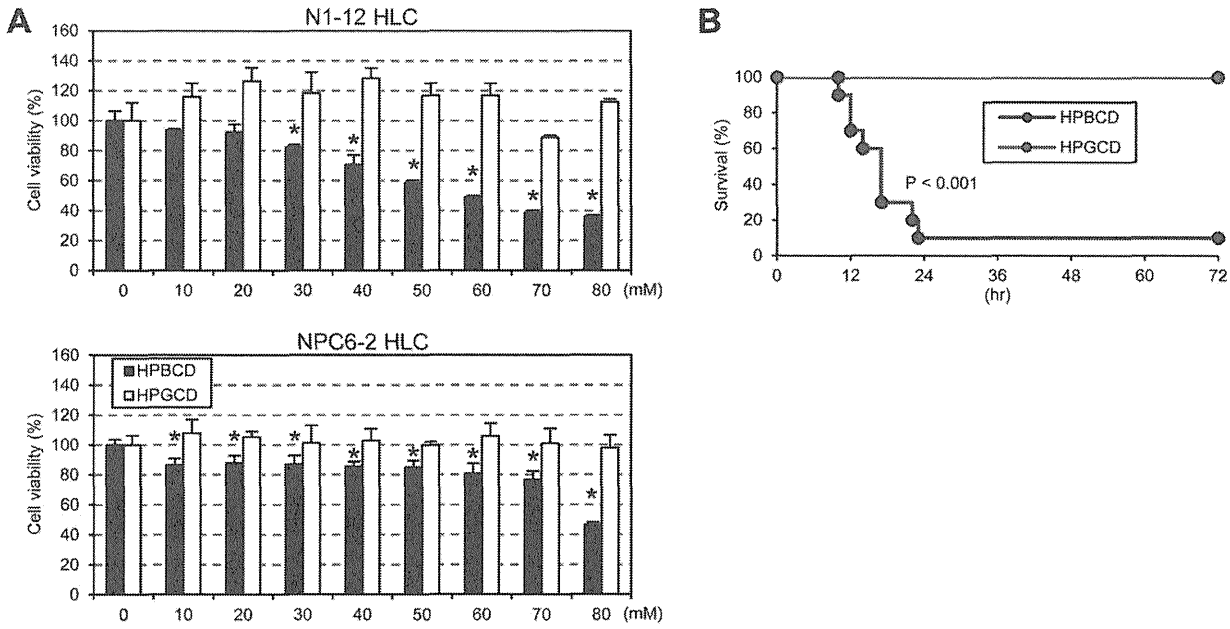

Figure 7. Toxicities of HPGCD are smaller than those of HPBCD. **(A):** In vitro cell toxicity testing of HLCs treated with HPBCD and HPGCD. Healthy donor- and NPC-derived iPSCs were induced into HLCs and the cell survival rates were determined with various HPBCD and HPGCD concentrations. Data are means ± SD of three independent experiments. *, $p < .01$, nontreatment versus treatment, Student's t test. **(B):** Acute toxicity test of normal mice given HPBCD and HPGCD injections. 14.4 mM HPBCD and HPGCD were injected into the subcutaneous tissues of 8-week-old mice ($n = 10$ each), and then survival rates were calculated. Almost all mice injected with HPBCD died up to 72 hours after the injection. In contrast, no mice died with the HPGCD injection. Abbreviations: HLC, hepatocyte-like cell; HPBCD, 2-hydroxypropyl-β-cyclodextrin; HPGCD, 2-hydroxypropyl-γ-cyclodextrin; NPC, Niemann-Pick disease type C.

accumulation in NPC-HLCs is consistent with these structural differences. Although both HPGCD and HPBCD treatments can reduce the cholesterol accumulation in HLCs and neural progenitors, some gene signatures altered with HPGCD treatment are shown to be different from those with HPBCD treatment. Both HPGCD and HPBCD treatments improved the abnormal molecular signatures in NPC-derived HLCs. In our system, Miglustat could not improve the cholesterol accumulation,

Figure 6. NPC model mice treated with HPGCD. **(A):** Injection schedule of HPGCD into NPC model mice. HPGCD was subcutaneously injected into NPC model mice once a week from 4 weeks to 8 weeks of age, and the mice treated with HPGCD were analyzed at 8.5 weeks old. Experiments were done twice (first; $n = 6$ each and second; $n = 4$ each). **(B):** Levels of AST and ALT in the serum of NPC model mice were markedly and significantly reduced with HPGCD treatment, compared to saline-treated mice. Data are means ± SD of two independent experiments (experiment 1: $n = 6$ each, experiment 2: $n = 4$ each). WT: wild type mice ($n = 4$), experiment 1 only. *, $p < .01$, saline versus HPGCD treatments, Student's t test. **(C):** Representative histological sections of liver from the NPC model mice with saline and HPGCD treatments. Liver histology was greatly improved with HPGCD treatment. Arrow heads indicate lipid-laden macrophages. Upper images: low magnification (×200), lower images: high magnification (×400). Scale bars = 50 μm. **(D):** Levels of cholesterol in the mice treated with HPGCD. The level of free cholesterol was markedly and significantly reduced in the livers of mice treated with HPGCD, compared to saline-treated controls (upper graph). Consistently, positive filipin staining was weaker in the HPGCD-treated mice than in saline-treated controls (lower images, representative data). Data are means ± SD of two independent experiments (Experiment 1: $n = 6$ each, Experiment 2: $n = 4$ each). *, $p < .01$, saline versus HPGCD treatments, Student's t test. Scale bars = 50 μm. **(E):** Representative histological sections of cerebellar vermis from the NPC model mice with HPGCD treatment. The Purkinje cells partially remained in HPGCD-treated mice (right images), comparing to saline-treated mice (left images). The sections were stained for calbindin immunoreactivity. The areas surrounded by the squares (upper images) are shown in the middle and lower images. Upper images: low magnification (×40), scale bars = 200 μm. Middle and lower images: high magnification (×200). Scale bars = 50 μm. **(F):** Abnormal autophagy was restored in NPC model mice with HPGCD treatment. The expression levels of LC3 and insoluble p62 in mice liver and brain were recovered to normal levels by treatments with HPGCD. The expression level was normalized to α-tubulin expression in each sample. The Western blot data (upper images) are summarized in the lower graphs. Data are means ± SD of four mice. #, $p < .05$; *, $p < .01$, saline versus HPGCD treatments, Student's t test. **(G):** Survival curve for mice with HPGCD treatment. HPGCD treatment prolonged the survival of NPC model mice. A total of 12 mice were injected with saline or HPGCD ($n = 6$ each) once a week from 4 weeks old. **(H):** Representative histological analyses of the major organs in the dead mice. A number of abnormal vacuoles were observed in the liver and kidney of saline-treated mice. In contrast, almost all organ histology did not exhibit marked abnormality in HPGCD-treated mice. HE staining. Scale bars: liver = 50 μm; lung = 100 μm; kidney = 50 μm; heart = 500 μm. P78, P102, P102: ages of dying or analyzing. **(I):** Representative histological sections of cerebellar vermis from the dead mice. Comparing to the heterozygous mouse (right images), the large defect of Purkinje cells was observed in both mice except for region X (left and center images). The sections were stained for calbindin immunoreactivity. Same mice as shown in Figure 6H. The areas surrounded by the squares (upper images) are shown in the middle and lower images. Upper images: low magnification (×40), scale bars = 200 μm. Middle and lower images: high magnification (×200). Scale bars = 50 μm. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HPGCD, 2-hydroxypropyl-γ-cyclodextrin; NPC, Niemann-Pick disease type C.

ATP level, and abnormal autophagy in HLCs and neural progenitors. These results suggest that HPGCD could remove the accumulation of cholesterol from NPC-derived HLCs by a different mechanism from that used by HPBCD and Miglustat, in that HPGCD and HPBCD treatments significantly altered different molecular signatures. The cluster analyses of genes in the abnormal signature revealed that HPGCD treatment could rescue the abnormal expression patterns of genes to a status closer to that of normal HLCs than HPBCD treatment. The result suggests that HPGCD treatment can correct the abnormal patterns of gene expression induced by NPC more effectively than HPBCD treatment.

We also demonstrated that the intermittent injections of HPGCD could partially rescue the liver injury typical of NPC model mice and prolong their survival. Consistently, the abnormal liver histology was also improved by HPGCD treatment, with an efficiency similar to that previously demonstrated for HPBCD treatment [44, 45]. However, we showed HPGCD to be safer than HPBCD in terms of acute toxicities in vitro and in vivo, and indeed, one of the most important requirements for drug candidates is no or acceptably low levels of intrinsic cytotoxicity. Additionally, our study revealed that high-dose treatments with HPGCD, but not HPBCD, had no effect on cell viability and mouse survival. Future in vitro lysis of isolated erythrocytes might provide a simple and reliable measure of cyclodextrin toxicity because the interaction between these drug molecules and the plasma membrane is the initial step any such cell damage. The hemolytic activity of the hydroxypropyl-cyclodextrins is reportedly in the order HPBCD > HPACD > HPGCD [43], and previous studies indicated that γ-cyclodextrin is safer than α- or β-cyclodextrin for acute intravenous administration in rats, in which the intravenous doses that are lethal to 50% of population (LD50 values) are 1,000, 788, and >3,750 mg/kg for α-, β-, and γ-cyclodextrins, respectively [46, 47]. These study findings are supported by all these previous results. In addition, HPGCD-based pharmaceutical products, such as eye drops, intravenous solutions, and intramuscular solutions, have been used clinically [48]. Therefore, although the safety data for HPGCD provided by this study are limited, our results indicated that HPGCD is a promising candidate for the treatment of NPC. Based on the results from the DNA array and safety examinations, HPGCD outperforms HPBCD as a potential treatment for NPC.

Disease-derived iPSCs generated using the SeV vector are highly suitable for studying disease because the vector never integrates into the host genome, and herein, we have thus established a novel cellular model of NPC using transgene-free iPSCs, and have revealed new drug candidate for the future therapy. Biosamples collected from NPC patients are essential for studying the mechanisms of diseases and developing new therapies, but tissue collection is difficult because of the limited numbers of patients and the inaccessibility of target cells such as liver and neural cells. The transgene-free iPSC lines developed in this study will become indispensable tools for facilitating the development of new and much-awaited therapeutic agents for treating NPC patients.

Conclusions

In summary, the disease model of NPC-derived iPSC lines is useful for studying the pathological events and HPGCD is a potential candidate as a future drug for NPC.

Acknowledgments

We would like to thank Toyoko Tsuru, Yumi Soejima, and Kousaku Ohno for providing NPC model mice and technical support. This study was supported in part by grants from the Ministry of Health, Labor, and Welfare of Japan, Precursory Research for Embryonic Science and Technology (PRESTO), Core Research for Evolutional Science and Technology (CREST), and Japan Science and Technology Agency (JST). N.F. is currently affiliated with the Department of Ophthalmology, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160–8582, Japan. H.F. is currently affiliated with the Department of Neurology, Kochi Medical School, Okatoyo-machi, Nangoku city, Kochi 783-8505, Japan.

Author Contributions

T.E.: designed all experiments, wrote and prepared the manuscript, and edited the manuscript; T.I.: designed the in vivo experiment involving HPGCD injections, provided the chemicals, and edited the manuscript; Y.I., M.S., M.H., K.Y., and N.N.: performed the experiments and analyzed data and edited the manuscript; N.F.: designed, produced, and provided the Sendai virus vectors and edited the manuscript; H.F., H.I., M.M., K.N., and F.E.: provided the patient samples and data and edited the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872.

2 Yu J, Vodyanik MA, Smuga-Otto K et al. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318:1917–1920.

3 Avilion AA, Nicolis SK, Pevny LH et al. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 2003;17:126–140.

4 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–676.

5 Nichols J, