つ広がっている。また、学生時代に新しい遺伝学に触れた新世代の医師は、遺伝性疾患に対する医療の受け入れが速いのが特徴である。これに伴い医療者から難聴者に遺伝的原因の話をする施設も近年増えており、近い将来に難聴の遺伝カウンセリングが広く普及すると予想している。

難聴者、難聴児の両親、医療者によく聞かれる質問について

a．どのような難聴者が遺伝性難聴なのか

受診した患者のほかにも、家族の中(両親、兄弟など)に患者と類似した難聴者がいる場合は、患者側も医療者側も遺伝性難聴を意識するし、その可能性は高いといえる。一方、受診患者以外に難聴者がいない場合(孤発例)は、患者側は遺伝性とは考えない。しかし、実際には遺伝性難聴では、このように家族歴がない患者が多い。これは、難聴のみを症状とする遺伝性難聴(非症候群性難聴)では、劣性遺伝(両親は健聴でその子どもの4人に1人が難聴)が80%と多いためである。したがって、家族歴にかかわらず、原因が不明の両側性難聴が先天性あるいは若くして発症するようであれば、遺伝性難聴を疑うべきである[2]。遺伝性難聴の程度はさまざまであり、最近では新生児聴覚スクリーニングの普及により、小児の軽-中等度難聴の早期診断が促進され、その中で遺伝的原因が判明する例も増加している[3]。

b．遺伝性難聴の原因がわかると治療はできるか

先天性あるいは慢性的に経過する感音難聴を正常聴覚に戻す根本的治療法はまだない。これは遺伝性難聴についても同様である。しかし、現在の主たる医学的対応である補聴器、人工内耳の選択には役立つ場合がある。人工内耳は内耳障害による高度難聴に有効であり、早期に実施するほど効果が高いことがわかっている。しかし乳幼児などでは詳細な聴覚検査ができず、人工内耳の適応があるかどうかの判断が困難な場合がある。このような場合、遺伝子解析で難聴の原因と病態がわかることは人工内耳の適応の決定に役立つ。また Auditory Neuropathy という難聴では、人工内耳が有効な場合と有効でない場合があるが、これも原因遺伝子の解析によって鑑別できる場合がある[4]。また症候群性難聴では、合併症の治療に役立つ場合がある。例えば、A3243G ミトコンドリア DNA 変異では、難聴に続いて比較的高い確率で糖尿病が出現するが、早期発見と治療で糖尿病やその合併症の悪化を予防することが可能な場合がある。

c．遺伝子解析とカウンセリングで患者の満足は得られるか、何か問題は生じないか

遺伝カウンセリングに対して患者の満足が得られるか、そして何か問題が生じないかは医療者が一般に最も懸念する点で、筆者も約10年前に開始した際に心配した。その後、約1,000人の遺伝子解析とカウンセリングの経験をしたが、事前の準備をしっかりして、遺伝

カウンセリングの原則を守ることで、これまで解決困難な問題は生じていない。そして原因の判明した難聴者と難聴児の両親の大部分から、遺伝子解析とカウンセリングを受けてよかったという反応を頂いてきた。難聴診断から1〜2年以内の患者の場合は、遺伝カウンセリングが可能であることを聞くと大部分が希望される。一方、先天性難聴で既に就学年齢に達している患者や、後天性難聴で聴力変化なく長期経過している患者あるいはその両親では、希望されない割合が高まる。

d．遺伝性難聴の遺伝子解析(検査)の内容は実施する施設によって違うのか

現時点では、費用負担、何種類の遺伝子を調べるか、遺伝子全体を調べるのか特定の変異のみを調べるのか、結果報告までの期間など、施設によってさまざまな違いがある。このため事前に内容を確認することをお勧めする。また、一施設で原因遺伝子が判明しなくても他の施設で判明する可能性があるので、そのような場合でも他の施設に相談してみる価値がある。

筆者が遺伝カウンセリングを行っている施設(国立病院機構東京医療センター耳鼻咽喉科、慶應義塾大学病院耳鼻咽喉科、国立成育医療センター耳鼻咽喉科)では、発症年齢、聴覚検査所見、家族歴などに応じて、調べるべき遺伝子の種類、遺伝子の部位あるいは特定の変異をアルゴリズムに従って決定する系統的遺伝子解析を行っており、現在は25種類の難聴遺伝子の解析を実施している[5]。多くの遺伝子は全体を調べるが、特定の部位あるいは変異のみを調べる場合もある。結果報告は遺伝子解析のための採血(年齢により5〜20 m*l*)から3ヵ月後である。難聴の遺伝的原因にはまだ不明の点も多く残されていることから、私たちは遺伝子解析を現時点では研究の一環として位置づけている。より詳細な内容に関しては筆者の総説を参照されたい[6][7]。他の施設からの検体の解析も受け付けているので、ご希望の方は筆者まで連絡されたい。(連絡先：国立病院機構東京医療センター聴覚障害研究室 松永達雄、tel 03-3411-0111、e-mail：matsunagatatsuo@kankakuki.go.jp)。

●おわりに

難聴遺伝子に限らず遺伝子とその異常に関する情報、そしてそれを解析する技術は日進月歩である。そのため診療に有用な情報を遺伝子解析からより多く、正確に、低価格で利用可能になっている[8]。遺伝性難聴はその多くが内耳障害によるものであるが、近年は外耳、中耳の奇形でも遺伝的原因の解明が進んでいる[9]。難聴の臨床にこれからますます遺伝子がかかわるのは避けることのできない流れと思われる。そのため、難聴診療にかかわる多くの皆様に遺伝子についての理解が広がり、臨床の場で活用されることを祈念している。

(松永達雄)

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Pediatr Nephrol
DOI 10.1007/s00467-012-2125-9

BRIEF REPORT

Alport-like glomerular basement membrane changes with renal-coloboma syndrome

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Received: 26 October 2011 / Revised: 20 January 2012 / Accepted: 23 January 2012

Abstract
Background Autosomal dominant mutations in paired box gene 2 (*PAX2*), on chromosome 10q24, are responsible for renal coloboma syndrome (RCS). The role of *PAX2* in glomerular basement membrane (GBM) formation and maintenance remains unknown.
Case-diagnosis We report a case of a 13-year-old Japanese girl who had both optic disk coloboma and renal insufficiency. Her father and sister also had both coloboma and renal dysfunction. Renal pathological findings revealed a basket-weave pattern of the GBM, which was compatible with Alport syndrome, but type IV collagen α5 staining was normal. The patient's findings of coloboma and renal dysfunction suggested that she had RCS, and genetic analysis revealed a *PAX2* heterozygous mutation in exon 2 (c.76dup, p.Val26Glyfsx27) without any mutations of *COL4A3*, *COL4A4*, and *COL4A5*, which are responsible for autosomal and X-linked Alport syndrome.
Conclusions *PAX2* mutations may result in abnormal GBM structure.

