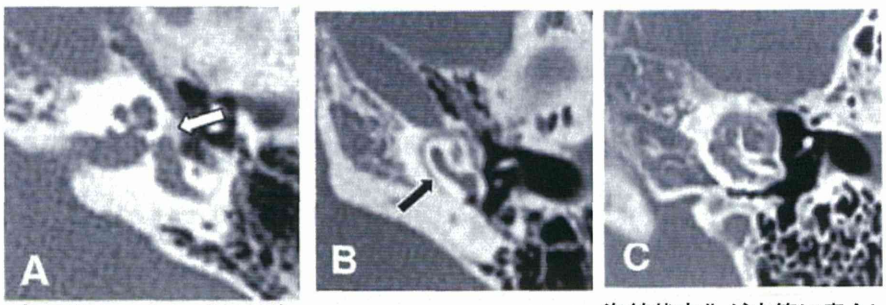
図2　側頭骨CTの評価方法
脱灰像の位置と範囲による分類

Grade1　　Grade2　　Grade3

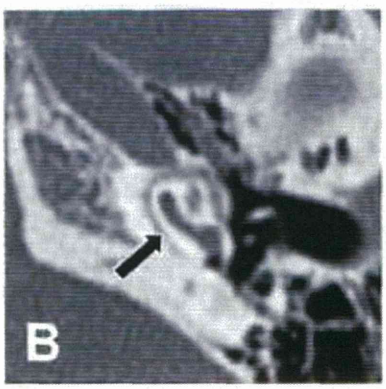


窓周囲のみに脱灰像を認めるもの。

海綿状変化が広範に癒合し、蝸牛に含有された状態。
CT上では、蝸牛との識別が困難。

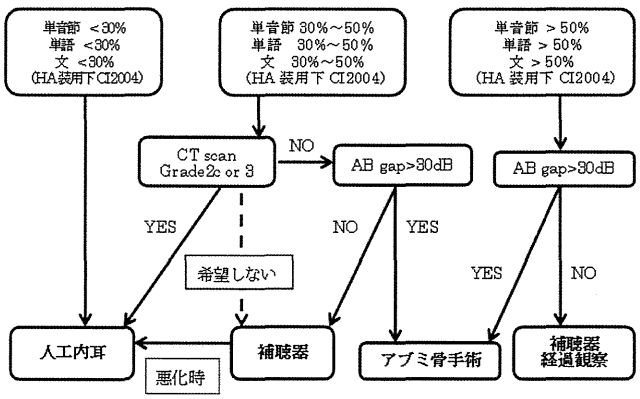
Rotteveelら：Otol Neurotol　2004

図3　Grade2のsub分類



≪2A ≫
蝸牛周囲にリング状に脱灰像（double ring sign）を認めるもの
≪2B ≫
基底回転の狭窄を認めるもの
≪2C ≫
2A 、2Bの両方の所見を認めるもの

図4　耳硬化症の診療ガイドライン



三澤建、熊川孝三、加藤央、武田英彦：人工内耳埋め込み術を施行した蝸牛型耳硬化症および van der Hoeve 症候群の長期成績と当院における治療戦略。
Otol Japan 23:841-87, 2013.

平成２６年度厚生労働科学研究費補助金
難治性疾患等政策研究事業（難治性疾患政策研究事業）
分担研究報告書

薬剤性難聴の診断基準・診療ガイドライン策定に向けて

研究分担者　山岨　達也（東京大学医学部耳鼻咽喉科）
研究協力者　樫尾　明憲（東京大学医学部耳鼻咽喉科）

研究要旨

アミノ配糖体や白筋製剤による薬剤性難聴は臨床的にも動物実験的にも知られているが、本邦における全国的な実態調査はされていない。今回東大病院における症例の検討と文献レヴューから全国調査として必要な項目、問題点を検討した。アミノ配糖体による薬剤性難聴の症例は母集団も少なく調査は困難と考えられた。白金製剤による難聴検索には化学療法施行前後の聴力評価が必要と考えられた。白金製剤の投与総量・放射線の併用の有無・年齢・他剤併用の有無重点的調査項目として全国調査を行うことが必要と考えられた。

Ａ．研究目的

抗生剤の一種であるアミノ配糖体や抗がん剤の一種である白金製剤などは内耳障害をきたすことが臨床的にも動物実験的にも知られている。しかしながら、本邦における薬剤性難聴の実態の詳細はまだ明らかとなっていない。今後、本邦における薬剤性難聴の実態を調査し、難聴の発生頻度・重症度などを検討することが必要である。本研究では東大病院における過去の薬剤性難聴症例を検討するとともに、白金製剤による難聴の文献的レヴューを行い、今後全国的な調査として必要な項目を検討することを目的とした。

Ｂ．研究方法

過去５年間に東大病院難聴外来を受診し、薬剤性難聴と診断された症例の聴力型、難聴原因、難聴発生の時期、進行の有無を検討した。また過去の文献報告を渉猟し、白金製剤伴う難聴の出現率・リスク因子などを調査した。

