn delivery problems, acoustic trauma, ototoxic drug use, and prenatal/postnatal infection [1]. Newborn hearing screening programs and subsequent early hearing loss detection have developed rapidly, leading to improved outcomes in cognitive and linguistic ability, and better educational management of deaf children [2]. Along with early discovery of hearing loss and subsequent intervention programs, much attention has been paid to the determination of the hearing loss etiology. Genetic testing, therefore, is now vital to enable a precise diagnosis, predict the severity of hearing loss, estimate associated abnormalities, select appropriate habilitation, prevent hearing loss, and provide improved genetic counseling. There has been considerable progress in the discovery of deafness genes, but clinical application still entails difficulties due to the genetic heterogeneity of deafness. Nearly 100 genes are thought to cause hereditary hearing loss, but a number of these may result in similar phenotypes that entail no abnormalities other than hearing loss. The costs and time required for screening genes one by one are prohibitive, but it is now thought that certain mutations

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are recurrent. A screening strategy that focuses on those frequently reported recurrent mutations, expected to be commonly encountered in the clinical setting, might be a breakthrough for clinical application.

Our series of studies has revealed genes responsible for Japanese deafness patients, and the differences in mutation spectrum between Japanese (who are probably representative of other Asian populations) and populations with European ancestry. To construct a deafness gene database for Japanese subjects and to establish an efficient genetic testing method, the current study 1) summarized the frequency and the spectrum of the mutations found in the Japanese deafness population in comparison with those reported in the populations of European ancestry, and 2) considered whether a diagnostic strategy based on a mutation/gene database followed by Invader assay would be adequate for genetic screening for such heterogeneous genetic disorders.

Genes responsible for deafness

Genes that were responsible for non-syndromic hearing loss in Japanese patients and the frequencies of mutations mainly based on our screening series are summarized in Table I.

We chose references that describe mutations and frequency in each gene in Japanese.

The major cause of hearing loss in Japanese patients and clinical relevance

The present review summarized the 15 genes responsible for non-syndromic hearing loss in Japanese patients (including some who may have visited an ENT clinic as a non-syndromic hearing loss patient), a number that suggests there may be many genes involved in deafness in Japanese patients. As predicted, there are many frequent and recurrent mutations within the reported mutations. So far, mutations in *GJB2*, *SLC26A4*, *CDH23*, and the 1555A→G mitochondrial mutation were found to be the major causes of hearing loss in Japanese patients. These mutations are prevalent and therefore commonly encountered in the clinic, and the clinical application of this information will be important.

GJB2

In large-scale screening using more than 1000 samples (1227 sensorineural hearing loss patients with various onsets and etiology), 11.3% (259/2454) of the alleles had some mutations. A total of 13 mutations in *GJB2* have so far been reported in Japanese subjects [3]. When restricting the subjects to only those with congenital SNHL, 18.3% (62/338) had at least one *GJB2* mutation detected by Invader assay [4]. Of the mutations identified, 235delC was the most frequent, followed by V37I, G45E, and Y136X [3–5].

GJB2 is currently known to be the most prevalent gene responsible for congenital hearing loss worldwide [1], and therefore is closely related to universal infant hearing screening programs. In our screening, approximately 20% of congenital subjects had at least one mutation in *GJB2*, confirming that *GJB2* is the major causative gene in Japanese deafness patients [5].

It has been known that prevalent *GJB2* mutations have a high ethnic predilection (see The connexin-deafness homepage; http://davinci.crg.es/deafness/): 35delG is quite common in the Caucasoid population, 167delT was reported as prevalent in Ashkenazi Jews, R143W in a restricted village in Africa, and 235delC in Asian populations. The 235delC mutation was found to be the most prevalent mutation in our screening, accounting for 37.6% (38/101) of the hearing-impaired persons [5].

Investigation regarding a founder effect for the 235delC mutation by means of single nucleotide polymorphism (SNP) analysis indicated that this mutation was derived from a common ancestor [3]. In contrast, the 35delG mutation, the most prevalent mutation in Caucasoid populations, was not detected in our population, indicating that this mutation is seldom seen in Japanese subjects.

There have been general rules that inactivating mutations (deletion mutations and stop mutations) show more severe phenotypes compared with those caused by non-inactivating mutations (missense mutations) [6–8]. As well as a highly accurate diagnosis, these genotype-phenotype correlation data could provide prognostic information to help decide the strategy of intervention with hearing, i.e. whether a child should receive cochlear implantation or hearing aids. For the patients with severe phenotypes who have *GJB2* mutations, genetic information would aid decision-making regarding cochlear implantation, because their hearing loss is of cochlear origin and therefore they are good candidates for implantation. In fact, cochlear implantation has resulted in remarkable improvements in auditory skills and development of speech production for patients with profound hearing loss associated with *GJB2* mutations [9].

SLC26A4

SLC26A4 is also a common responsible gene in Japanese patients associated with enlarged vestibular

Table I. Responsible genes reported in Japanese patients (in order of reported year).

Genes	References	Frequency
Mitochondrial *3243A→G*	Goto et al., 1990	0.3% (1/319 Usami et al., 2000 [15]) – 3% (3/100 Oshima et al., 1999 [26]) of the SNHL patients who visited outpatient clinics
Mitochondrial *1555A→G*	Hutchin et al., 1993	3% (11/319 Usami et al., 2000 [15]) – 5% (7/138 Noguchi et al., 2004 [18]) of the SNHL patients who visited outpatient clinics 33% (7/21 Usami et al., 2000 [15], 2/6 Noguchi et al., 2004 [18]) of the SNHL patients due to aminoglycoside injection 10% (14/140) of patients who received cochlear implantation 57% (13/22) of patients who received cochlear implantation due to aminoglycoside-induced hearing loss (Usami et al., 2000 [15])
MYO7A	Liu et al., 1997	Not available (single DFNA11 family)
POU3F4	Hagiwara et al., 1998	Not available (single DFN3 family)
GJB2	Fuse et al., 1999	11.3% (259/2454) alleles $n=1227$ SNHL patients (Ohtsuka et al., 2003 [3]) 18.3% (62/338) congenital SNHL (Abe et al., 2007 [4])
SLC26A4	Usami et al., 1999 [11]	90% (9/10) Pendred syndrome families 78% (25/32) non-syndromic hearing loss Associated with EVA families (Tsukamoto et al., 2003 [12])
KCNQ4	Akita et al., 2001 [22]	1/16 ADSNHL (Akita et al., 2001 [22])
Mitochondrial *7511T→C*	Ishikawa et al., 2002	Not available (single maternally inherited family)
TECTA	Iwasaki et al., 2002 [23]	Not available (single mid-frequency involved family)
WFS1	Komatsu et al., 2002	3/182 ADSNHL 3/10 low-frequency ADSNHL 0/64 ARSNHL (Fukuoka et al., 2007 [24])
COCH	Usami et al., 2003 [21]	1/23 ADSNHL 0/20 Meniere's disease (Usami et al., 2003 [21])
CRYM	Abe et al., 2003 [25]	2/192 congenital SNHL (Abe et al., 2003 [25])
KIAA1199	Abe et al., 2003 [27]	4/192 congenital SNHL (Abe et al., 2003 [27])
COL9A3	Asamura et al., 2005 [28]	2/147 SNHL (Asamura et al., 2005 [28])
CDH23	Wagatsuma et al., 2007 [14]	5/64 AR congenital SNHL (Wagatsuma et al., 2007 [14]).

SNHL, sensorineural hearing loss; AD, autosomal dominant; AR, autosomal recessive.

aquaduct (EVA). Based on genetic evaluations, the historical concept of two distinct categories of disease, Pendred syndrome and non-syndromic hearing loss associated with EVA, has changed and they should be considered and treated as a continuum of diseases caused by the same gene [10,11]. The prevalent association of *SLC26A4* mutations in these patients (90% Pendred syndrome, 78% non-syndromic hearing loss associated with EVA) indicates the importance of this gene in the pathophysiology of this category of hearing impairment [12]. Our recent study confirmed clinical characteristics of 38 patients with EVA caused by biallelic *SLC26A4* mutations. These included congenital (100%), fluctuated (92.3%), and progressive (92.3%) hearing loss usually associated with vertigo (70.6%) and/or goiter (27.8%) during long-term follow-up [13]. Among the mutations identified, H723R was the most common. There was great variation regarding hearing loss severity even with the same mutations, suggesting that other factors may contribute to such variability. Unlike in the case of *GJB2*, phenotype cannot be predicted from the genotype [13]; however, the clarification of clinical

features will enable more appropriate genetic counseling and proper medical management for these patients.

