を受診した新生児・乳幼児のうちANSDと診断され，当院で人工内耳埋込術を行った*OTOF*遺伝子変異を認める3例の人工内耳装用効果について検討した。検討項目は，術前の裸耳および補聴器装用下でのCOR閾値と人工内耳装用下でのCOR閾値または遊戯聴力検査閾値，術前後のIT-MAIS点数，術後の音声理解言語数，音声表出語彙数とした。

対象の概要を表1に示す。初診時月齢平均11.3カ月（0カ月～2歳4カ月，男2例，女1例），3例とも基礎疾患，難聴家族歴は認めず，DPOAE両側正常，ABRはクリック105dBnHLで両側無反応であった。聴性定常反応検査（ASSR），条件詮索反応聴力検査（COR）も行ったが無反応の場合は閾値として最大測定音圧に5dB加算した値をとり，3分法平均（500，1k，2kHz）閾値を求めた。またMRI（3-D CISS撮影）を行い蝸牛神経は正常であることを確認している。

ABR検査は日本光電MEB-2204（Neuropack）により測定した。鎮静下に検査を行い，刺激にはクリック音を用いて10dBステップで閾値を求めた。DPOAEはOAE analyzer ER-32（Grason-Stadler社製）またはILO292（Otodynamics社製）を用いて記録した。DPOAEの刺激音圧はL1＝65dB SPL，L2＝55dB SPLで，また測定条件はOAE analyerおよびILO292ともにデフォルトの設定通りとした。両耳ともOAE analyerでpassと判定されたもの，あるいはILO292で測定9周派数（1～6kHz）のうち8周波数以上がノイズレベルより5dB以上高いものをDPOAE正常とした。ASSR検査にはGrason-Stadler社製Auderaを使用した。ASSRの刺激音は250，500，1k，2k，4kHzのAM/FM複合音を用い鎮静下に行った。ASSRは推定聴力レベルではなく，実際の閾値（反応の得られた最小の刺激音圧）について検討したが，250Hzでは110dBHL，500Hzでは120dBHL，1～4kHzでは125dBHLで反応がなければ無反応とした。また10dBステップで閾値を求めたが，1～4kHzについては120dBHLで反応がない場合，125dBHLでも測定を行った。COR検査は250Hzでは95dBHL，500Hzでは100dBHL，1～4kHzでは110dBHLで反応がなければ無反応とした。

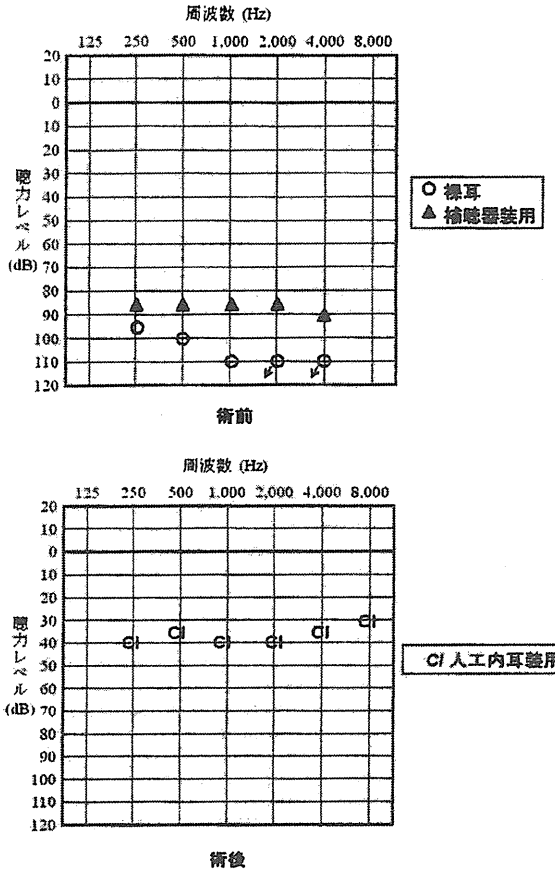
なお，*OTOF*遺伝子変異について検討したが，同定方法は現在他誌に投稿中である。

症例 1

周産期異常なく自然分娩にて出生，新生児聴覚スクリーニング（自動ABR）で異常を指摘され月齢0カ月で当科を受診した。DPOAE両側正常，ABRはクリック105dBnHLに対し両側無反応，ASSR閾値（1歳0カ月）は会話域平均が右103dBHL，左113dBHLであった。COR閾値（2歳0カ月）は108dB，補聴器装用下85dBであった。補聴器装用は11カ月から開始している。発達検査（1歳7カ月）では姿勢運動・言語社会面で軽度遅れを認め，自閉傾向を指摘されていた。手話を用いていたが模倣が多く自発的に使用することは少なかった。術前IT-MAISは0点であった。2歳5カ月で右人工内耳埋込術（メドエル社PULSAR，電極は全て挿入）を施行し，神経反応テレメトリーは良好に測定された。音入れ2カ月後（2歳8カ月）人工内耳装用下COR閾値は38dBであった。しかし，その後反応が悪い時期があり3歳0カ月時に人工内耳のコードの断線が発見されたため，人工内耳が使用されていなかった期間が数カ月あったと考えられた。3歳2カ月（術後7カ月）ではIT-MAIS2点，3歳7カ月（術後11カ月）では人工内耳装用下COR閾値は38dB

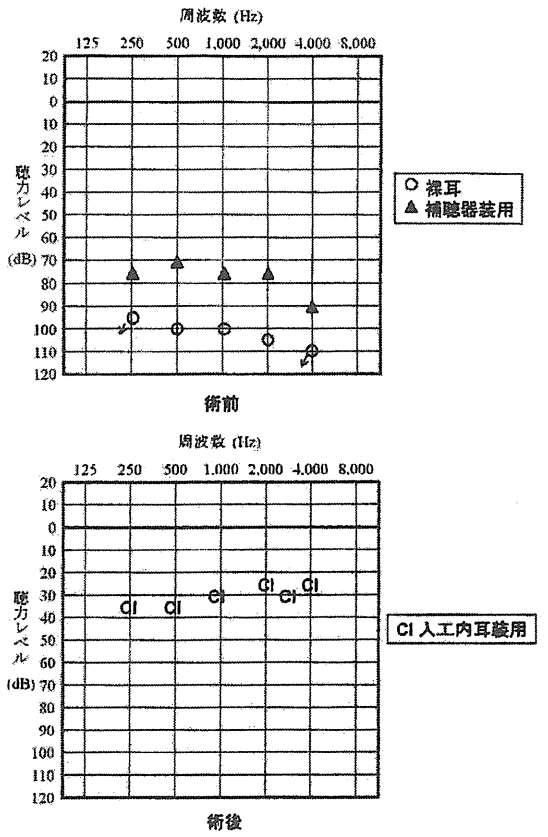
表1

症例	性別	初診時月齢	基礎疾患	ABR閾値（dBnHL）	DPOAE	ASSR右閾値（dBHL）	ASSR左閾値（dBHL）	COR閾値［HA装用］（dB）
1	M	0	なし	両側無反応	両側正常	103	113	108［85］
2	F	28	なし	両側無反応	両側正常	97	107	102［73］
3	M	6	なし	両側無反応	両側正常	100	107	102［63］



	術前	術後７カ月
IT-MAIS	０点	２点

図１



	術前	術後８カ月
IT-MAIS	０点	28点

	術後６カ月	術後８カ月
音声理解語彙	57語	102語
音声表出語彙	26語	61語

図２

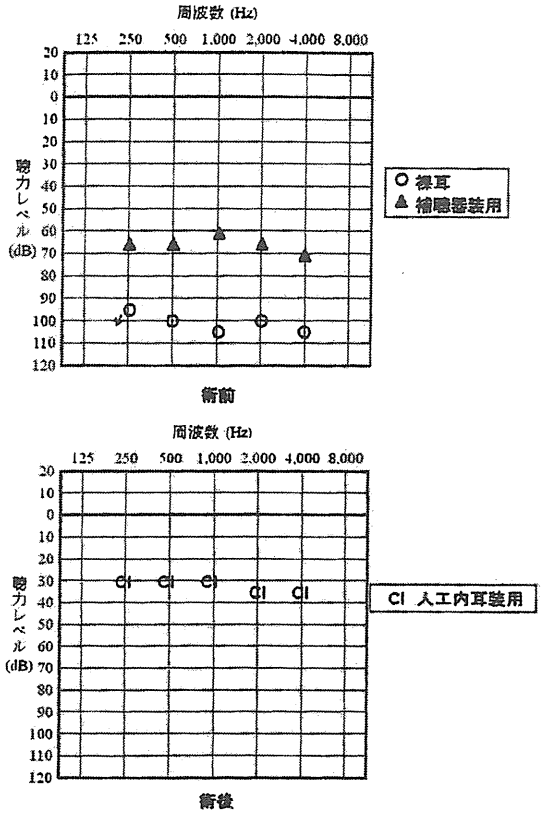
(図１)，音への反応は良く，リング音の検知，音の模倣は可能であり，楽器音の聞き分けも可能となり，音声の模倣も可能となってきている。