（倫理面への配慮）

本研究は東大病院倫理委員会の承認を得て施行した。個人の情報は**匿名化を行い**、資料は鍵のかかる場所に保管管理を行った。発表に際しては個人が特定できない形で行った。

Ｃ．研究結果

2010 年より 2014 年に当科難聴外来を受診して薬剤性難聴の診断を受けた物は８名であった。原因薬剤は抗がん剤４名（白金製剤３名、シクロホスファミド１名）、抗結核

治療薬 2 名（ストレプトマイシン・カナマイシン各 1 名)、心臓術後感染に対するアミカシン 1 名、原因不明熱に対するストレプトマイシン 1 名であった。聴力型の多くは高音漸傾型を呈したが、一部水平、谷型聴力も存在した。アミノ配糖体による薬剤性難聴は心臓術後感染を除いて全例 20 年以上昔に投薬された陳旧例であった。抗がん剤による難聴者は薬剤投与後 2 日と比較的短期に発症したものから投薬 2 ヶ月後に難聴を自覚したものもおり、発症までの期間は多岐にわたった。

D．考察

東大病院における薬剤性難聴の症例数は非常に少なかった。アミノ配糖体については現在使用ようとも制限されており、母集団が少ないためと予想される。従って、アミノ配糖体における難聴の詳細な評価は、本邦では困難ことが予想される。一方で、抗がん剤（特に白金製剤）については現在も多くの患者に使用されている。文献的には白金製剤による難聴の出現率は、24%～79 % に生じるとも報告されており（Theunissen et. al. Ann Otol Rhinol Laryngol. 2014)、当院における症例数と大きな開離が存在した。この原因としては当科では化学療法を施行する患者に対して、ルーチンでの聴力評価がなされていなかった点が考えらえる。文献を詳細に検討すると、難聴は観察されるもの日常生活に影響の無い症例も多く、その場合難聴を訴えることをせず検査がなされていないという可能性がある。また、抗がん剤治療の対象となる患者の多くは老人性難聴も合併しており、難聴があっても薬剤に起因するものかどうか評価が困難である点なども一因であろう。これにらついては、多施設からのデータの解析を待つ必要があると思われる。

白金製剤に伴う難聴の危険因子は白金製剤の投与総量・放射線の併用の有無・年齢・多剤併用の有無（Yasui N, et al. J. Pediatr Hematol Oncol. 2014）などが挙げられており、今後全国的な調査で重点的に調査する必要があると考えられた。また、白金製剤使用中止後も進行することも報告されている（Einarsson EJ, et al. Int J Audiol. 2010)。当科における症例も発症時期が多岐にわたっており同様の傾向がうかがわれ、長期の経時的聴力経過観察が必要だと考えられた。

薬剤耳毒性の重症度分類基準については Common Terminology Criteria for Adverse Events version 4 (CTCAEv4) や、the American Speech Language Hearing Association (ASHA) system が海外では提唱されている。しかしながら、聴力閾値による分類であり、日常生活における QOL の低下は反映されていない。抗がん剤投与中止は生命予後に影響を及ぼすので、聴力レベルよりも QOL の低下の重症度という面からの重症度分類を検討するべきであると考えられる。

抗がん剤の使用は耳鼻咽喉科以外でも頻繁に施行されているが、施設においては聴力検査を行うこともできない場合もある。重症度分類・ガイドラインを作成すること

は訴訟の問題へとも発生する危険性をはらんでおり、慎重に行う必要がある。

E．結論

東京大学耳鼻咽喉科における薬剤性難聴症例の検討と文献的レヴューから、今後全国調査を行う点での問題点を検討した。アミノ配糖体による薬剤性難聴は母集団も少なく、調査は困難と思われた。白金製剤による難聴検索には化学療法施行前後の聴力評価が必要と考えられた。白金製剤の投与総量・放射線の併用の有無・年齢・多剤併用の有無重点的調査項目として全国調査を行うことが必要と考えられた。

F．研究発表

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H．知的財産権の出願・登録状況（予定を含む。）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

平成２６年度厚生労働科学研究費補助金
難治性疾患等政策研究事業（難治性疾患政策研究事業）
分担研究報告書

薬剤性難聴の診断基準・診療ガイドライン策定に向けて

研究分担者　中川　尚志（福岡大学医学部耳鼻咽喉科）

Ａ．研究目的

難治性疾患等政策研究事業の一環として、薬剤による聴力障害の臨床像を明らかにする。

Ｂ．研究方法

薬剤性難聴の臨床情報データベースを作成し、本事業の研究分担者、研究協力者が所属する医療機関より患者データを収集する。本年度は臨床情報データベースを作成するために、臨床および動物を用いた基礎実験で対象になるであろう文献の検索を行う。