Keywords Renal-colombia syndrome · PAX2 · Glomerular basement membrane · Type IV collagen · Podocyte

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Introduction

Renal-coloboma syndrome (RCS, OMIM 120330) is a rare autosomal dominant disorder associated with paired box gene 2 (*PAX2*, 10q24) heterozygous mutations [1]. PAX2 is a nuclear transcriptional factor and is highly conserved among species [2]. In the fetal period, PAX2 is expressed in the otic and optic vesicles, spinal cord, hindbrain, mesonephros, and metanephros in the embryonic kidney. PAX2 is one of the central regulators for early-stage kidney development, but the precise mechanisms of PAX2 for kidney development have not been fully clarified. RCS is characterized by ocular and renal abnormalities. Renal malformations include hypoplasia, dysplasia, vesicoureteral reflux (VUR), multicystic dysplastic kidney, and horseshoe kidney [3]. Renal histopathological findings in RCS have been reported, including oligomeganephronia that is induced by a reduction in nephron number in the RCS kidney. However, there are no previous reports of obvious glomerular basement membrane (GBM) changes as evaluated by electron microscopy. We report here for the first time remarkable GBM changes with RCS due to *PAX2* mutation, which are similar to those found in Alport syndrome.

Published online: 21 February 2012

Case report

Patient 1

A 6-year-old Japanese girl visited our hospital because of mild proteinuria. We found that she also had bilateral optic disk coloboma (Fig. 1a). At the age of 13 years, laboratory findings showed that her blood urea nitrogen (BUN) level was 26 mg/dl, serum creatinine (SCr) level was 0.97 mg/dl, and creatinine clearance (CrCl) was decreased to 59.1 ml/min/1.73 m^2. Urinary protein was slightly increased (0.3 g/day), especially urinary β2 microglobulin (1,000 μg/l, normal range <230 μg/l), but she had no hematuria. Ultrasonography revealed left renal atrophy (kidney size 64× 35 mm), but the right kidney size was normal (81× 37 mm). Her eye and renal abnormalities were compatible

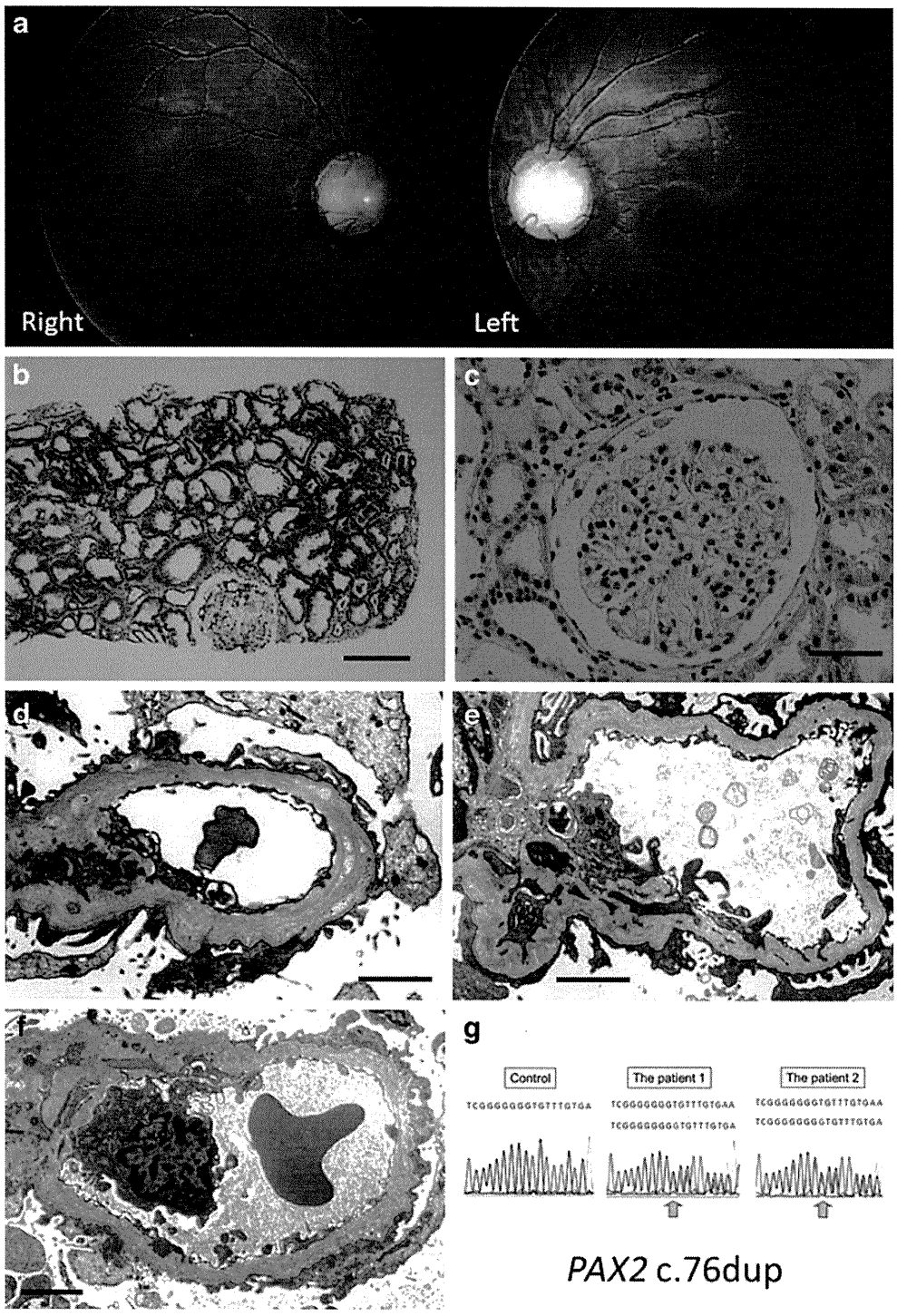

Fig. 1 Fundus photographs from patient 1 (**a**): bilateral optic discs are enlarged. Renal pathological findings in patient 1 (**b**–**c**): Light microscopy shows that the number of glomeruli is small (**b**), periodic acid-methenamine-silver (PAM) staining, original magnification ×100, *scale bar*=100 μm), but hypertrophy or proliferative lesions cannot be seen in the glomeruli (**c**), periodic acid-Schiff (PAS) staining, original magnification ×400, *scale bar*=30 μm). Electron microscopy shows thickening, which is compatible with a basket-weave appearance in the glomerular basement membrane (GBM) in patient 1 (**d**) and patient 2 (**e**). GBM findings are similar to a genetically confirmed case of Alport syndrome by *COL4A3* homozygous mutation (**f**) (original magnification ×5,000, *scale bar*=2 μm). Genetic analysis (**g**) shows that both patients have a *PAX2* mutation (c.76dup)

with RCS, but the precise cause of her renal insufficiency was unknown.