症　例　２

周産期異常なく自然分娩にて出生し，言語発達遅滞，難聴を主訴に前医を受診した。ABR はクリック 105dBnHL に対し両側無反応，DPOAE 両側正常であり，発達検査（２歳３カ月）では言語社会面で中等度の遅れを認めた。２歳４カ月で当科を受診し，ASSR 閾値（２歳６カ月）は会話域平均が右 97 dBHL，左 107dBHL，COR 閾 値（２歳８カ 月）は裸耳 102dB，補聴器装用下 73dB であった。補聴器装用は２歳５カ月から開始している。術前 IT-MAIS ０点であった。２歳11カ月で左人工内耳埋込術を施行し（メドエル社 PULSAR，電極は全て挿入），神経反応テレメトリーは良好に測定された。術後の音反応は良好であり，術後８カ月の人工内耳装用下の遊戯聴力検査閾値は左 30dBHL，IT-MAIS 28点，音声理解言語102語，音声表出語彙61語となった(図２)。

症　例　３

自然分娩にて出生（妊娠３カ月時切迫流産，39週５日）し，新生児聴覚スクリーニング（自動 ABR）で異常を指摘され，６カ月で当科を受診し，DPOAE 両側正常，ABR はクリック 105dBnHL に対し両側



	術前	術後４カ月
IT-MAIS	10点	26点

	術後４カ月
音声理解語彙	30語
音声表出語彙	11語

図３

無反応であった。ASSR閾値（２歳９カ月）は会話域平均が右100dBHL，左107dBHLであり，COR閾値（２歳10カ月）は裸耳102dB，補聴器装用下63dBであった。発達検査（３歳０カ月）で言語社会面は境界域であり，コミュニケーションは手話を用いており術前の手話表出語彙数は260語，IT-MAIS 10点であった。３歳４カ月で右人工内耳手術埋込術（メドエル社PULSAR，電極は全て挿入）を施行し，神経反応テレメトリーは良好に測定された。３歳９カ月ではIT-MAIS 26点，音声理解言語30語，音声表出11語となり，術後８カ月（４歳０カ月）での人工内耳装用下の遊戯聴力検査閾値は32dB，現在自発的な発語も増加してきている（図３）。

考　察

ANSDは画像上では蝸牛，蝸牛神経および聴覚中枢路にも異常を認めず，内有毛細胞と蝸牛神経の間のシナプスの伝達に問題があるという説と蝸牛神経がなんらかの原因で同期発火しないために神経伝導が生じないという説[2,7,8]がある。

これまでの報告ではANSDに対して人工内耳成績は不良であるとする報告[9)10)]もみられるが，人工内耳により良好な聴取能が得られたことが多く報告[11~14)]されている。Snallopら[11)]は３歳～６歳で人工内耳埋込術を行ったDPOAE正常反応でABR無反応の高度感音難聴症例５例中３例は，電話でのコミュニケーションが可能になったと報告している。Maddenら[12)]，Bussら[13)]も小児ANSDに人工内耳埋込術が聴覚の獲得に効果的であると報告している。Ranceら[14)]は通常の症例と同等の結果は必ずしも期待できないが，人工内耳により良好な聴力を提供できるとしている。

今回の我々の検討でも人工内耳装用下での聴力閾値は良好であるとの結果が示されたが，症例１は症例２，３に比べ言語発達については劣っている。症例１は術前から軽度発達遅滞，自閉傾向を認めており，自発的な手話は少なく模倣がほとんどであった。人工内耳埋込術に関しても，術前に家族に十分な効果が得られない可能性を説明した上で手術を施行している。術後は場面設定をすれば音の検知は可能となっているが自発的な環境音への反応が悪いためIT-MAISは低く，音声の模倣は可能であるものの理解，表出するまでには至っていない。一方，症例２，３では言語面以外での発達遅滞はなく，術前から自発的な手話を用いており，術後も言語理解語彙，表出語彙も経過とともに増加している。一般的な補聴器，人工内耳装用と同じく患児に発達障害を認める場合には十分な人工内耳の効果が得られない可能性があると考えられた。３症例とも人工内耳埋込術後の経過がまだ１年未満と短いが，言語発達には個々の差はあるものの人工内耳装用下での聴力閾値は良好となっている。言語発達については今後も長期の訓練，評価が必要であると考える。

また，*OTOF*遺伝子変異によるANSD患者において人工内耳が有用であったという報告もなされて

いる[15)16)]。*OTOF* 遺伝子は内有毛細胞に発現されているOtoferlin蛋白をエンコードしている遺伝子であり，シナプス小胞のシナプス前膜への結合に関与している。内有毛細胞と蝸牛神経間のシナプスにおける神経伝達物質の放出に異常が生じることで聴力障害を来たすと考えられる。*OTOF* 遺伝子変異によるANSDでは蝸牛神経が保たれていることから理論的には蝸牛神経を直接刺激する人工内耳は有用であると考えられる。今回の3症例でもDPOAEが正常であり神経反応テレメトリーが良好に測定されていることから外有毛細胞とラセン神経節細胞の機能は正常であり，内有毛細胞もしくは内有毛細胞と蝸牛神経の間のシナプスに障害あると推測された。ANSDにおいて *OTOF* 遺伝子の診断がされた場合，障害部位が推定でき人工内耳装用効果が期待されるため，ANSDに対する人工内耳埋込術の選択をするうえで本遺伝子診断が役立つ可能性があると考えられる。

おわりに

ANSDは1996年に報告されて以来，多くの報告がなされるようになり，小児ANSDに対する人工内耳の有用性を示すものも報告されるようになった。しかし，ANSDには様々な病態・原因があるとされており，難聴の程度や補聴器・人工内耳の効果もまだ一定していない。今回，我々はANSDの原因遺伝子の一つとされる *OTOF* 遺伝子変異をもつ小児3症例に対して人工内耳が有効であったものを経験した。今後，ANSDに対する人工内耳の適応や効果については，ANSDの病態・原因ごとに十分検討される必要があると考えられる。

Results of cochlear implantation in auditory neuropathy spectrum disorder in three children with mutation in the *OTOF* gene.

Takuya ohara[1)], Hidenobu Taiji[1)], Noriko Morimoto[1)], Tomoko Honmura[1)], Tatsuo Matsunaga[2)]

[1)]Department of Otorhinolaryngology, National Center for Child Health and Development
[2)]Department of Otorhinolaryngology, National Hospital Organization Tokyo Medical Center

Auditory neuropathy spectrum disorder (ANSD) is a hearing disorder caused by desynchronized neural discharges of the auditory nerve. It is a retrocochlear hearing disorder identified by abnormal or complete absence of auditory brainstem responses (ABR) and presence of evoked acoustic otoemissions (OAE). Some reports have shown that cochlear implantation may be successful for rehabilitation of children with ANSD. One report has shown that mutations of the *OTOF* gene, which encodes otoferlin, cause non-syndromic ANSD. Herein, we report on three children with ANSD who underwent cochlear implantation at the National Center for Child Health and Development (Japan). We report the patient characteristics, preoperative audiological profiles, and postoperative performances of the children with cochlear implants. They were diagnosed as having ANSD based on the detection of normal DPOAE, but absent ABR. Mutation of the *OTOF* gene was also detected. They had no or very poor open-set speech recognition, both with and without appropriate hearing aids, and benefited from cochlear implantation. Cochlear implantation is expected to be a useful modality for language development in ANSD children with mutation of the *OTOF* gene.

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東京都世田谷区大蔵2-10-1
成育医療研究センター耳鼻咽喉科
大原卓哉
Takuya Ohara
Department of Otorhinolaryngology
National Cener for Children Health and Development
2-10-1 Okura Setagaya-ku Tokyo, 157-8535 Japan

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RESEARCH ARTICLE Open Access

Systematic analysis of mitochondrial genes associated with hearing loss in the Japanese population: dHPLC reveals a new candidate mutation

Hideki Mutai[1], Hiroko Kouike[1], Eiko Teruya[1], Ikuko Takahashi-Kodomari[1], Hiroki Kakishima[2], Hidenobu Taiji[3], Shin-ichi Usami[4], Torayuki Okuyama[2] and Tatsuo Matsunaga[1*]

Abstract

Background: Variants of mitochondrial DNA (mtDNA) have been evaluated for their association with hearing loss. Although ethnic background affects the spectrum of mtDNA variants, systematic mutational analysis of mtDNA in Japanese patients with hearing loss has not been reported.

Methods: Using denaturing high-performance liquid chromatography combined with direct sequencing and cloning-sequencing, Japanese patients with prelingual (N = 54) or postlingual (N = 80) sensorineural hearing loss not having pathogenic mutations of m.1555A > G and m.3243A > G nor *GJB2* were subjected to mutational analysis of mtDNA genes (*12S rRNA*, $tRNA^{Leu(UUR)}$, $tRNA^{Ser(UCN)}$, $tRNA^{Lys}$, $tRNA^{His}$, $tRNA^{Ser(AGY)}$, and $tRNA^{Glu}$).