（倫理面への配慮）

福岡大学病院臨床研究審査委員会に研究案を提出、承認を得た（整理番号 14-11-08）。

Ｃ．研究結果

過去に動物実験で内耳毒性が報告されている薬剤は、消毒薬としてはグルコン酸クロールヘキシジンとポピドンヨード、ブロー液、ゲンチアナバイオレットがあった。それぞれ薬物濃度と投与時間に依存していた。抗生剤としてはアミノ配糖体とマクロライド系抗菌薬の文献があった。マクロライド系抗菌薬の内耳毒性は一過性であった。アミノ配糖体抗菌薬は薬剤の種類によって蝸牛および前庭と障害の程度が異なった板。また腎機能障害や騒音曝露後、高齢、併存する難聴によって毒性が増強し、特に利尿剤との併用で内耳毒性が発現しやすくなる。抗がん剤としてはシスプラチンをはじめとする白金製剤が聴力障害を生じることが報告されており、シスプラチンでは総投与量は 200mg/m^2 を超えるときに聴力障害が発現する危険性が高くなり、不可逆性となりやすいとの臨床データが示されていた。

Ｄ．考察

薬剤性内耳障害には投与薬剤の濃度、総投与量、投与期間もしくは時間を記載することが必要である。また前庭を含めた関連する症状と患者背景、併用薬の情報が障害の程度を評価する情報として有用である。薬剤性難聴のデータベースを作成するときは留意する必要がある。

Ｅ．結論

薬剤性難聴の臨床情報データベースの作成を行った。作成に必要な項目や因子の文献検索を行い、作成に反映させた。

F．研究発表

1.　論文発表

該当なし。

2.　学会発表

該当なし。

H．知的財産権の出願・登録状況（予定を含む。）

1．特許取得

該当なし。

2．実用新案登録

該当なし。

3.その他

該当なし。

Ⅲ.　研究成果の刊行に関する一覧表

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Ⅳ.　研究成果の刊行物・別刷

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ORIGINAL ARTICLE

Frequency of mitochondrial mutations in non-syndromic hearing loss as well as possibly responsible variants found by whole mitochondrial genome screening

Takuya Yano, Shin-ya Nishio, Shin-ichi Usami and the Deafness Gene Study Consortium[1]

Mutations in mitochondrial DNA (mtDNA) are reported to be responsible for the pathogenesis of maternally inherited hearing loss. Complete mtDNA sequencing may detect pathogenic mutations, but whether they are indeed pathogenic can be difficult to interpret because of normal ethnic-associated haplogroup variation and other rare variations existing among control populations. In this study, we performed systemic mutational analysis of mtDNA in 394 Japanese patients with hearing loss. Two different cohorts were analyzed in this study: Cohort 1, 254 maternally inherited patients; and Cohort 2, 140 patients with various inheritance modes. After screening of the entire mtDNA genome with direct sequencing, we evaluated the frequency of previously reported mutations and the frequency and pathogenicity of the novel variants. As a result, the 'Confirmed' mitochondrial mutations were found predominantly in Cohort 1 rather than in Cohort 2 (14.6 vs 0.7%). 1555A>G ($n=23$) is the most common mutation, followed by the 3243A>G ($n=11$) mutations. On the basis of prediction analysis, we detected 10 novel homoplasmic mitochondrial variants. After further classification, the 3595A>G and 6204A>G variants were found to be new candidate mutations possibly associated with hearing loss.
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Keywords: mitochondrial mutation; non-syndromic hearing loss

INTRODUCTION

Hearing impairment is one of the most common sensory handicaps, with a frequency of at least 1/1000 at birth, and 50% of these cases can be attributed to genetic causes. Furthermore, causative mitochondrial DNA (mtDNA) mutations have been found in 5–10% of patients with postlingual non-syndromic hearing loss.[1]

Among mitochondrial mutations, 1555A>G mutations in the mitochondrial *12S rRNA* are found frequently (0.6–5.3%, depending on the ethnic group) in aminoglycoside-induced and late-onset non-syndromic hearing loss.[2–4] A 1494C>T mutation in *12S rRNA* is also associated with aminoglycoside-induced and non-syndromic hearing loss.[5] A 3243A>G mutation in the $tRNA^{Leu(UUR)}$ is associated with maternally inherited diabetes combined with deafness,[6] and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), which frequently present with hearing loss. 7445A>C/G/T,[7,8] 7472insC and 7510T>C[9] mutations in the $tRNA^{Ser(UCN)}$ are also associated with aminoglycoside-induced or non-syndromic hearing loss.

Moreover, additional mutations in *12S rRNA* (827A>G,[10] 961T>C, 961delT+Cn, 1005T>C and 1095T>C[11]) have been reported as mitochondrial hearing loss mutations. Although there were growing numbers of reports of various novel mtDNA mutations associated with hearing loss, most focused on a few limited nucleotide positions or only the *12SrRNA* region.[12] Therefore, we conducted a whole mitochondrial genome mutational analysis by direct sequencing using samples from 254 maternally inherited and 140 non-syndromic Japanese hearing loss probands with various inheritance modes, and summarized the frequencies of the mutations, as well as the spectrum and phenotypes found in the hearing loss patients with mtDNA mutations.