Patient 2

Patient 2 is patient 1's elder sister, and she had left renal atrophy detected soon after birth. At the age of 4 months, eye abnormalities including optic disk coloboma and macular hypoplasia were found. At the age of 10 months, mild proteinuria (0.4 g/day) and VUR were detected, and at the age of 5 years, she received a surgery for VUR. However, her proteinuria did not disappear and her renal function deteriorated. She had a renal biopsy performed at the age of 6 years, and electron microscopy showed that the glomeruli had diffuse thickening accompanied by a basket-weave formation of the GBM, which was compatible with Alport syndrome. She was diagnosed with Alport syndrome on the basis of renal histological findings; however, hematuria never appeared during her course, and genetic analysis was not performed at that time.

Family history

The patients' father also had coloboma and renal failure, but his funduscopic findings and cause of his renal dysfunction were unknown because he died after receiving four renal transplantations. The paternal grandfather had left renal atrophy, but his renal function was preserved and he did not have coloboma. Others in the family showed no renal disorder. None of their relatives had hearing loss.

Renal histology and genetic analysis

To confirm the cause of renal insufficiency, we performed renal biopsy in patient 1. Light microscopy findings showed that the kidney was oligonephronic but not enlarged (Fig. 1b, c). Immunofluorescent staining showed no significant abnormalities. Electron microscopy showed thickening and thinning of the GBM (Fig. 1d), similar to patient 2 (Fig. 1e). The patients' GBM findings are similar to a genetically confirmed case of Alport syndrome (Fig. 1f). The eye abnormality, oligonephronic kidney, and family history indicated that patient 1 suffered from RCS. To confirm this diagnosis, we performed genetic analysis of *PAX2* for both patients and their paternal grandfather after obtaining informed consent. We detected a c.76dup heterozygous mutation in exon 2 of *PAX2* (Fig. 1g) in the siblings but not the grandfather. This frameshift mutation induces an amino acid change from valine to glycine and introduces a premature stop codon (p.Val26Glyfsx27). To exclude the possibility of complicating Alport syndrome, we performed type IV collagen staining of glomeruli for patient 1 and observed that the α5 chain [α5 (IV)] staining pattern was normal. Furthermore, we sequenced the genes *COL4A4* (2q35-q37), *COL4A3* (2q36-q37), and *COL4A5* (Xq22), which are responsible for autosomal recessive, dominant, and X-linked Alport syndrome. No mutations or significant variants were detected in either patient. Based on the presence of a heterozygous *PAX2* mutation in both patients with normal immunohistochemistry for α5 (IV) and the absence of significant sequence variation in any of the genes encoding type IV collagen proteins found in the GBM, we concluded that the GBM changes resulted from *PAX2* haploinsufficiency in our patients.

Discussion

We identified that our patients had a *PAX2* heterozygous mutation in exon 2 (c.76dup, p.Val26Gly fsx27). Although there is no genotype–phenotype correlation in RCS, this is the most frequent mutation of *PAX2* [4]. This frameshift mutation leads to haploinsufficiency of the PAX2 protein. The *Pax2*$^{1Neu\ +/-}$ mutant mouse is a model of RCS that has a heterozygote 1-bp insertion in *PAX2* [5], [6], and it has been reported that heterozygous mutations of *PAX2* induce apoptotic cells in the fetal kidney and reduce branching of the ureteric bud. As a result, *PAX2* heterozygous mutations induce renal hypoplasia [7]. Oligomeganephronia is induced by renal hypoplasia and *PAX2* mutation [8], and there are few reports regarding the association with oligomeganephronia and GBM changes [9]. Although the number of glomeruli in patient 1 was decreased, glomerular enlargement was not observed. The causes of our patients' renal insufficiency are unknown, but reducing renal mass may induce this condition.

Laminin, type IV collagen α3 (α3 [IV]) chain, α4 (α4 [IV]) chain, and α5 (IV) are major components of the GBM. Laminin is produced by both podocytes and endothelial cells, and α3 (IV), α4 (IV), and α5 (IV) originate only from podocytes [10]. A host of transcription factors, especially WT1 and PAX2, play a significant role in modulating podocyte maturation. Although PAX2 is essential for embryonic renal formation, a decrease in PAX2 and increase in WT1 in the embryonic kidney are also necessary for further differentiation of podocytes [11]. Therefore, *PAX2* mutation may result in abnormal GBM production in podocytes, but further investigations are required to clarify this issue.

In conclusion, this is the first report of Alport-like GBM changes in RCS due to *PAX2* mutation. It is unknown whether *PAX2* haploinsufficiency leads to GBM changes, as observed in the siblings in this study. Our observations may lead to an improved understanding of the pathogenesis of RCS.

Disclosure The authors declare that they have no competing financial interests and no funding sources to disclose.

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Molecular Genetics and Metabolism 104 (2011) 560–565

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Newborn screening for Pompe disease in Japan

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ARTICLE INFO

Article history:
Received 14 July 2011
Received in revised form 5 September 2011
Accepted 5 September 2011
Available online 10 September 2011

Keywords:
Pompe disease
Acid alpha-glucosidase
Dried blood spots
Newborn screening
Genetic polymorphism

ABSTRACT

Pompe disease is caused by a deficiency of acid alpha-glucosidase (GAA) that results in glycogen accumulation, primarily in muscle. Newborn screening (NBS) for Pompe disease has been initiated in Taiwan and is reportedly successful. However, the comparatively high frequency of pseudodeficiency allele makes NBS for Pompe disease complicated in Taiwan. To investigate the feasibility of NBS for Pompe disease in Japan, we obtained dried blood spots (DBSs) from 496 healthy Japanese controls, 29 Japanese patients with Pompe disease, and five obligate carriers, and assayed GAA activity under the following conditions: (1) total GAA measured at pH 3.8, (2) GAA measured at pH 3.8 in the presence of acarbose, and (3) neutral glucosidase activity (NAG) measured at pH 7.0 without acarbose. The % inhibition and NAG/GAA ratio were calculated. For screening, samples with GAA<8% of the normal mean, % inhibition>60%, and NAG/GAA ratio>30 were considered to be positive. Two false positive cases (0.3%) were found, one was a healthy homozygote of pseudodeficiency allele (c.1726G>A). The low false-positive rate suggests that NBS for Pompe disease is feasible in Japan.

1. Introduction

Pompe disease (glycogen storage disease type II) is an autosomal recessive lysosomal storage disorder characterized by deficiency of lysosomal enzyme acid alpha-glucosidase (GAA, EC3.2.1.20). Pompe disease is progressive, with massive accumulation of glycogen in lysosomes. The clinical manifestations are variable, depending on age at onset, range of organ involvement, and rate of progression. Patients with infantile-onset Pompe disease have severe cardiomegaly and generalized skeletal muscle weakness, and die before the age of 2 years old. Patients with juvenile- and adult-onset Pompe disease have mainly skeletal muscle involvement and often progress to respiratory failure [1,2].

Enzyme replacement therapy with recombinant human GAA can be used to treat patients with Pompe disease and has been shown to prolong survival, reverse cardiomyopathy, and improve motor function [3–5]. The best motor function outcomes have been achieved after early initiation of enzyme replacement therapy, which underscores the need for early diagnosis [4,6–8]. However, early diagnosis of Pompe disease is difficult because of the low index of suspicion and the lack of specificity in early symptoms. Until recently, demonstration of deficient GAA activity in dried blood spots (DBSs) was not possible because of interference from the isoenzyme maltase glucoamylase (MGA), which is abundant in neutrophils. However, the identification of acarbose as an effective inhibitor of MGA has permitted evaluation of GAA activity in blood samples, including DBSs, on filter paper [5,9–13]. These methods were shown to reliably identify patients with Pompe disease in a large pilot program in Taiwan [13].

It is reported that pseudodeficiency allele reduces GAA activity but causes no symptoms of the disease [14,15]. Pseudodeficiency alleles are known as c.1726G>A (p.G576S) variant in cis with c.2065G>A (p.E689K), also as c.[1726A; 2065A]. The alleles almost segregate together. Substitution c.2065G>A (p.E689K) reduces GAA activity by 50% at most. On the contrary, substitution c.1726G>A (p.G576S) reduces the activity to such extent that it falls into the patient range. Pseudodeficiency allele is more frequent in Japanese population than in other populations studied to date [14], and this may complicate issue in NBS of Pompe disease with Japanese population. According to the article of NBS in Taiwan, the presence of the GAA pseudodeficiency alleles made NBS in Taiwan complicated [15]. Therefore, NBS may require modification to distinguish a healthy homozygote of pseudodeficiency allele from Pompe disease patients by using a new cut-off value in the screening procedure in Japanese population. Herein, we report the GAA activity and presence of the sequence variant c.1726G>A (p.G576S) in the GAA gene in 520 DBSs collected from 400 healthy Japanese newborns, 96 healthy adults, 29 patients with Pompe disease, and 5 obligate carriers.

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doi:10.1016/j.ymgme.2011.09.002

2. Materials and methods

2.1. Samples

DBSs (Advantec®, ToyoRoshi Kaisha Ltd., Japan) were obtained from 29 Japanese patients with Pompe disease (two infantiles, 14 juveniles and 13 adult phenotypes; age range: 3 months–67 years old), 496 healthy Japanese controls (400 newborns aged 3–5 days old, 96 adults aged 18–62 years old) and 5 obligate carriers (33–46 years old). DBSs were dried at room temperature and stored at −20 °C in plastic bags until analysis. Informed consent was obtained from all subjects or family members. All samples were prepared and analyzed in accordance with the protocols approved by the ethics committee of the National Center for Child Medical Health and Development.

2.2. Reagents

A Big Dye Terminator kit was purchased from Applied Biosystems (Foster City, CA, USA). Acarbose, 4-methylumbelliferone (4MU) and 4-methylumbelliferyl-αD-glucopyranoside (4MUG) were purchased from Sigma-Aldrich (St. Louis, MO). Ampdirect™ Plus was obtained from Shimadzu (Kyoto, Japan) and rTaq DNA polymerase was purchased from Takara (Shiga, Japan). Other chemicals (all from Sigma-Aldrich or Wako, Osaka, Japan) were of reagent grade.

2.3. Frequency of pseudodeficiency alleles

Both pseudodeficiency alleles c.[1726A; 2065A] almost segregate together. Substitution c.2065G>A (p.E689K) reduces GAA activity by 50% at most. On the contrary, substitution c.1726G>A (p.G576S) reduces the activity to such extent that it falls into the patient range. Therefore, we tested for the presence of the s sequence variant only c.1726G>A (p.G576S).

A 1.2-mm diameter disk from each DBS was obtained using Uni-Core™ and placed in a sample tube, into which 10 μL Ampdirect™ Plus (including polymerase chain reaction (PCR) buffer and dNTPs), 0.5 units rTaq DNA polymerase, and a set of specific primers (each 1.0 μmol/L) were added to produce a reaction mixture of total volume 20 μL. The sequences 5′-AGG GAG GGC ACC TTG GAG CCT G-3′ and 5′-GCA GAG GCC CCA ACC TTG TAG G-3′ were designed as forward and reverse primers for amplification of the single nucleotide polymorphism (SNP) c.[1726G] of the *GAA* allele. PCR was performed using denaturation at 95 °C for 10 min; 30 cycles of amplification with denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s; and final extension at 72 °C for 7 min. DNA sequencing analysis was performed on amplified products by direct sequencing with the Big Dye Terminator kit on an ABI PRISM 3100 DNA Genetic Analyzer (Applied Biosystems).

The DBSs were also analyzed for the sequence variant c.1726G>A (p.G576S) in the GAA gene using PCR-RFLP (Restriction Fragment Length Polymorphism). Sample preparation and PCR was performed as described above, using the sequences 5′-AGG GAG GGC ACC TTG GAG CCT G-3′ and 5′-GGG AGG CGA TGG CTT CGG TCA AG-3′ as the forward and reverse primers, respectively, for amplification of the SNP c.[1726G] of the *GAA* allele. For PCR-RFLP, the PCR-amplified product (5 μL) was placed in a sample tube, into which 14 μL of Neb buffer (50 nM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 1 mM DTT; pH 7.9) and 1 μL of *AluI* enzyme were added to give a reaction mixture of total volume 20 μL. This mixture was then incubated for 4 h at 37 °C.

2.4. GAA activity

For determination of GAA activity, a modified protocol based on the fluorometric assay developed by Chamoles et al. [9] was used. Briefly, a disk of 3.0 mm in diameter obtained from each DBS by Uni-Core™ was placed in a sample tube and 360 μL of water was added, followed by gentle mixing for 1 h at 4 °C on a rocking platform. A 70 mM stock solution of the synthetic substrate 4MUG in dimethyl sulfoxide (Wako) was prepared in advance. Substrate solutions consisting of 1.4 mM 4MUG at pH 3.8 and pH 7.0 were prepared in 40 mM aqueous sodium acetate buffer and 40 mM sodium acetate buffer, respectively. Enzyme reactions at pH 3.8 and pH 7.0 were carried out in 50 μL of substrate solution, 10 μL of deionized water, and 40 μL of DBS extract. For enzyme reactions in the presence of an inhibitor, the water was replaced with 10 μL of 2.7 μM acarbose. These reagents were incubated for 20 h at 37 °C with covering with sealing film (Sumitomo Bakelite Co.). The DBS extract for blanks was incubated separately and combined with the other reagents at the end of the incubation period, immediately followed by addition of 200 μL of 150 mM ethylene diamine triacetic acid (EDTA) (pH 11.5) to all wells.

A 4MU standard curve was prepared on every plate by mixing aqueous standards (100 μL per well) in the range 0.00 to 3.13 μM with EDTA solution (200 μL per well). Eight different standards per curve were used in duplicate. The relative fluorescence (excitation 355 nm, emission 460 nm) of each well was measured using a Wallac 1420-011 Multilevel Counter (PerkinElmer, Turku, Finland). The fluorescence readings were corrected for the blank and the results were compared with the fluorescence of a 4MU calibrator. The absolute amount of whole blood per spot cannot be determined accurately, but is comparable among samples. Therefore, the enzymatic activities are expressed as nmol of substrate hydrolyzed per punch per hour.

For Pompe disease screening, three assays were performed: (1) total GAA activity (tGAA) measured at pH 3.8, (2) GAA activity measured at pH 3.8 in the presence of acarbose, and (3) total neutral glucosidase activity (NAG) measured at pH 7.0 without acarbose. The % inhibition [(tGAA–GAA)/tGAA] and NAG/GAA ratio were calculated using data from these assays.

3. Results

3.1. Frequency of pseudodeficiency allele

The presence of the sequence variant c.1726G>A (p.G576S) in the GAA gene was examined in DBSs using DNA sequencing analysis and PCR-RFLP. The results were as follows: among Pompe disease patients (n = 29), 16 (55.1%) had sequence c.1726G/G (G/G), 10 (35%) had c.1726G/A (G/A), and 3 (10%) had c.1726A/A (A/A); among obligate carriers (n = 5), 3 (60%) had G/G, 2 (40%) had G/A, and among 496 healthy controls, 332 (67%) had G/G,149 (30%) had G/A, and 15 (3.0%) had A/A. These data are similar to those in previous reports. The A/A allele occurred at a significantly higher frequency (10%) in the patients than in controls (3%).

3.2. GAA activity

GAA activities measured with 4MU in the presence of acarbose in DBSs were as follows. The average GAA activity in the 496 healthy controls was 21.6 pmol/punch/h (range 1.3–63.4, Fig. 1A). The distribution of GAA activity based on the frequency of the three alleles is shown in Fig. 1B. A cut-off point for the GAA activity of 8% of the normal average (1.7 pmol/punch/h) identified 29 patients (100%), 5 healthy homozygotes of pseudodeficiency allele (33%), 1 obligate carrier (20%), and no healthy control without pseudodeficiency allele.

3.3. NAG/GAA ratio

The averages (range) of the NAG/GAA ratios were 5.0 (0.9–30.8) for healthy control without pseudodeficiency allele, 25.2 (14.6–57.6) for healthy homozygotes of pseudodeficiency allele, 22.3 (11.1–39.5) for obligate carrier, and 89.4 (41.9–222.3) for patients with Pompe disease. A cut-off value of 30 identified 29 patients (100%), 12 healthy

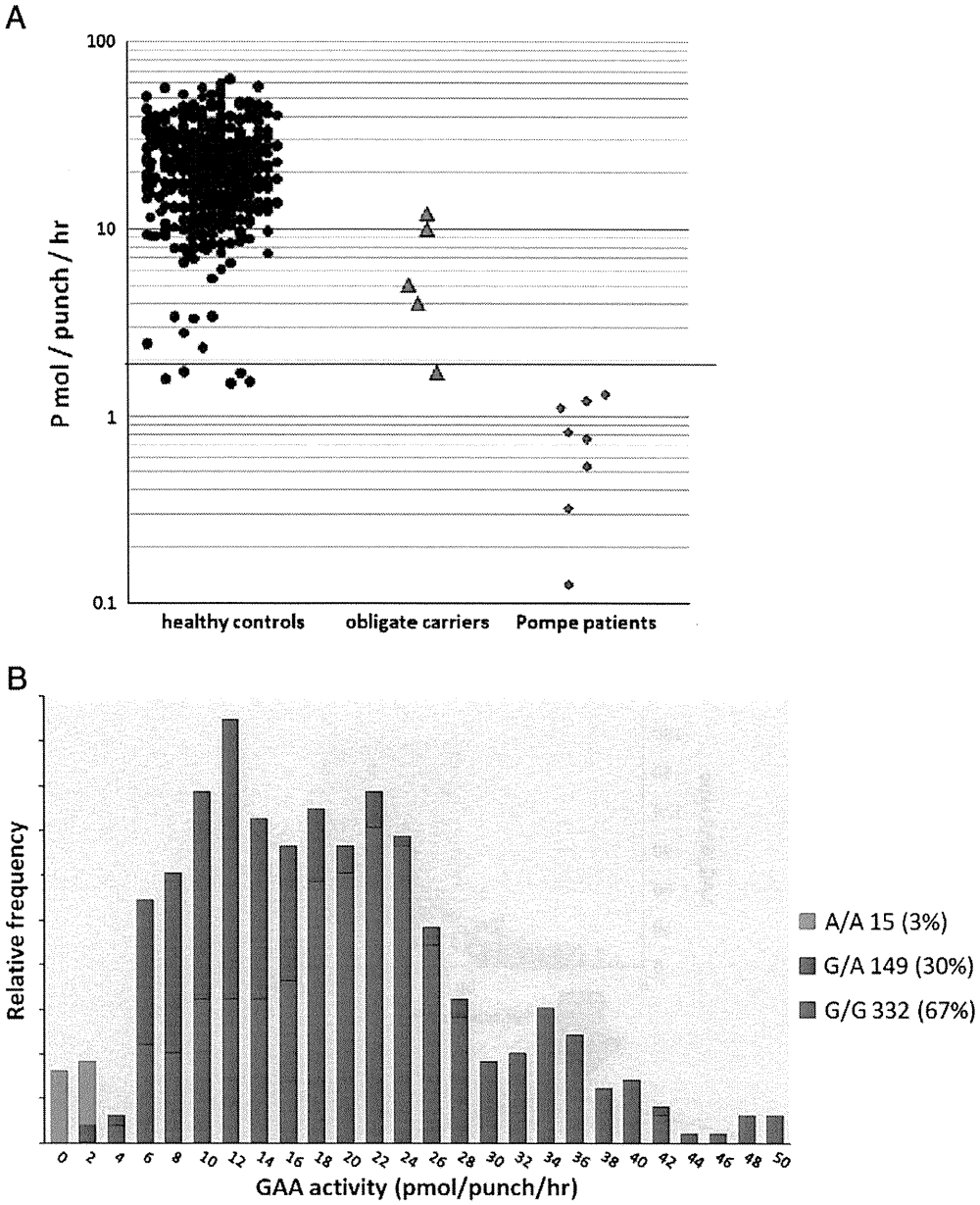

Fig. 1. Correlation between GAA activity (pmol/punch/h) and genotype. (A) A log scale of GAA activity (pmol/punch/h) in healthy controls, obligate carriers, and patients with Pompe disease. A cut-off point shows with red bar (1.7 pmol/punch/h). (B) GAA activity (pmol/punch/h) in G/G (no sequence variant of c.1726G>A in the GAA gene) G/A (heterozygote sequence variant), and A/A (homozygote sequence variant) cases.

Table 1
Positive results in screening for healthy controls, obligate carriers, and patients with Pompe disease.

Cut-off point		Healthy controls (with pseudodeficiency allele)	Obligate carriers	Pompe patients
Single screening	GAA (% of nM/mean)<8%	1.0% 5/496 (5)	20% 1/5	100% 29/29
	NAG/GAA>30	2.4% 12/496 (10)	40% 2/5	100% 29/29
	% inhibition>60	2.8% 14/496 (12)	20% 1/5	100% 29/29
Combination screening (double marker screening)	% inhibition>60 NAG/GAA>30 Meet the above condition	1.0% 5/496 (5)	20% 1/5	100% 29/29
Combination screening (triple marker screening)	GAA (% of nM/mean)<8 % NAG/GAA>30 % inhibition>60 Meet the above condition	0.20% 1/496 (1)	20% 1/5	100% 29/29

homozygotes of pseudodeficiency allele (80%), 1 obligate carrier (20%), and 2 healthy controls without pseudodeficiency allele (0.4%) (Table 1).

3.4. % inhibition

The averages (range) for % inhibition were 38.0 (8.0–68.9)% for healthy control without pseudodeficiency allele, 64.4 (44.7–77.6)% for healthy homozygotes of pseudodeficiency allele, 48.7 (24.7–64.3)% for obligate carriers, and 83.2 (71.3–97.2)% for patients with Pompe disease. A cut-off value of 60% identified 29 patients (100%), 12 healthy homozygotes of pseudodeficiency allele (80%), 1 obligate carrier (20%), and 2 healthy controls without pseudodeficiency allele (0.4%) (Table 1).

3.5. Double-marker screening with NAG/GAA ratio and % inhibition

All patients with Pompe disease had a NAG/GAA ratio >30 and % inhibition >60%. A cut-off of a combination of NAG/GAA ratio >30 and % inhibition >60% identified 29 patients (100%), 5 healthy homozygotes of pseudodeficiency allele (33%), 1 obligate carrier (20%), and no healthy control without pseudodeficiency allele (Table 1). Thus, a combination of the two cut-off values resulted in only 6 false positive cases.

3.6. Triple-marker screening with NAG/GAA ratio, % inhibition and GAA activity

A three-dimensional graph of GAA activity, NAG/GAA ratio and % inhibition is shown in Fig. 2. Triple-marker screening with these parameters (GAA activity<8%, NAG/GAA ratio>30, % inhibition>60%) decreased the number of false-positive cases to 2: one healthy homozygote of pseudodeficiency allele and one obligate carrier.

3.7. Calculation of false positive rate

All Pompe disease patients, one healthy homozygote of pseudodeficiency allele, and one obligate carrier screened positive using our algorithm of GAA activity<8%, % inhibition>60%, and NAG/GAA ratio>30. Therefore, the false-positive rate in this retrospective study was 0.3%, with the assumption that the frequency of patients with Pompe disease in Japan is 0.001% and that the frequency of the pseudodeficiency allele in Japan is 3%.

4. Discussion

This is the first report to show that mass screening for Pompe disease in Japanese population can be performed with a false-positive rate of

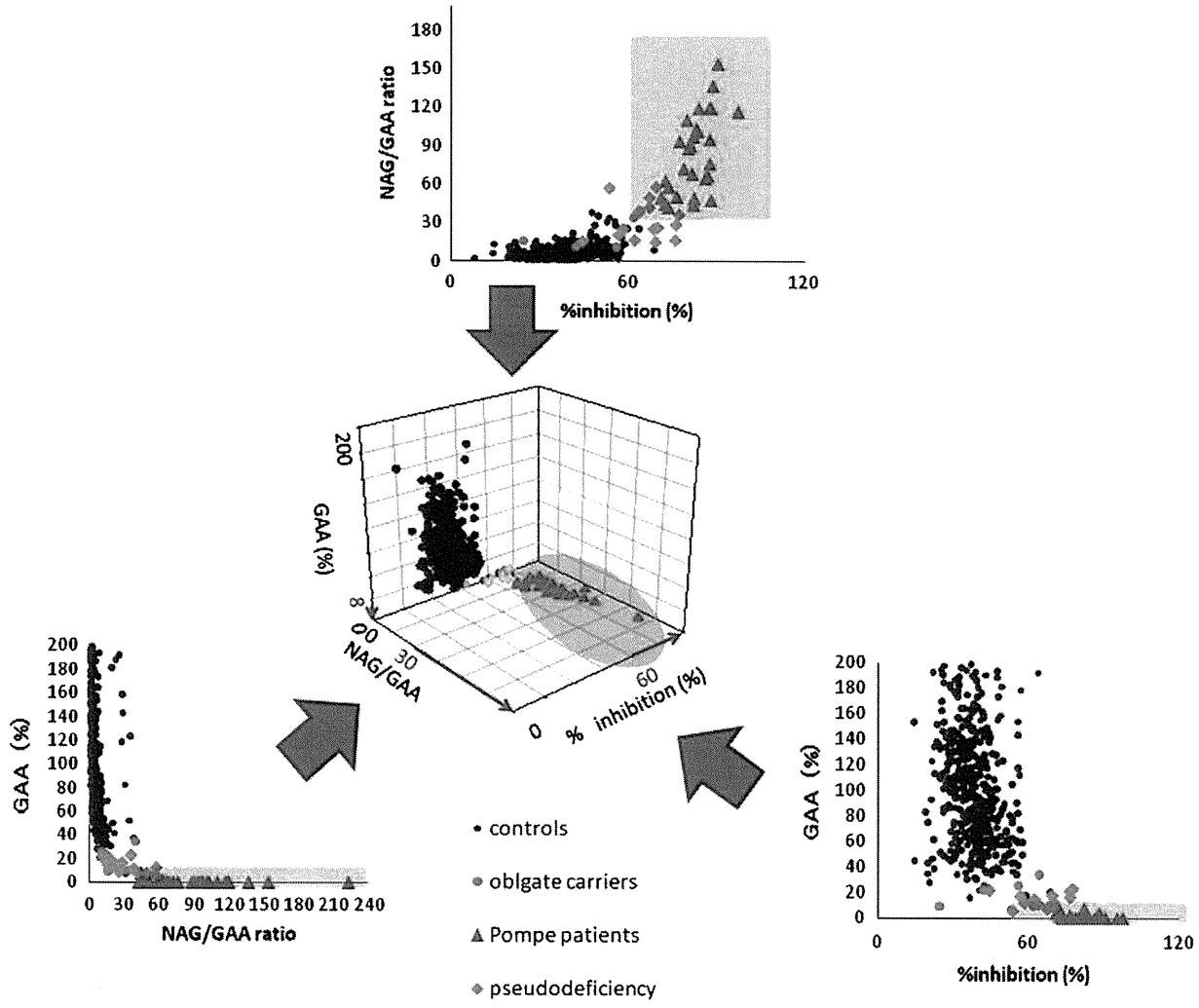

Fig. 2. (Center) 3D plot of combination (triple-marker) screening using the NAG/GAA ratio (Y axis), % inhibition (X axis) and GAA activity (Z axis) for healthy control without pseudodeficiency allele (n = 481, blue circles), obligate carriers (n = 5, green triangles), healthy homozygotes with pseudodeficiency allele (n = 15, light blue squares), and patients with Pompe disease (n = 29, red squares). (Lower left) Relationship of GAA activity with NAG/GAA ratio. (Lower right) Relationship of GAA activity with % inhibition. (Upper) Relationship of NAG/GAA ratio with % inhibition. The cut-off line was 8% for GAA activity, 30 for NAG/GAA ratio, and 60% for % inhibition as pink area.