Results: We discovered 15 variants in *12S rRNA* and one homoplasmic m.7501A > G variant in $tRNA^{Ser(UCN)}$; no variants were detected in the other genes. Two criteria, namely the low frequency in the controls and the high conservation among animals, selected the m.904C > T and the m.1105T > C variants in *12S rRNA* as candidate pathogenic mutations. Alterations in the secondary structures of the two variant transcripts as well as that of m.7501A > G in $tRNA^{Ser(UCN)}$ were predicted.

Conclusions: The m.904C > T variant was found to be a new candidate mutation associated with hearing loss. The m.1105T > C variant is unlikely to be pathogenic. The pathogenicity of the homoplasmic m.7501T > A variant awaits further study.

Background

Hearing loss manifests in more than 1 in 1000 persons at birth, and the frequency increases subsequently to 3 in 1000 by 4 years of age [1,2]. Approximately 50 to 70% of congenital and childhood deafness is estimated to be due to genetic mutations. In adults, the prevalence of hereditary hearing impairment has been estimated to be approximately 3.2 in 1000 [3]. Some of the mitochondrial DNA (mtDNA) genes, such as *12S rRNA*, $tRNA^{Leu(UUR)}$, and $tRNA^{Ser(UCN)}$, are known to be responsible for hereditary hearing loss [4]. Among them, the m.1555A > G mutation in *12S rRNA* is found relatively frequently (0.6-16%, depending on the ethnic group) in aminoglycoside-induced, congenital, and late-onset nonsyndromic hearing loss [4,5]. The m.1494C > T mutation in *12S rRNA* is also associated with aminoglycoside-induced and nonsyndromic hearing loss [6,7]. The m.3243A > G mutation in $tRNA^{Leu(UUR)}$ is associated with late-onset nonsyndromic hearing loss [8,9], maternally inherited diabetes and deafness (MIDD) [10,11], and mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes (MELAS), which frequently presents with hearing loss [12,13]. The m.7445A > C/G/T [14-16], 7472insC [17], and 7510T > C mutations [18] in $tRNA^{Ser(UCN)}$ are also associated with

* Correspondence: matsunagatatsuo@kankakuki.go.jp
[1]Laboratory of Auditory Disorders, Division of Hearing and Balance Research, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan
Full list of author information is available at the end of the article

aminoglycoside-induced, nonsyndromic, or syndromic hearing loss.

In addition, many other variants in *12S rRNA* have been proposed to be associated with hearing loss [4]. Some variants such as m.827A > G [19,20], 961T > C [21], 961delT + Cn [21,22], 1005T > C [22], and 1095T > C in *12S rRNA* [22-26] are not definitively related to hearing loss, because they have been found in subjects with normal hearing and/or are not conserved among mammals [19,27-30]. Moreover, a variety of mitochondrial haplogroups often localize in specific ethnic groups, making it difficult to determine whether the mtDNA variants are associated directly with diseases, indirectly as risk factors, or simply with rare subhaplogroups [31-34]. Accumulating reports of various novel mtDNA mutations associated with hearing loss prompted us to evaluate these variants in patients with hearing loss in Japan, where mtDNA mutation studies have focused on a few limited nucleotide positions [35,36].

A single cell contains hundreds of mitochondria, and the mtDNA in each mitochondrion is occasionally heterogeneous, a feature called heteroplasmy [37]. The proportion of pathogenic mutations of heteroplasmic mtDNA is considered to be one of the reasons for the wide range of severity of phenotypes seen in patients with mitochondrial-related diseases, such as those reported in the case of the m.3243A > G mutation [38-40]. Denaturing high-performance liquid chromatography (dHPLC) is a sensitive method to detect heteroplasmic mutations that can be overlooked by simple direct sequencing and comparison of the scanned peaks or restriction fragment length polymorphism-PCR [28,41]. In this study, we conducted a systematic mutational analysis of mtDNA by dHPLC combined with direct sequencing and cloning-sequencing in samples from Japanese patients with hearing loss.

Methods

Subjects

Subjects with bilateral sensorineural hearing loss were recruited by the National Tokyo Medical Center and collaborating hospitals. Subjects' medical histories were obtained and physical examinations were performed to exclude those subjects with syndromic symptoms, diseases of the outer or/and middle ear, and environmental factors related to hearing loss such as history of infectious diseases, premature birth, and newborn meningitis. Patients with a history of use of ototoxic drugs were included in the study because these drugs are known to be associated with mitochondrial hearing loss. Prior to this study, the patients were confirmed not to have the m.1555A > G and m.3243A > G mutations or not to be diagnosed as having *GJB2* -caused hearing loss, as assessed by restriction fragment length polymorphism-PCR or together with direct sequencing if the heterozygotic 235delC mutation was detected in *GJB2* [42,43]. The 134 subjects were classified into prelingual hearing loss (onset before 5 years old, 20 males and 34 females) or postlingual hearing loss (onset at 5 years old or later, 31 males and 49 females) [1]. The control group consisted of 137 unrelated Japanese individuals with normal hearing as examined by pure-tone audiometry. All subjects or their parents gave prior informed consent for participation in this study. This study was approved by the ethics committee of National Tokyo Medical Center.

Screening for mtDNA mutations by dHPLC

DNA was extracted from blood samples using the Gentra Puregene DNA isolation kit (QIAGEN, Hamburg, Germany). Initially, whole mtDNA from each patient was amplified in three overlapping fragments (1351-8197, 6058-12770, and 11706-2258) [44] by LATaq DNA polymerase (TaKaRa BIO, Shiga, Japan). PCR was conducted at 94°C for 1 min followed by 30 cycles of 98°C for 10 s and 68°C for 6.5 min. Then, using the PCR products as templates, variants were analyzed by the Mitoscreen assay kit (Transgenomic, Glasgow, UK). We amplified the genes *12S rRNA*, $tRNA^{Leu(UUR)}$, $tRNA^{Ser(UCN)}$, $tRNA^{Lys}$, $tRNA^{His}$, $tRNA^{Ser(AGY)}$, and $tRNA^{Glu}$, for which mutations were reported to be associated with hearing loss on the Hereditary Hearing Loss Homepage [45] when the study was started. The PCR products using primer sets MT4 (for *12S rRNA*), MT6 ($tRNA^{Leu(UUR)}$), MT10 ($tRNA^{Ser(UCN)}$), MT11 ($tRNA^{Lys}$), MT15 ($tRNA^{His}$ and $tRNA^{Ser(AGY)}$), and MT18 ($tRNA^{Glu}$) were incubated with the appropriate restriction enzymes, incubated for heteroduplex formation either with reference PCR products to detect homoplasmy or with their own PCR products to detect heteroplasmy, then analyzed by dHPLC (WAVE system, Transgenomic) according to the manufacturer's protocols.

The reference mtDNA was derived from a Japanese individual with normal hearing. Sequencing of the entire reference mtDNA revealed 750A > G and 1438A > G polymorphisms, and the mtDNA sequence was otherwise comparable to the revised Cambridge Reference sequence (AC_000021) [46,47]).

DNA sequencing

When homoplasmic or heteroplasmic variants were detected, the PCR product was subjected to direct sequencing by the BigDye Terminator ver. 3 cycle sequencing kit and ABI genetic analyzer 3730 (Life Technologies, Carlsbad, CA). To sequence *12S rRNA*, an additional nested PCR product (656-1,266) was amplified with primers F (5'-tggtcctagcctttctattagctctt-3') and R (5'-tggcggtatataggctgagca-3'). To sequence $tRNA^{Ser}$

$^{(UCN)}$, an additional nested PCR product (7,209-7,609) was amplified with primers F (5'-atgccccgacgttactcg-3') and R (5'- acctacttgcgctgcatgtg-3'). To determine the proportion of heteroplasmic 1005T > C variant in the *12S rRNA*, the nested PCR (656-1,266) product was cloned and sequenced. Nested PCR was carried out by replacing AmpliTaq Gold DNA polymerase with PrimeSTAR DNA polymerase, which has 3'-proofreading activity (TaKaRa BIO), followed by the Zero Blunt TOPO PCR cloning kit (Life Technologies). We sequenced 54 clones derived from the proband mtDNA and 24 clones derived from the mtDNA of each of five siblings. Sequencing data were analyzed by SeqScape ver2.6 (Life Technologies) and DNASIS Pro (Hitachisoft, Tokyo, Japan). The sequencing results for each patient were compared with the revised Cambridge Reference sequence to identify mtDNA variants. The uniqueness of each mutation was evaluated by comparison with the mtSNP database [48], MITOMAP [49], and the Uppsala mtDB database [50].

Prediction of pathogenicity of mtDNA variants

The variants were evaluated based on double selection as proposed by Leveque and coworkers [51], with modification. Initially, we measured the frequencies of each variant found in the controls in our study (N = 137) and in the mtSNP database (N = 672, including: centenarians in Gifu, centenarians in Tokyo, type 2 diabetes mellitus patients (without or with vascular disorders), overweight young adult males, non-overweight young adult males, Parkinson's disease patients, and Alzheimer's disease patients in Japan). The variants with a frequency of more than 3% in one of the groups were considered as non-pathologic polymorphisms. We used a frequency threshold lower than that previously used (4%) [51] because the mtSNP database of Japanese individuals and the controls reflect the patient ethnic group background more closely than the mtDB and therefore requires a lower frequency threshold to exclude polymorphisms. The nucleotide conservation in each gene from human and 50 mammalian species was evaluated by ClustalW. The additional file lists the mammalian species and the accession numbers of the mtDNA (Additional File 1: Table S1). The variant frequencies in the mtDB were calculated to determine if the low variant frequencies measured in the controls reflect rare haplotypes in the Japanese population and are more common worldwide. All the variants were also analyzed with PhyloTree (mtDNA tree Build 10) [52] to search for previously characterized variants in haplogroups. Pathogenicity of the variants was also evaluated by predicting the secondary structures of the mitochondrial transcripts with or without the variant using Centroid Fold [53,54].

Results

dHPLC screening and subsequent direct sequencing in the patients identified 12 homoplasmic or heteroplasmic variants in *12S rRNA* and 1 homoplasmic variant in $tRNA^{Ser(UCN)}$ (Table 1). In addition, the 3 homoplasmic variants, m.752C > T, 1009C > T, and 1107T > C in *12S rRNA* were detected in the controls by direct sequencing. All the patients and the controls appeared to have the non-pathogenic m.750A > G and 1438A > G variants, as previously noted [49]. No $tRNA^{Glu}$, $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$, $tRNA^{His}$, or $tRNA^{Ser(AGY)}$ variants were detected. Table 1 lists the number of patients found with each variant, the frequencies of the variants in the controls and among Japanese individuals with various clinical conditions (mtSNP, N = 672), previous reports of the variants, and the frequencies of the variants in the mtDB. We evaluated two criteria, namely that the frequency of the variants be < 3% in both the controls and in the Japanese database (mtSNP) and that the variant nucleotide conserved by >50% among the 51 mammalian species we considered [51]; based on this analysis, two *12S rRNA* variants, m.904C > T and 1005T > C, were selected as candidate pathogenic mutations and subjected to further study. Although the homoplasmic m.7501T > A variant in $tRNA^{Ser(UCN)}$ did not meet the conservation criteria, it was also subjected to further study because several other $tRNA^{Ser(UCN)}$ mutations have been reported to be associated with hearing loss, whereas the m.7501T > A variant has not been studied for its pathogenicity.

A novel homoplasmic m.904C > T variant in the*12S rRNA* was found in a 46-year-old female patient (Figure 1A). She did not possess additional mtDNA pathogenic mutations and showed prelingual, progressive hearing loss with tinnitus. The patient was suspected of hearing impairment as early as 4 years old and was diagnosed with sensorineural hearing loss at age 11. The audiometric examination showed mild hearing loss at low frequencies and no response at 1 kHz and higher frequencies (Figure 1B). She had no response to an otoacoustic emission test, indicating dysfunction of the auditory outer hair cells. The patient had no history of treatment with ototoxic drugs and did not suffer from any other symptoms. The siblings also suffered from prelingual, severe hearing loss (with similar ages of onset and severity), but their parents had normal hearing (Figure 1A). The patient bore two children with normal hearing. DNA samples were not obtained from other family members. The secondary structure of the variant 12S rRNA predicted by Centroid Fold suggested that substitution of C > T (transcribed as U) at position 904 of the *12S rRNA* results in gross structural alteration of the transcript region that includes nucleotide positions 862 to 917, in addition to truncation of the

Table 1 Mitochondrial DNA variants identified in this study

Gene	Mutation	Homo/ heteroplasmy	prelingual HL (N = 54)	Late-onset HL (N = 80)	Controls (N = 137)	freq in controls (%)	Japanese (N = 672)[a]	freq in Japanese (%)	conservation index[b]	Previous report[c]	mtDB[c] (N = 2704)	freq in mtDB (%)
12S rRNA	663A > G	homoplasmy	3	5	2	**1.5**	48	7.1	**29/51**	yes	86	3.2
	709G > A	homoplasmy	7	7	12	8.8	125	18.6	19/51	yes	444	16.4
	750A > G	homoplasmy	54	80	137	100.0	no data	no data	**49/51**	yes	2682	96.7
	752C > T	homoplasmy	0	0	9	6.6	17	2.5	44/51	yes	20	0.7
	827A > G	homoplasmy	4	3	3	**2.2**	25	3.7	**48/51**	yes	54	**2.0**
	904C > T	homoplasmy	1	0	0	**0.0**	0	**0.0**	**48/51**	none	0	**0.0**
	961insC	homoplasmy	1	0	3	**2.2**	1	**0.1**	9/51	yes	37	**2.0**
	961delT+ Cn	both	0	1	4(2)[d]	**2.9**	no data	no data	9/51	yes	no data	no data
	1005T > C	both	1	1(1)	1	**0.7**	1	**0.1**	**33/51**	yes	7	**0.3**
	1009C > T	homoplasmy	0	0	1	0.7	1	0.1	9/51	yes	2	0.1
	1041A > G	homoplasmy	0	4	5	3.6	11	**1.6**	**26/51**	yes	14	**0.5**
	1107T > C	homoplasmy	0	0	6	4.4	29	4.3	30/51	yes	34	1.26
	1119T > C	homoplasmy	1	2	7	5.1	20	3.0	20/51	yes	26	1.0
	1382A > C	homoplasmy	0	1	11	8.0	62	9.2	**38/51**	yes	65	**2.4**
	1438A > G	homoplasmy	54	80	137	100.0	662	98.5	**46/51**	yes	2620	96.9
$tRNA^{Ser}$ (UCN)	7501T > A	homoplasmy	0	3	0	**0.0**	1	**0.1**	15/51	yes	1	**0.0**

Mitochondrial gene variants that met the criterion for association with hearing loss (HL) are underlined and in bold type. [a]Data from the mtSNP database [48]. [b]Based on the results of the multiple alignment by ClustalW. See Additional File 1: Table S1 for information on the species used to calculate the sequence conservation. [c]Uppsala mtDB database [50]. [d]Each number in parentheses indicates the number of individuals with a heteroplasmic variant.

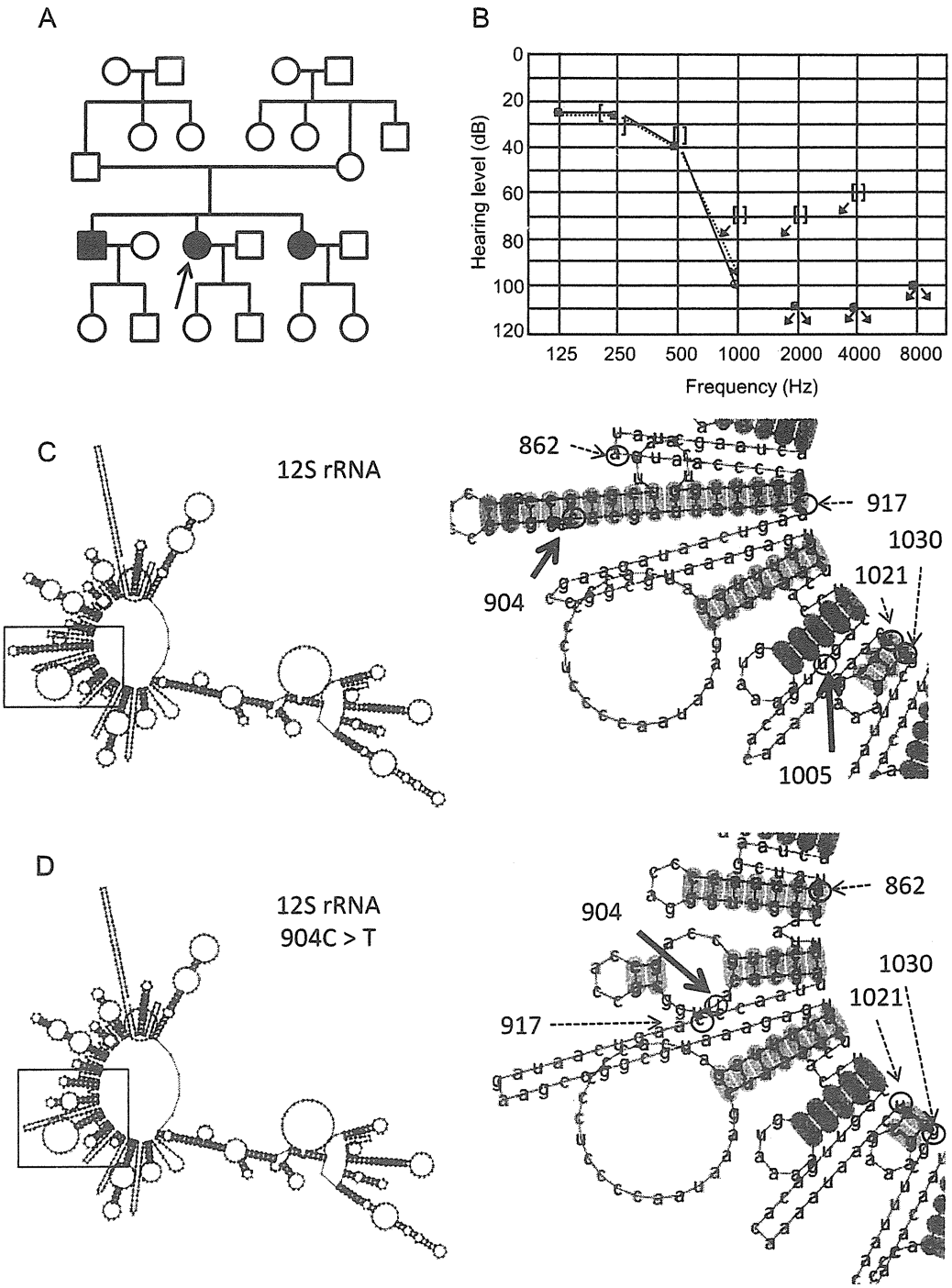

Figure 1 Pedigree of a family carrying the m.904C > T variant. (A) Pedigree of a family carrying the homoplasmic m.904C > T variant. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (B) Audiogram of the proband of m.904C > T. Open circles with the line indicate the air conduction thresholds of the right ear; the X's with dotted line indicate the air conduction thresholds of the left ear; [, bone conduction thresholds of the right ear;], bone conduction thresholds of the left ear. Arrows indicate the scale-out level of hearing loss. (C, D) Secondary structures of wild-type 12S rRNA (C) and 12S rRNA with the m.904C > T (D) predicted by Centroid Fold. To the right is shown an enlargement of the region of predicted secondary structures surrounding nucleotide positions including 904 and 1005 (bold arrows with red circles). Positions 862, 917, 1021, and 1030 are marked by dashed arrows with black circles for easy comparison of the structural changes. Each predicted base pair is indicated by a gradation of color (red to blue) corresponding to the base-pairing probability from 1 (red) to 0 (blue) according to Centroid Fold.

stem-like structure from positions 1021 to 1030 (Figure 1C and 1D), implicating a significant role for 904C in 12S rRNA folding.

The homoplasmic m.1005T > C variant in the *12S rRNA* was found in a male patient with prelingual, severe hearing loss (Figure 2A, B). The patient's spouse had prelingual hearing loss owing to measles, and their child also had prelingual hearing loss. The m.1005T > C variant was not detected in the patient's spouse or daughter. DNA samples were not obtained from other family members.

The heteroplasmic m.1005T > C variant together with the homoplasmic mutation m.709G > A was detected in a male patient from a consanguineous marriage of parents with normal hearing (Figure 2C). In the proband (III:3), onset of hearing loss and diabetes mellitus occurred in his 40s. Among his five siblings, four (III:1, 2, 4, 6) also showed adult-onset hearing loss between age 20 and 50 years, but they did not have diabetes mellitus. The fifth sibling suffered from infantile paralysis and died at age 6 (III:5). Cloning of the fragment of *12S rRNA*, which demonstrated apparent heteroduplex formation (Figure 2D, arrow), yielded 12 of 54 clones (22%) with the m.1005T > C variant. However, the m.1005T > C variant was not detected in 24 clones derived from the mtDNA from each of these siblings, indicating that the variant was absent in the siblings or the frequency was less than 4%. The audiograms showed severe to profound hearing loss in the siblings III:1, 2, 3, and 4 (Figure 2E, F, 3A, B). The secondary structure of the *12S rRNA* variant predicted by Centroid Fold indicated that the m.1005T > C induces a gross structural alteration in the transcript, including nucleotide positions 862 to 917 (Figure 1C and 3C).

Three patients appeared to carry the homoplasmic m.7501T > A variant in $tRNA^{Ser(UCN)}$ (Figure 4A, C, E). One female patient suffered from episodic vertigo from age 27 years followed by tinnitus and fluctuant, moderate progressive hearing loss, and she had no familial history of hearing loss (Figure 4A, B). Another female patient suffered from tinnitus beginning at age 24 years and had been exposed to streptomycin from age 36 to 37 for treatment of tuberculosis (Figure 4C, D). She suffered from fluctuant, moderate hearing loss from her 50s and had no familial history of hearing loss. The third patient was a male from a consanguineous marriage of parents with normal hearing and showed nonprogressive, severe hearing loss from childhood without tinnitus or vertigo (Figure 4E, F). Later, he was also found to have X-linked spinal and bulbar muscular atrophy (SBMA/Kennedy-Alter-Sung disease/Kennedy's disease). In this family, six of seven siblings showed hearing loss. Family members other than the proband did not participate in this study. According to the secondary structure prediction by Centroid Fold, the m.7501T > A in $tRNA^{Ser(UCN)}$ (which is transcribed as U in the reverse direction) causes an elongation of the D-arm in the transcript by reducing the size of the D-loop of $tRNA^{Ser(UCN)}$ (Figure 4G, H), which might affect biosynthesis of mitochondrial proteins [55].

Discussion

In our study, screening of mtDNA by dHPLC and direct sequencing detected 15 variants in *12S rRNA* and 1 variant in $tRNA^{Ser(UCN)}$. Comparison of the variant frequencies in controls, assessment of nucleotide conservation among mammalian species, and structural analysis of the transcript was used to select candidate mutations associated with hearing loss. No variants in $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$, $tRNA^{His}$, $tRNA^{Ser(AGY)}$, or $tRNA^{Glu}$ were detected in the subjects studied here, suggesting that the mutations in these genes associated with hearing loss are not common in the Japanese population.

To our knowledge, the homoplasmic m.904C > T variant in *12S rRNA* has not been reported elsewhere. Lack of symptoms in the maternal relatives does not exclude mitochondrial transmission, because penetrance of *12S rRNA* mutations can be extremely low, as seen in the m.1555A > G associated with hearing loss [56]. Conservation of the nucleotides among mammals and gross alteration of the predicted secondary structure of the *12S rRNA* transcript suggest that the m.904C > T variant might affect auditory function by changing the efficiency with which mRNAs are transcribed to yield mitochondrial proteins.

A patient with the homoplasmic m.1005T > C variant in the *12S rRNA* had a child with prelingual hearing loss. The inheritance of hearing loss in the child is likely due to the transmission of an autosomal mutation, not mtDNA, from the male proband. Therefore, the data for this family may not provide unequivocal information about the pathogenicity of the m.1005T > C variant [4,22,27,30].

Identification of the heteroplasmic m.1005T > C variant in a patient with hearing loss is a novel finding, because this variant has been known only as homoplasmic [22,27,30,34]. We did not verify that the heteroplasmic m.1005T > C variant was correlated with hearing loss because four of five siblings of the proband had hearing loss without carrying the variant, whereas it might be associated with diabetes mellitus. However, it is difficult to exclude the possibility of association of the heteroplasmic variant detected in blood samples with mitochondrial diseases such as deafness. Frequencies of heteroplasmy of mtDNA vary considerably among tissues in the same individual (for instance, [37,57,58]). Therefore, it is possible that the frequency of the m.1005T > C variant in the inner ear cells of the

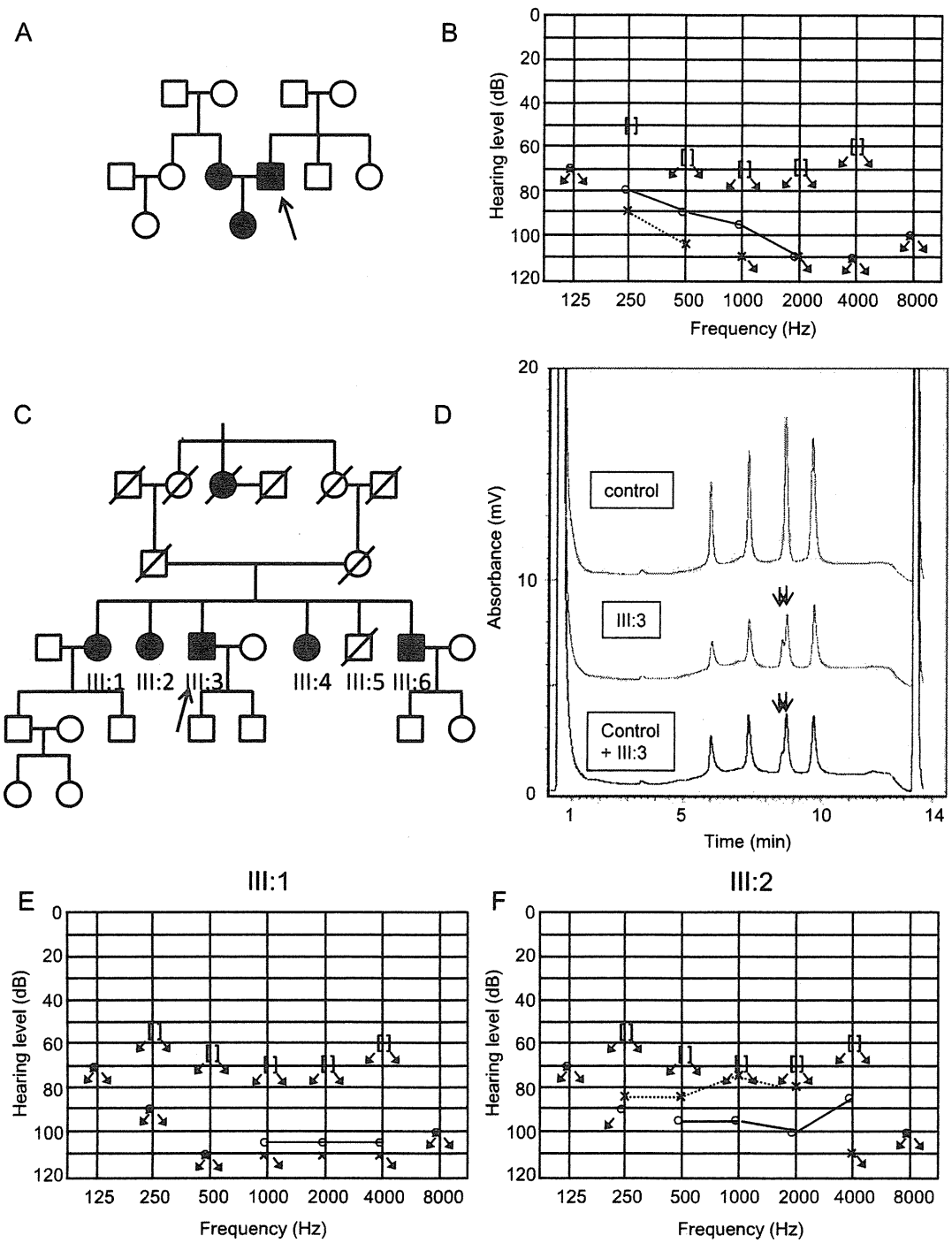

Figure 2 Pedigrees of families carrying the m.1005T > C variant. (A,B) Pedigree of a family carrying the homoplasmic m.1005T > C (A), and the audiogram of the proband (B). (C-F) Pedigree of a family carrying heteroplasmic m.1005T > C (C), and the chromatogram of dHPLC of the MT4 fragment of the proband (D). The arrows indicate split peaks of the fragment owing to the heteroplasmic m.1005T > C. Audiograms of the siblings (III:1, 2) are shown in (E-F).

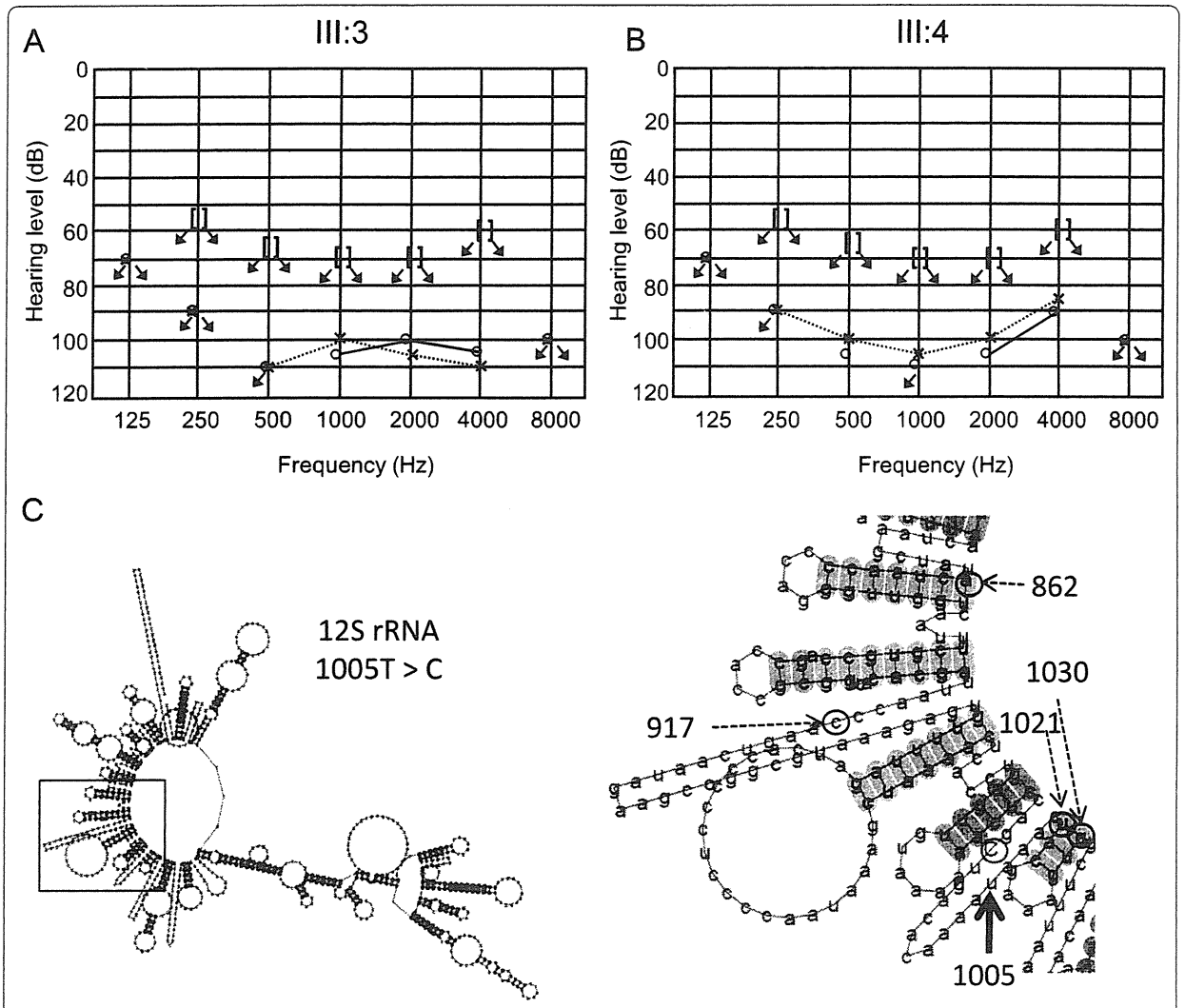

Figure 3 Pedigrees of families carrying the m.1005T > C variant (continued). (A,B) Audiograms of the siblings (III: 3, 4) of a family carrying the heteroplasmic m.1005T > C (Figure 2C). (C) Predicted secondary structure of the *12S rRNA* transcript with the m.1005T > C. To the right is shown an enlargement of the region of predicted secondary structures surrounding nucleotide position 1005.

siblings is much higher than in the blood cells and thus may underlie the hearing loss.

Another finding in this study is that three patients with postlingual hearing loss had the homoplasmic m.7501T > A variant in $tRNA^{Ser\ (UCN)}$. Various mutations in $tRNA^{Ser(UCN)}$, such as m.7445A > G [15,16], 7472insC [17,59], 7505T > C [60], 7510T > C [18], and 7511T > C [51,59,61], are associated with various types of hearing loss (syndromic or nonsyndromic, prelingual or late-onset), raising the possibility that the m.7501T > A variant, reported elsewhere without detailed investigation [33], is also associated with hearing loss. The low conservation of the variation at this position (29% among mammals) does not support the pathogenicity of the variant, in contrast to the much higher conservation at m.7472A (61%), 7505A (98%), 7510T (78%), and 7511T (98%). On the other hand, the m.7501T > A variant is predicted to modify the secondary structure of the D-arm in the $tRNA^{Ser(UCN)}$ transcript; the D-arm is important for the stability of the transcript and the general rate of mitochondrial protein synthesis [55]. Further investigation, such as haplogroup analysis or generating lymphoblastoid cell lines to measure endogenous respiration rates, may help to define the pathogenicity of the m.7501T > A variant.

All other variants found in this study, such as m.827A > G, 961insC, and 961delT + Cn, which have been discussed elsewhere with respect to their pathogenicity [21,22,27,30,62], were considered to be non-pathologic polymorphisms because they were found frequently in

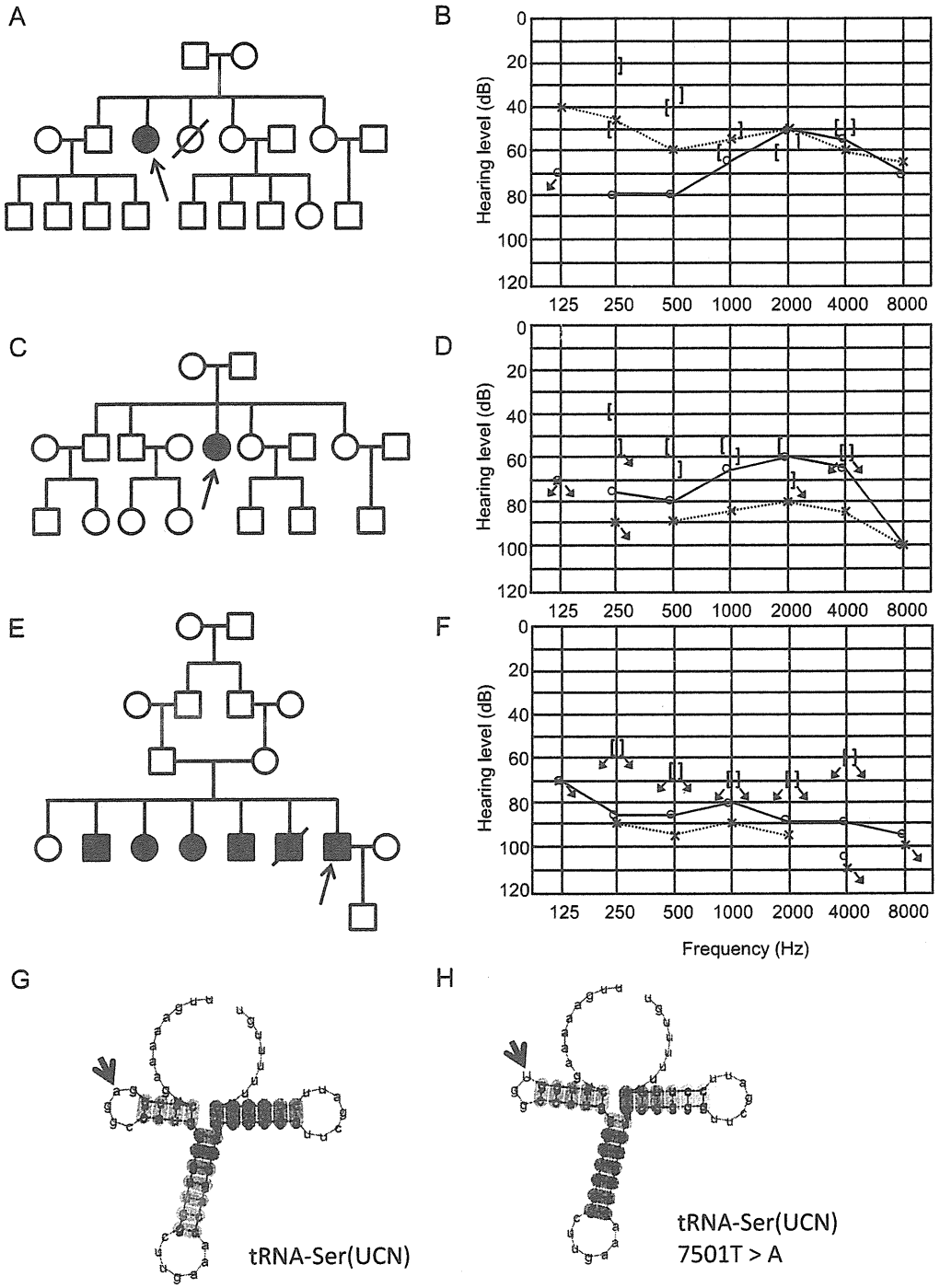

Figure 4 Pedigrees of families carrying the m.7501T > A variant. (A-F) Pedigrees of three families carrying the homoplasmic m.7501T > A, and audiograms of the probands (A and B, C and D, E and F). (G,H) Predicted secondary structure of the *tRNA*$^{Ser(UCN)}$ transcript (G) and the tRNA$^{Ser(UCN)}$ with m.7501T > A (H). Because the gene is transcribed in the reverse direction, thymine at 7501 (G) and adenine (H) are indicated as a and u, respectively (bold arrows).

the controls. The other variants, m.663A > G, 709G > A, 750A > G, 752C > T, 1009C > T, 1041A > G, 1107T > C, 1119T > C, 1382A > C, and 1438A > G, were frequently detected in the controls and considered to be nonpathogenic polymorphisms, which is in consistent with a previous report [27]. The spectrum of variants of mitochondrial genes in Japanese individuals was similar to that in a Chinese population [27], for which most of the variants detected in this study (other than the m.904C > T and 7501T > A) have been reported. In contrast, the spectrum was dissimilar to those in other ethnic groups such as the Polish population [19,63]. Our results indicate that ethnic background should be taken into consideration when studying the pathogenicity of mtDNA variants based on their frequencies in controls.

Conclusions

We sought to detect mitochondrial variants other than m.1555A > G or 3243A > G mutations, which are known to be related to hearing loss, by dHPLC, direct sequencing, and cloning-sequencing in samples from Japanese patients with hearing loss. The homoplasmic m.904C > T variant in *12S rRNA* was considered to be a new candidate mutation associated with hearing loss. The pathogenicity of the m.7501T > A variant in $tRNA^{Ser(UCN)}$ remains inconclusive, and other variants identified in this study, including the heteroplasmic m.1005T > C variant, are not positively associated with hearing loss. No variants were detected in the in $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$, $tRNA^{His}$, $tRNA^{Ser(AGY)}$, and $tRNA^{Glu}$.

Additional material

Additional file 1: Table S1. List of animal species and the accession numbers of the mtDNA (GenBank) used to calculate nucleotide conservation.

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Author details

[1]Laboratory of Auditory Disorders, Division of Hearing and Balance Research, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan. [2]Department of Clinical Laboratory Medicine, National Center for Child Health and Development, Tokyo, Japan. [3]Division of Otorhinolaryngology, Department of Surgical Subspecialties, National Center for Child Health and Development, Tokyo, Japan. [4]Department of Otorhinolaryngology, Shinshu University School of Medicine, Nagano, Japan.

Authors' contributions

HM participated in cloning and sequencing, data analysis, and drafted the manuscript. HKo, ET, ITK, and HKa established and conducted the dHPLC analysis, sequencing, and data analysis. HT, SU, and TO coordinated the study and helped with gene analysis. TM planned and organized the study, examined patients, analyzed data, and helped draft the manuscript. All the authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Otoferlin遺伝子変異が確認された小児難聴症例の検討

仲野　敦子[*]、有本友季子[*]、松永達雄[**]、工藤　典代[***]
[*]千葉県こども病院 耳鼻咽喉科
[**]国立病院機構東京医療センター 耳鼻咽喉科
[***]千葉県立保健医療大学 衛生学部

Hearing impairments and otoferlin gene mutations in children
Pedicatric hearing impairments and OTOF

Atsuko Nakano[*], Yukiko Arimoto[*], Tatsuo Matsunaga[**], Fumiyo Kudo[***]
[*] Division of Otolaryngology, Chiba Children's Hospital
[**] Department of Otolaryngology, National Institute of Sensory Organs National Tokyo Medical Center
[***] Division of Nutrition, Chiba Prefectural Healthcare College

The otoferlin (OTOF) gene is known to be involved in autosomal recessive hearing impairment. OTOF mutations are considered to be a major cause of inherited auditory neuropathy (AN).

A total of 4 children with hearing impairments who were suspected of having AN based on audiological findings and language development delays were studied. All 4 had abnormal auditory brain-stem response (ABR) and normal distortion product otoacoustic emissions (DPOAE), at least on one side. None had inner ear malformations nor other complications or risk factors for AN (e. g., hyperbilirubinemia). Mutations in the OTOF gene were detected in 3 of the 4 cases: compound heterozygous mutations in 1 case and only 1 mutant allele in 2 cases.

The case with the compound heterozygous mutations had passed the newborn-hearing screening test (NHS) and had normal DPOAE at the first test, but an abnormal DPOAE at 5 years of age. Another case passed the NHS using an auto-ABR in the left ear and referred in the right ear, and had normal DPOAE in the left ear and abnormal DPOAE in the right ear. Profound hearing loss with little benefit from use of hearing aids was present in 2 cases. Mild to moderate hearing loss with some benefit received from hearing aids in language acquisition was present in 1 case.

It could be very helpful to investigate mutations in the OTOF gene in order to diagnose AN. Furthermore, detection of mutations in the OTOF gene should lead to appropriate management (such as cochlear implants). However, the recent report also suggested that AN-related mutations in the OTOF have case-by-case differences and that some cases of undiagnosed AN may exist due to abnormal DPOAE. Children with AN should be correctly diagnosed and managed in order to mitigate language development delay.