MATERIALS AND METHODS

Subjects

Two cohorts were used in this study: Cohort 1, 254 Japanese maternally (or possibly autosomal dominant with affected mother and one or more affected children) inherited sensorineural hearing loss (SNHL) subjects; and Cohort 2, 140 Japanese SNHL subjects with various inheritance modes (14 autosomal dominant or mitochondrial inherited, 126 autosomal recessive inherited or sporadic cases), both collected from 33 ENT departments nationwide in Japan. All subjects gave prior written informed consent for participation in the

Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Japan
[1]Participating institutions: see Acknowledgments.
Correspondence: Professor S Usami, Department of Otorhinolaryngology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.
E-mail: usami@shinshu-u.ac.jp
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project, which was approved by the ethical committee of each hospital. The control group consisted of 192 unrelated Japanese healthy individuals with normal hearing evaluated by auditory testing.

Mutation analysis

Whole mtDNA from each patient was amplified into two long fragments, A and B, by LA Taq DNA polymerase (TaKaRa BIO, Shiga, Japan) as described elsewhere.[13] In brief, each genomic DNA sample was amplified by long PCR for 1 min at 94 °C, followed by 30 three-step cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 6 min, with a final extension at 72 °C for 5 min, ending with a holding period at 4 °C.

After the PCR amplification, resulting products were purified and direct sequenced with ABI Big Dye terminators and ABI 3130 autosequencer (Applied Biosystems, Carlsbad, CA, USA). Sequencing reaction was performed with 50 primers for the whole mitochondrial genome, designed with mitoSEQr Resequencing System (Applied Biosystems).

Sequencing data were analyzed by SeqScape ver2.6 and SeqAnalysis (Applied Biosystems). The sequencing result from each patient was compared with the rCRS (Reversed Cambridge Reference Sequence) to identify mtDNA mutations. Mitochondrial DNA mutations included in the mtSNP (http://mtsnp.tmig.or.jp/mtsnp/index.shtml), MITOMAP (http://www.mitomap.org/MITOMAP) or Uppsala mtDB (http://www.genpat.uu.se/mtDB/) databases were excluded as non-pathogenic variants when we search to novel variants.

We evaluated mutations according to evaluation criteria derived from a previous report by Zaragoza *et al.*[14]

Prediction of pathogenicity of mtDNA mutations

Initially, we measured the frequencies of each mutation found in healthy controls in our study ($n=192$) and in the mtSNP database ($n=2153$, including: centenarians in Gifu and Tokyo, type 2 diabetes mellitus patients (with or without vascular disorders), overweight and non-overweight young adult males, Parkinson's disease patients and Alzheimer's disease patients in Japan). The nucleotide conservation in each gene from humans and 60 mammalian species (*Artibeus jamaicensis*, NC_002009; *Balaenoptera musculus*, NC_001601; *Balaenoptera physalus*, NC_001321; *Bos taurus*, NC_006853; *Canis familiaris*, NC_002008; *Cavia porcellus*, NC_000884; *Cebus albifrons*, NC_002763; *Ceratotherium simum*, NC_001808; *Chalinolobus tuberculatus*, NC_002626; *Dasypus novemcinctus*, NC_001821; *Didelphis virginiana*, NC_001610; *Dugong dugon*, NC_003314; *Echinops telfairi*, NC_002631; *Echinosorex gymnura*, NC_002808; *Equus asinus*, NC_001788; *Equus caballus*, NC_001640; *Erinaceus europaeus*, NC_002080; *Felis catus*, NC_001700; *Gorilla gorilla*, NC_001645; *Halichoerus grypus*, NC_001602; *Hippopotamus amphibious*, NC_000889; *Hylobates lar*, NC_002082; *Isoodon macrourus*, NC_002746; *Lama pacos*, NC_002504; *Loxodonta africana*, NC_000934; *Macaca sylvanus*, NC_002764; *Macropus robustus*, NC_001794; *Mus musculus*, NC_005089; *Myoxus glis*, NC_001892; *Nycticebus coucang*, NC_002765; *Ochotona collaris*, NC_003033; *Ornithorhynchus anatinus*, NC_000891; *Orycteropus afer*, NC_002078; *Oryctolagus cuniculus*, NC_001913; *Ovis aries*, NC_001941; *Pan paniscus*, NC_001644; *Pan troglodytes*, NC_001643; *Papio hamadryas*, NC_001992; *Phoca vitulina*, NC_001325; *Physeter catodon*, NC_002503; *Pongo pygmaeus*, NC_002083; *Pongo pygmaeus* abelii, NC_002083; *Pteropus dasymallus*, NC_002612; *Pteropus scapulatus*, NC_002619; *Rattus norvegicus*, NC_001665; *Rhinoceros unicornis*, NC_001779; *Sciurus vulgaris*, NC_002369; *Soriculus fumidus*, NC_003040; *Sus scrofa*, NC_000845; *Tachyglossus aculeatus*, NC_003321; *Talpa europaea*, NC_002391; *Tarsius bancanus*, NC_002811; *Thryonomys swinderianus*, NC_002658; *Trichosurus vulpecula*, NC_003039; *Tupaia belangeri*, NC_002521; *Ursus americanus*, NC_003426; *Ursus arctos*, NC_003427; *Ursus maritimus*, NC_003428; *Volemys kikuchii*, NC_003041; *Vombatus ursinus*, NC_003322) was evaluated by the ClustalW method or the mtSNP database (mtSAP Evaluation; http://mtsnp.tmig.or.jp/mtsnp/search_mtSAP_evaluation.html). The mutations were considered to be possibly pathogenic if the original amino acid or base was conserved in >50% of the species (31 or more of 61 species).[15]

RESULTS

Direct sequence screening of the 254 probands of Japanese maternally inherited SNHL families and 140 non-syndromic hearing loss probands with various severities of hearing loss revealed 634 single-nucleotide polymorphisms in whole mitochondrial genome. Among those single-nucleotide polymorphisms, 19 were previously reported as associated with hearing loss: 792C>T ($n=1$), 827A>G ($n=10$), 856A>G ($n=3$), 961T>C ($n=3$), 1005T>C ($n=2$), 1095T>C ($n=1$), 1310C>T ($n=3$), 1494C>T ($n=1$), 1555A>G ($n=23$), 3243A>G ($n=11$), 3398T>C ($n=1$), 3421G>A ($n=2$), 5628T>C ($n=1$), 7511T>C ($n=3$), 8108A>G ($n=1$), 8348A>G ($n=1$), 11696G>A ($n=4$), 14693A>G ($n=1$) and 15927G>A ($n=4$) (Tables 1 and 2). In this study, based on the MITOMAP database, status was considered to be 'Confirmed' if at least two or more independent laboratories had published reports on the pathogenicity of a specific mutation (Table 1). More ambiguous substitutions were categorized as 'Unclear,' 'Reported' or 'Point mutation/polymorphism' (Table 2). 'Reported' status indicates that one or more reports have considered the mutation as possibly pathologic. 'Point mutation/

Table 1 'Comfirmed' mitochondrial mutations associated with sensorineural hearing loss found in this study

								Case					
Allele	*Locus*	*Status*[a]	*Disease*	*Total (/394)*	*Cohort 1 (/254)*	*Cohort 2 (/140)*	*Control (/192)*	*Hearing characteristics*	*Progression of hearing loss*	*Tinnitus*	*Vertigo*	*Associated symptom*	*Reference*
C1494T	12S rRNA	Confirmed	SNHL	1	0	1	0	High frequency	1/1	1/1	0/1	0	5
A1555G	12S rRNA	Confirmed	SNHL	23	23	0	0	High frequency	15/21	13/16	6/16	0	2
A3243G	tRNA ^{Leu} (UUR)	Confirmed	SNHL/DM/FSGS/ Cardiac dysfunction	11	11	0	0	Flat	10/10	6/10	6/10	Diabetes mellitus (8/10)	6
T7511C	tRNA $^{Ser\ (UCN)}$	Confirmed	SNHL	3	3	0	0	High frequency	1/2	3/4	0/4	0	23
Total					37/254 (14.6%)	1/140 (0.7%)			27/34	23/31	12/31		

Abbreviations: DM, diabetes mellitus; FSGS, focal segmental glomerulosclerosis; SNHL, sensorineural hearing loss.
[a]Based on the MITOMAP database; 'Confirmed' status indicates that at least two or more independent laboratories have published reports on the pathogenicity of a specific mutation.

Table 2 Ambiguous-status mitochondrial substitutions associated with sensorineural hearing loss found in this study

								Case					
									Progression				
				Total	Cohort 1	Cohort 2	Control	Hearing	of hearing			Associated	
Allele	*Locus*	*Status*[a]	*Disease*	*(/394)*	*(/254)*	*(/140)*	*(/96)*	*characteristics*	*loss*	*Tinnitus*	*Vertigo*	*symptom*	*Reference*
C792T	12S rRNA	Reported	SNHL	1	1	0	0	Flat	1/1	1/1	1/1	0	24
A827G	12S rRNA	Conflicting reports	SNHL	10	5	5	1	High frequency	4/11	6/11	2/11	0	10
A856G	12S rRNA	Reported	SNHL/LHON/AD	3	3	0	0	Flat	1/1	1/1	1/1	0	25
T961C	12S rRNA	Unclear	SNHL/LVNC	3	3	0	2	Profound	1/1	1/1	1/1	0	26
T1005C	12S rRNA	Unclear	SNHL	2	1	1	1	Low frequency	2/2	1/1	1/1	0	26
T1095C	12S rRNA	Unclear	SNHL	1	1	0	0	Flat	1/1	1/1	1/1	0	11
C1310T	12S rRNA	Reported	SNHL	3	0	3	0	unknown	1/3	0/3	0/3	0	24
T3398C	ND1	Reported	SNHL/DM/HCM/ GDM/LVNC/ Cardiomyopathy	1	1	0	0	Profound	1/1	1/1	0/1	0	27
G3421A	ND2	Reported	SNHL	2	1	1	0	Profound	1/1	1/1	0/1	0	28
T5628C	$tRNA^{Ala}$	Reported	SNHL/CPEO	1	1	0	1	Profound	1/1	0/1	1/1	0	29
A8108G	CO2	Reported	SNHL	1	1	0	0	Low frequency	1/1	1/1	1/1	0	30
A8348G	$tRNA^{Lys}$	Reported	SNHL/Cardiomyopathy/HT	1	0	1	0	Low frequency	1/1	0/1	1/1	0	31
G11696A	ND4	Reported	SNHL/LHON/LDYT/ HT	4	0	4	2	Profound	1/4	1/4	0/4	0	32
A14693G	$tRNA^{Glu}$	Reported	SNHL/MELAS/LHON/ HT	1	0	1	1	Profound	0/1	0/1	0/1	0	33
G15927A	$tRNA^{Thr}$	Point mutation/ Polymorphism	SNHL/MS	4	1	3	4	High frequency	3/4	0/4	0/4	0	34
Total					19/254 (7.5%)	19/140 (13.6%)			20/34	15/33	10/33		

Abbreviations: AD, Alzheimer's disease; DM, diabetes mellitus; FSGS, focal segmental glomerulosclerosis; HT, hypertension; LDYT, Leber's hereditary optic neuropathy and dystonia; LHON, Leber hereditary optic neuropathy; LVNC, left ventricular non-compaction; MELAS, mitochondrial encephalomyopathy lactic acidosis, and stroke-like episodes; MIDD, maternally inherited diabetes and deafness; MS, multiple sclerosis; SNHL, sensorineural hearing loss.
'Point mutation/Polymorphism' status indicates that some published reports have determined the mutation to be a non-pathogenic polymorphism.
[a]Based on the MITOMAP database; 'Reported' status indicates that one or more reports have considered the mutation as possibly pathologic.

Table 3 Ten novel mitochondrial SNPs

Location	*Mutation*	*Conservation in 61 species (base) (/61)*	*Conservation rate (base) (%)*	*Amino-acid change*	*Conservation in 61 species (amino acid) (/61)*	*Conservation rate (amino acid) (%)*	*Amino-acid number/all amino acid of locus*	*Control (/192)*	*Mode of inheritance*	*Type of hearing loss*
16S rRNA	2069T>C	16	31.4	—	—	—	—	0	AD or Mit[a]	High frequency
16S rRNA	2285T>C	22	43.1	—	—	—	—	0	AD or Mit[a]	High frequency
16S rRNA	2285T>G	22	43.1	—	—	—	—	0	Sporadic	Dish shaped
16S rRNA	2634T>C	34	66.7	—	—	—	—	0	Sporadic	Profound
ND1	3595A>G	54	88.5	Asn>Asp	54	88.5	97/318	0	AD or Mit[a]	High frequency
COI	6204A>G	61	100	Ser>Gly	61	100	101/513	0	AD or Mit[a]	High frequency
ATPase6	9124A>G	60	98.4	Thr>Ala	59	96.7	200/226	0	Sporadic	Unilateral
ND4L	10680G>A	59	96.7	Ala >Thr	59	96.7	71/98	0	Sporadic	Unknown
ND5	13153A>G	44	72.1	Ile >Val	35	57.4	273/603	0	Sporadic	High frequency
Cytb	15003G>C	61	100	Gly >Ala	61	100	86/380	0	Sporadic	Profound

Abbreviation: SNPs, single-nucleotide polymorphisms
[a]AD or Mit; autosomal dominant inheritance or maternal inheritance.

polymorphism' status indicates that some reports have determined the mutation to be a non-pathogenic polymorphism. In all, 14.6% (37/254) of the patients in Cohort 1 (maternally inherited patients) were associated with the 'Confirmed' mutations. Only 0.7% (1/140) of the patients had the 'Confirmed' mutations in Cohort 2 (patients with various inherited modes) (Table 1). Ambiguous-status substitutions were associated in 7.5% (19/254) of Cohort 1, in contrast to 13.6% (19/140) of Cohort 2 (Table 2).

With regard to the audiogram configuration, various types were found. In all, 69% (79% in Cohort 1 and 59% in Cohort 2) of the patients had progressive hearing loss and 59% (74% in Cohort 1 and 45% in Cohort 2) had tinnitus, while 34% (39% in Cohort 1 and 30% in Cohort 2) of the patients were associated with vertigo (Tables 1 and 2). Concerning clinical symptoms other than hearing loss, 80% (8/10) of the patients with the 3243A>G mutation had diabetes mellitus, but no other clinical symptoms were noticed (Table 1).

Ten novel variants that were not included in the public mtDNA databases were found in this study and they were located in the *16S rRNA*, *ND1*, *COI*, *ATPase6*, *ND4L*, *ND5*, and *Cytb* regions (Table 3). All new variants were found in only one different family each.

Four of the novel variants were found in the *16S rRNA* gene: 2069T>C, 2285T>G, 2285T>C and 2634T>C. Although the 2634T>C variant had a high conservation rate (66.7%), the 2069T>C, 2285T>G and 2285T>C variants had low conservation rates: 31.4, 43.1 and 43.1%, respectively.