CDH23

Mutations in the *CDH23* gene are known to be responsible for both Usher syndrome type ID (USH1D) and non-syndromic hearing loss (DFNB12), and the molecular confirmation of the *CDH23* gene, encoding a molecule that forms the lateral links between the stereocilia, has become important in the diagnosis of these conditions.

We have identified 6 patients (from 5 families) with mutations in *CDH23* out of 64 recessive hearing loss families, indicating that these mutations are one of the major known genetic causes of hereditary hearing loss in Japanese subjects 14]. A total of 17 variants (possible disease-causing mutations) were found in this screening. Among them, four mutations were confirmed to be responsible for deafness by segregation study. Although it is difficult to screen this gene because of the many exons as well as polymorphisms, much attention should be paid to it when performing genetic testing of hearing loss patients. Similar to *GJB2* and *SLC26A4*, the *CDH23* mutation spectrum in Japanese is very different from that found in Caucasians [14], indicating a possible founder effect.

1555A→G mutation in the mitochondrial 12S rRNA

The 1555A→G mitochondrial mutation has been identified as the most prevalent mitochondrial mutation [15]. This mutation has been reported to be associated with aminoglycoside injection, although there have been some patients with no history of injection [16,17]. In our series of screenings in the hearing impaired population, the 1555A→G mitochondrial mutation was found in approximately 3–5% of outpatients and 33% of subjects with a history of aminoglycoside injection [15,18]. In addition, it has also been found in congenital/early onset sensorineural hearing loss patients, suggesting that it is important to screen for this mutation in patients with that condition [4]. Although the mitochondrial 1555A→G mutation has been thought to transmit in a homoplasmic state, recent reports have shown heteroplasmy cases [4,19]. For the family members who do not have hearing loss, it is important to prevent severe hearing loss by avoiding use of aminoglycoside antibiotics; therefore we distribute a drug use warning card to them [20].

Less frequent genes

In contrast to the four genes discussed above, the occurrence of mutations of the *COCH* [21], *KCNQ4* [22], *TECTA* [23] *WFS1* [24], and *CRYM* [25] genes in our group of deaf subjects was low (Table I). The 3243A→G mutation in the mitochondrial tRNA (Leu(UUR)) was found in 0.3% of SNHL patients in our screening [15] but 3% in another report [26], the higher figure being due to sampling bias, indicating that further evaluation of this mutation is needed. A few patients with *COL9A3,* and *KIAA1199* have also been found, but due to the rather small families, replicable data are needed to confirm whether these mutations are really pathologic [27,28]. There are still only small numbers of autosomal dominant sensorineural hearing loss (ADSNHL) families whose responsible genes have been identified. Continuous collection and analysis of clinical data are necessary to clarify how these genes are involved in Japanese ADSNHL.

Comparison with the mutation spectrum

As shown in Figures 1–3, spectrums of *GJB2*, *SLC26A4*, and *CDH23* mutations found in the Japanese population were quite different from those reported in populations with European ancestry. This suggests that each racial population may have a unique epidemiology of deafness genes and there are specific common founder mutations, for example, 35delG, 235delC, R143W in the *GJB2* gene, and H723R in the *SLC26A4* gene. SNP analysis proved a founder event for the origin of the prevalent mutations in the Japanese [3,29]. These ethnic-specific mutation spectrums indicate that an individual's ethnic background should be borne in mind when genetic testing is performed.

Screening strategy based on recurrent mutations

The reason for the recurrence of some mutations has been explained either by the existence of common founders or a mutational hot spot. SNP analysis has proved that 235delC in the *GJB2* gene and H723R in the *SLC26A4* gene are common founder mutations, thus explaining their frequency [3,29]. Different combinations of *GJB2, SLC26A4* and *CDH23* mutations exist in the Japanese, indicating that ethnic background should be considered when performing genetic testing. On the other hand, there are certain hot spots for frequent genes, for example, in the *KCNQ4* gene, a mutation (W276S) has been found independently in three families originating from Europe and Japan. Haplotype analysis using