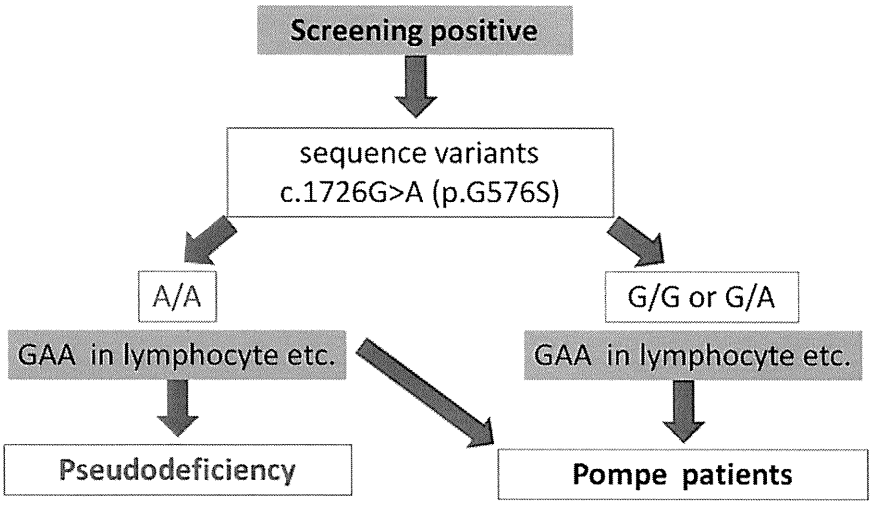

Fig. 3. Diagnostic flow-chart after a positive screen for Pompe disease in Japan.

approximately 0.3%. The improved understanding of the natural history of Pompe disease and the efficacy of enzyme replacement therapy [3–8] has increased the importance of mass screening. The American College of Medical Genetics (ACMG) score of 772 points (i.e. <1000 points) was calculated in 1968 [16], before the appearance of enzyme replacement therapy, and at that time mass screening for Pompe disease was not considered appropriate. However, the positive outcomes of enzyme replacement therapy and the development of screening methods with lower false-positive rates suggests that this score should now be >1000 points. Therefore, we suggest that mass screening for Pompe disease should now be considered to be appropriate.

Screening tests for the diagnosis of lysosomal storage diseases generally include evaluation of in vivo lysosomal enzyme activities and the amount of stored materials. For enzyme activities of Pompe disease, decreases in GAA activity can be measured in skeletal muscle, fibroblast cells, purified lymphocytes, leucocytes, and dried blood on filter paper. For stored materials of Pompe disease, accumulation of the level of hexose tetrasaccharide (Glc4), can be measured in urine. However, this is not disease-specific, since increased Glc4 is also observed in diseases such as glycogen storage disease (types III and VI), Duchenne muscular dystrophy, and acute pancreatitis [17]. Therefore, we performed the screening based on measurement of GAA activity in dried blood on filter paper.

We previously suggested that mass screening for Pompe disease in Japan may be more difficult than that in other countries because of the higher percentage of the population with the pseudodeficiency allele. Very few cases with pseudodeficiency allele are found in Europe and the United States, but diagnosis of Pompe disease in a case with reduced GAA activity in dried blood is still often confirmed by measuring the enzyme activity in skeletal muscles and fibroblast cells, and by genetic testing. In Fig. 3, we propose a diagnostic flowchart. If polymorphism analysis of p.G576S reveals the AA homozygote, the condition may be healthy homozygotes of pseudodeficiency allele or Pompe disease patients, even if the enzyme activity is reduced. Pompe patients can carry the pseudodeficiency alleles (consistent with our data). Not all healthy homozygotes of pseudodeficiency allele are false positives. Therefore, the diagnostic flowchart we presented here cannot diagnose all Pompe disease patients, especially for patients with juvenile- and adult-onset Pompe disease. Additional tests including sequencing analysis of GAA gene and GAA activity in lymphocyte may be necessary. However, we still believe this method is very useful for NBS, because in this study our main purpose is to find patients with infantile-onset Pompe disease before the disease becomes severe and irreversible.

Neonatal screening is currently expanding worldwide. Screening for abnormalities of amino-acid metabolism (approximately eight diseases), organic acid metabolism (approximately eight diseases), and fatty acid metabolism (approximately nine diseases) using tandem mass spectrometry has been implemented nationwide (i.e., almost all newborns are covered as a national policy) in the United States, Canada, Germany, Austria, Taiwan, and Singapore, and is partially implemented (i.e., implemented in some states only, for a fee, or in the study phase) in the United Kingdom, Italy, Spain, South Korea, China, and Argentina. This worldwide expansion has also included screening for lysosomal storage diseases, and a method that can simultaneously identify several diseases, including mucopolysaccharidosis, Fabry's disease, and Pompe disease, has been developed [18]. Before the advent of enzyme replacement therapy, these diseases were severe and progressive conditions that had no cure [19,20]. However, the efficacy of enzyme replacement therapies has increased the importance of screening for treatable lysosomal storage diseases.

In this study, we found that mass screening could be performed with a false-positive rate of approximately 0.3%. The results of our study suggest that screening for Pompe disease can be successfully implemented in Japanese population.

Acknowledgment

This work was supported by a grant from Japan Health Sciences Foundation.

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BMC Medical Genetics

RESEARCH ARTICLE Open Access

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

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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

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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.

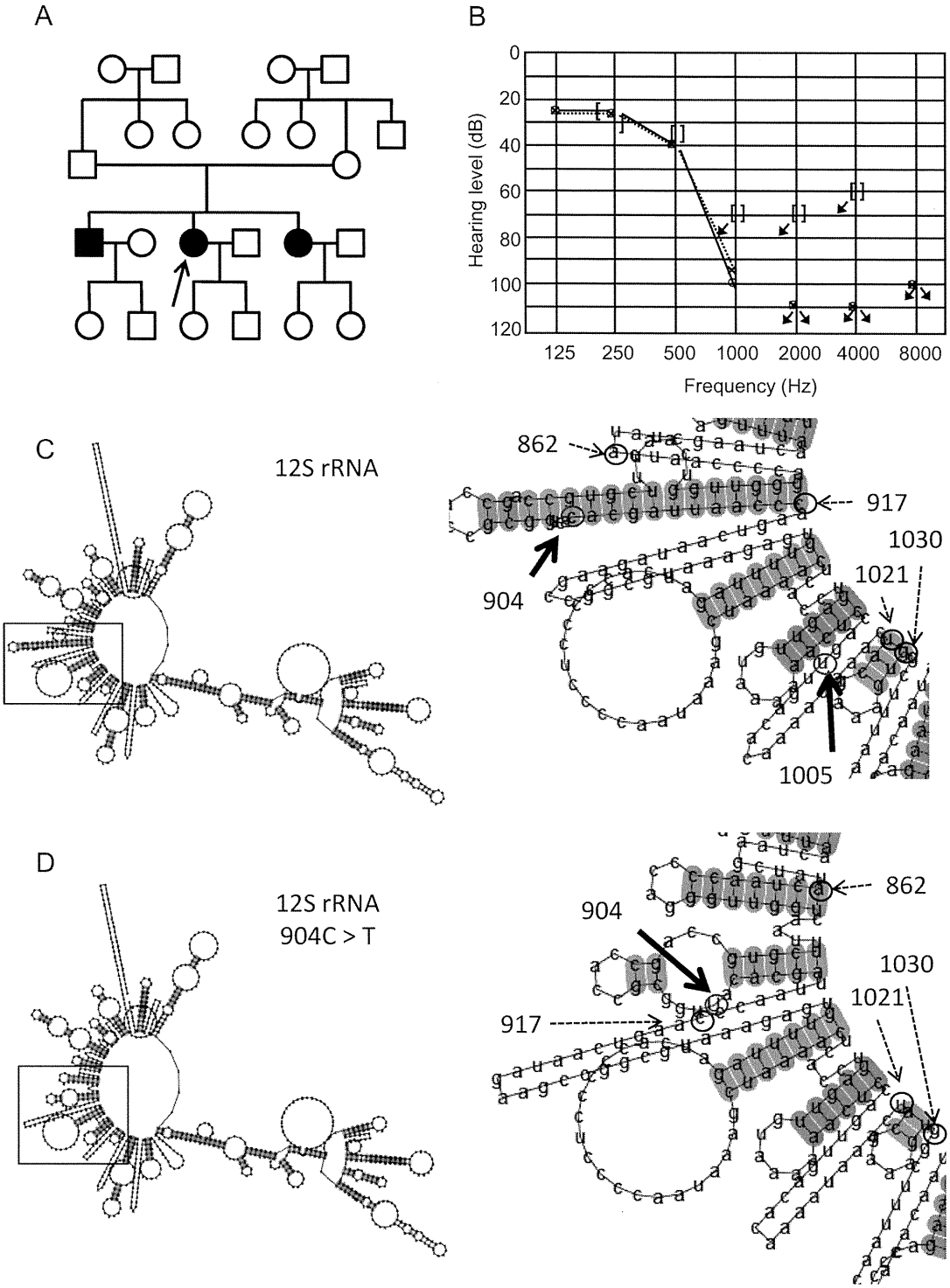

Figure 1 Pedigree of a family carrying the m.904C > T variant. (A) Pedigree of a family carrying the homoplasmic m.904C > T variant. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (B) Audiogram of the proband of m.904C > T. Open circles with the line indicate 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

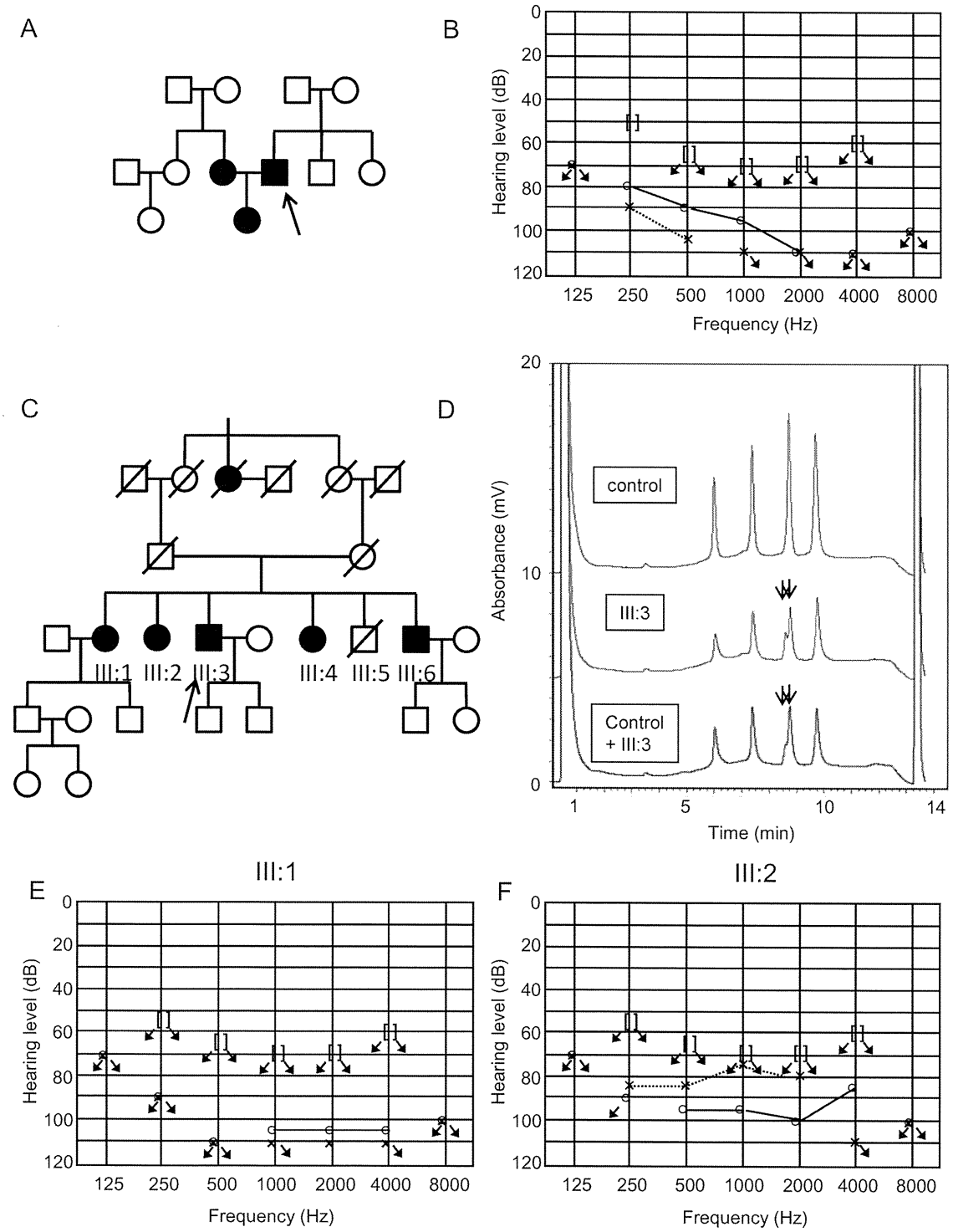

Figure 2 Pedigrees of families carrying the m.1005T > C variant. (A,B) Pedigree of a family carrying the homoplasmic m.1005T > C (A), and the audiogram of the proband (B). (C-F) Pedigree of a family carrying heteroplasmic m.1005T > C (C), and the chromatogram of dHPLC of the MT4 fragment of the proband (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).