Table 4 Conservation rate of 'Confirmed' mitochondrial mutations

Location	*Mutation*	*Conservation in 61 species (base) (/61)*	*Conservation rate (base) (%)*
12S rRNA	1494A>G	61	100.0
12S rRNA	1555A>G	56	91.8
$tRNA^{Leu\ (UUR)}$	3243A>G	60	98.4
$tRNA^{Leu\ (UUR)}$	3291T>C	58	95.0
$tRNA^{Ser\ (UCN)}$	7445A>G	42	68.9
$tRNA^{Ser\ (UCN)}$	7511T>C	60	98.4
$tRNA^{Lys}$	8363G>A	49	80.3
$tRNA^{His}$	12147G>A	61	100.0
$tRNA^{Glu}$	14709T>C	58	95.0

The remaining six novel variants were located in the protein coding regions: 3595A>G in *NADH dehydrogenase 1* gene (MTND1 (MIM 516000), 6204A>G in *cytochrome oxydase I* gene (MTCOI (MIM 516030)), 9124A>G in *ATPase 6* gene (MTATP6 (MIM 516060)), 10680G>A in *NADH dehydrogenase 4L* gene (MTND4L (MIM 516004)), 13153A>G in *NADH dehydrogenase 5* gene (MTND5 (MIM 516005)) and 15003G>C in *cytochrome b* gene (MTCYB (MIM 516020)).

These variants are found in very well-conserved gene positions (57.4–100%).

The conservation rates in all 'Confirmed' mtDNA mutations were high (Table 4).

However, as in Table 3, the 9124A>G, 10680G>A, 13153A>G and 15003G>C variants were found in sporadic cases which are not generally compatible with mitochondrial deafness. On the basis of the above evaluations, we categorized 3595A>G, and 6204A>G as possibly pathogenic mutants, and the remaining eight others as uncertain pathogenic mutants.

The homoplasmic mutation 3595A>G in the *ND1* was found in a 4-year-old male patient with prelingual, severe hearing loss of high frequencies (Figure 1). He was suspected to have hearing impairment when he was about 1 year old, but ABR testing and Computed Tomography resulted in a diagnosis of normal hearing. However, when he was 3 years old, his mother again suspected that he had hearing impairment and testing confirmed it. The mother, who had the same mutation, also had hearing impairment as well as progressive bilateral tinnitus and occasional vertigo from childhood.

The homoplasmic mutation 6204A>G in the *COI* gene was found in a 62-year-old male with mild hearing loss of high frequencies (Figure 2). He noticed his hearing loss at the age of 50 and suffered

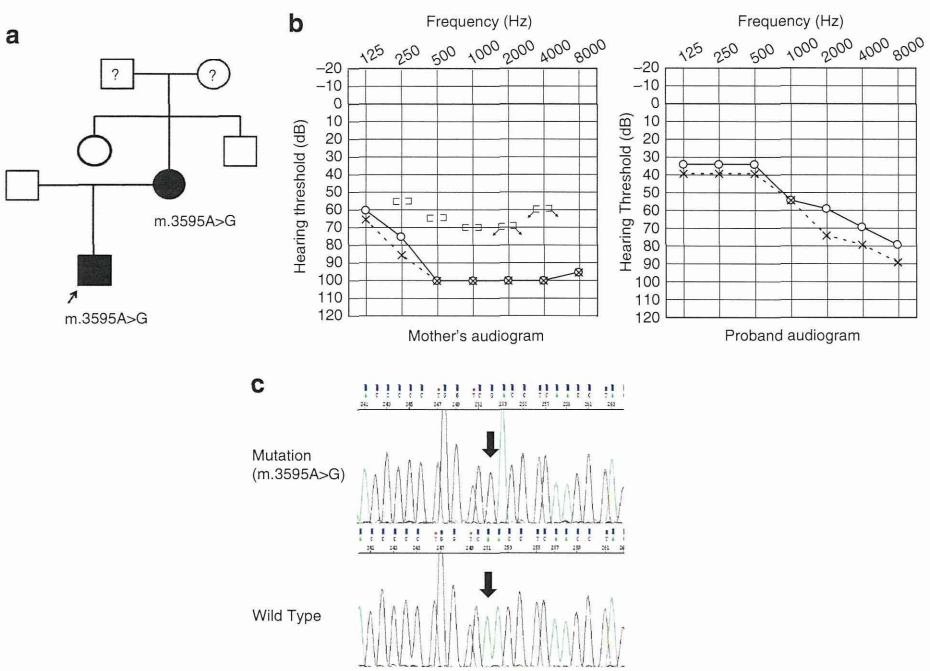

Figure 1 Clinical features of the proband carrying the homoplasmic 3595A>G variant. (**a**) Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (**b**) Audiograms of the proband and mother. (**c**) Electropherogram depicting the 3595A>G sequence and its flanks. Arrow indicates the position of the 3595A>G variant.