Key words：auditory neuropathy, OTOF, DPOAE
和文キーワード：auditory neuropathy, OTOF, DPOAE

論文要旨

Auditory Neuropathy（AN）が疑われた難聴小児に対して、難聴遺伝子解析を行い、3症例にOTOF遺伝子の変異を確認した。1症例は2アレルに、2症例は1アレルにのみOTOF遺伝子変異が同定されたが、3症例ともABRの結果とDPOAEの結果に乖離があり、言

語発達の面でも通常の内耳性難聴児とは異なる経過であった。3症例中1例は、新生児聴覚スクリーニング両側パスで、他の1例は一側要精査例であった。DPOAEは、初診時は正常でも徐々に異常となっている例や、初診時から一側は異常であった例も認められた。

新生児聴覚スクリーニングの普及により早期にANと診断される難聴児の経過は様々であり、DPOAEが異常となる例も確認され、遺伝子解析がAN診断の一助となる可能性があることが示唆された。

はじめに

1996年に純音聴力検査閾値に比べて語音弁別能が著しく悪く、DPOAEは正常で、ABRは無反応、画像上病変を認めない疾患がAuditory nerve disease、Auditory Neuropathy（AN）として報告された[1),2)]。その後新生児聴覚スクリーニング（NHS）の普及により、NHSで見逃される難聴児としてABRは異常であるがDPOAEが正常パターンを示す症例がANとして注目された。最近それらの中には多彩な病態が含まれていることが確認され、Auditory Neuropathy Spectrum Disorder（ANSD）という概念が提唱されている[3)]。2003年にはANの原因遺伝子の一つとしてOtoferlin（OTOF）遺伝子変異が同定されており、OTOF遺伝子変異による難聴症例は、内有毛細胞シナプスの障害による難聴で、内耳奇形を認めず、外有毛細胞機能、蝸牛神経機能は正常であるため人工内耳の効果があると報告されている[4),5)]。

我々の施設で経過観察をしている難聴症例のうち、聴力像や経過よりANを疑い、難聴遺伝子解析においてGJB2遺伝子には変異を認めず、OTOF遺伝子に変異を認めた3症例の臨床経過を検討して報告する。

対　象

千葉県こども病院で経過観察している両側難聴症例のうち、ABRで無反応あるいは高度難聴の所見を呈し、DPOAEが両側あるいは一側が正常で、側頭骨CTで異常を認めなかった5症例のうち難聴遺伝子解析を行った4症例を対象とした。OTOF遺伝子解析はDPOAEが両側異常の難聴症例でも臨床的にはANが疑われた症例にも施行したが、他の症例では変異が確認されなかったため、今回の対象からは除外した。

方　法

聴力は、2－3ヶ月毎に、年齢・発達に応じてBOA、COR、遊戯聴力検査を実施して経過観察を行った。ABR、ASSR、DPOAEは診断時に実施し、必要に応じて再検査を施行した。

難聴遺伝子解析は、GJB2遺伝子解析、ミトコンドリア遺伝子解析を直接シークエンス法により実施し、病的変異を認めなかった場合、疑われる難聴遺伝子解析を行っている。今回はANが疑われる症例でありOTOF遺伝子解析を実施した。OTOF遺伝子に関しては全エクソンとエクソン近傍のイントロン領域を直接シークエンス法で解析した。

なお、遺伝子解析に関しては千葉県こども病院倫理委員会で審査、承認されており、事前に保護者にインフォームドコンセントを行い、文書により同意を得て行った。

結　果

対象とした4症例中3症例にOTOF遺伝子変異を認めた。各症例の概要を表1にまとめた。4症例とも他の合併奇形や高ビリルビン血症等のリスクファクターのない児であった。NHSを受けていた2症例のうち1例は両側パスであり、1例は一側のみパスであった。4症例とも、DPOAEは初診時は両側あるいは一側で正常であったためにANを疑ったが、両側正常であった2例は経過観察中に施行した再検査で両側異常に変化していた。

表1　対象症例

症例	性別	初診時年齢	NHS	ABR（dBnHL）	DPOAE	純音聴力検査（dB）	OTOF遺伝子解析結果
1	F	1y10m	両pass	両105無反応	正常→異常	右110 左105	変異あり（2アレル）
2	F	2y5m	未	両105無反応	右正常 左一部正常	右115 左105	変異あり（1アレル）
3	M	0y3m	右refer 左pass	両105無反応	右異常 左正常	40 (COR)	変異あり（1アレル）
4	F	1y7m	未	右105無反応 左70（I波のみ）	正常→異常	右85 左108	変異なし

OTOF遺伝子変異を認めた3症例について以下に提示する。

症例1：初診時1歳10カ月女児

生育歴、既往歴、家族歴：特記すべきことなし

現病歴：NHS両側パス（検査方法は不明）。生後7カ月頃音への反応が悪いことに気づき近医を受診し、ABR両側無反応のため、人工内耳手術目的に某大学病院に紹介となった。ASSR検査は右60－70dB、左80－90dBで、中等度難聴の診断となり人工内耳適応とはならず補聴器装用開始となった。1歳10カ月の時点で聴覚管理目的に当院へ紹介となった。

初診時検査結果：CORは条件付けができなかった。ABR検査では両側105dBnHLでV波確認できずDPOAEは両側正常であった（図1）。

経　過：補聴器装用、聾学校での療育を開始するが、補聴器常時装用に至るまで1年以上を要した。聴力検査は音への反応が不良であり、CORの閾値は安定しなかった。6歳時には純音聴力検査が可能となり検査上は補聴効果が見られているが、音声のみでの言語の聞き取りは困難である（図2）。DPOAEは徐々に変化し、3歳7カ月の時点では2KHz以上の高音域のみが正常反応で低音域は異常となり、5歳2カ月以降は異常パターンとなっている。DPOAEはほぼ左右差なく同時に変化が見られた。OTOF遺伝子解析にて2アレルにミスセンス変異が確認された。

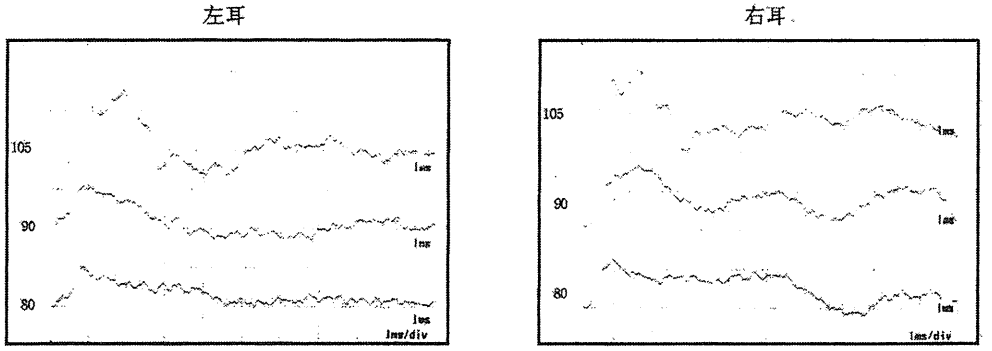
症例2：初診時2歳4カ月女児

生育歴、既往歴、家族歴：特記すべきことなし

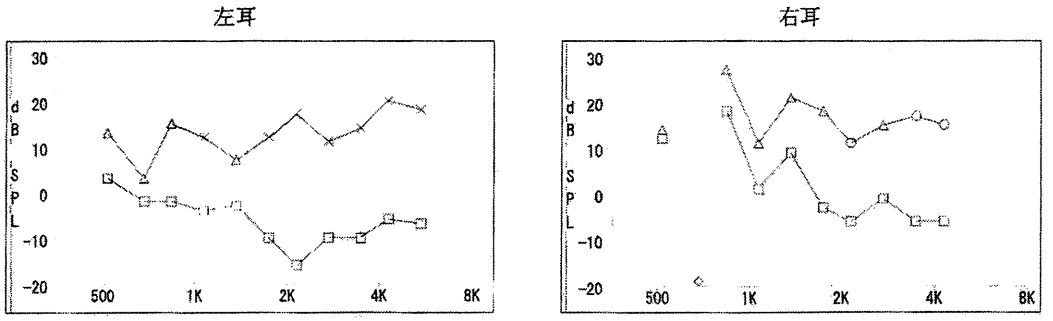
現病歴：NHSは上手くできないとのことで中止されていた。（詳細は不明）1歳半頃、音への反応の悪さと言葉の遅れに気付き、難聴の疑いにて当院を受診。初診時に有意語は見られなかった。

初診時検査結果：CORでは反応がほとんど確認できなかった。ABR検査では両側105dBnHLでV波確認できず、DPOAEは右正常、左は一部正常であった。

経　過：補聴器装用でも補聴効果が見られず、CORの条件付けもなかなかできなかったが、5歳頃より遊戯聴力検査が可能となった（図3）。聴力検査上の装用閾値とは異なり音声の聞き取りは困難であった。OTOF遺



(a) 初診時のABR結果



(b) 初診時のDPOAE結果

図1　症例1の初診時検査結果

(a) 初診時ABR検査結果：左右とも通常のI波より潜時の短い位置に波形が見られるが、I、V波は確認できなかった。
(b) 初診時DPOAE検査結果：正常DPOAE。