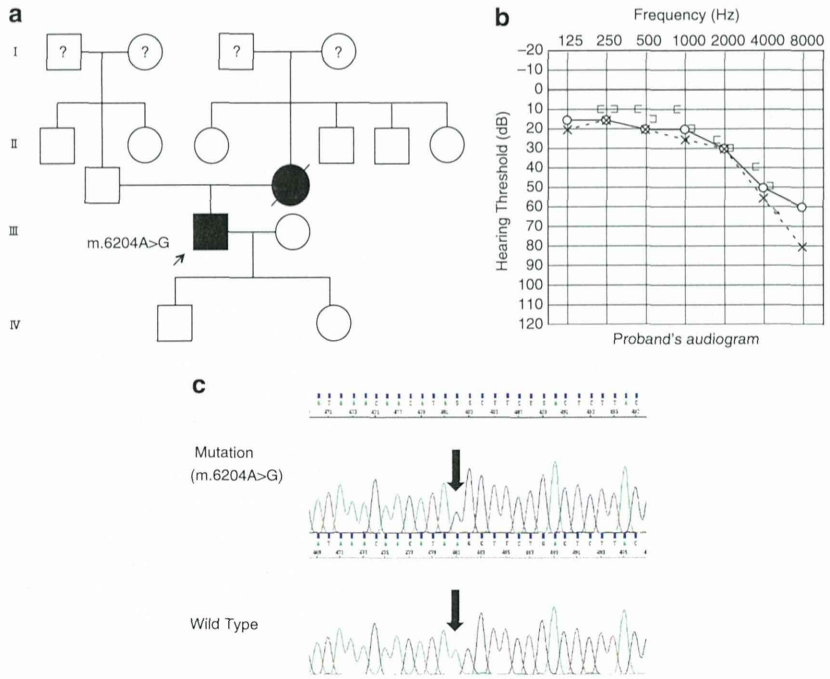

Figure 2 Clinical features of the proband carrying the homoplasmic 6204A>G variant. (**a**) Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (**b**) Audiogram of the proband. (**c**) Electropherogram depicting the 6204A>G sequence and its flanks. Arrow indicates the position of the 6204A>G variant.

from tinnitus, and mild diabetes mellitus. His mother also had hearing impairment that gradually progressed with age. DNA samples were not obtained from other family members.

DISCUSSION

Nineteen known mitochondrial mutations were found predominantly in the maternally inherited group (Tables 1 and 2). Clarification of pathogenecity of mitochondrial substitutions was hampered by low penetrance (probably due to heteroplasmy). Therefore, based on the MITOMAP database, they were classified as 'Confirmed' or 'Ambiguous-status' substitutions (Tables 1 and 2). The 'Confirmed' mitochondrial mutations were found predominantly in Cohort 1 rather than in Cohort 2 (14.6 vs 0.7%), supporting the pathogenicity of these mutations. Frequencies of 1555A>G and 3243A>G mutations were significantly high, indicating that these two mutations are important causes of maternally inherited hearing loss. In general, patients with these mitochondrial mutations showed more or less similar clinical characteristics, that is, progressive hearing loss with tinnitus (Table 1).

Among the 10 novel variants (Table 3), two, the *ND1* mutation 3595A>G and *COI* mutation 6204A>G, are thought to be possibly pathogenic, because (1) they are found in autosomal dominant or maternal inheritance (some of the others are found as sporadic cases); (2) the conservation rate of the variation at the position among mammals is at least over 50%, as is the conservation rate in all confirmed mtDNA mutations associated with phenotypes (Table 4) and (3) they are associated with high frequency hearing loss; the characteristic hearing type of mitochondrial hearing loss. These mutations affected a conserved nucleotide in the mitochondrial gene in primates and other species and had a conservation index of >50% (88.5 and 100%, respectively). None of these mutations were found in the controls or in the databases, further indicating that they are associated with hearing loss, however, no conclusion can be drawn without enzymatic analysis. Unfortunately, this study was a retrospective study using collected DNA samples from 1995 to 2012, so it was impossible to contact the patients and to get muscle or living samples from them. Therefore, enzymatic analysis of these mtDNA samples was not feasible.

In this study, we found one novel possibly pathogenic mutation in the ND1 hydrophobic arm region, in a patient with a homoplasmic 3595A>G mutation and hearing loss of the high frequencies from age 3 without complications. The family members of this patient did not have diabetes mellitus.

On the other hand, the novel possibly pathogenic mutation 6204A>G was located in the *COI* gene. The amino-acid conservation rate of this position was 100% (61/61 mammals). In previous reports, more than 20 pathogenic mutations in the *MT-ND1* gene were reported in patients with LHON (Leber's hereditary optic neuropathy) and MELAS. Also, *ND1* mutation-related hearing impairment has been reported: 3308T>C causing MELAS with deafness,[16] 3395A>G causing hypertrophic cardiomyopathy with profound SNHL,[17] and 3396T>C and 3421G>A causing maternally inherited diabetes and deafness.[18,19] Three COI mutations related to hearing loss have also been reported (7443A>G,[20] 7444G>A[21] and 7445A>G[7,8]). Our results taken with these previous reports support the possibility that mutations in the ND1 and COI regions are associated with hearing impairment.

Most of the mtDNA mutations associated with hearing loss indicate low penetrance explained as a mild biochemical defect indicating that the mutation itself is not sufficient to produce the clinical phenotype. Thus, other modifying factors including nuclear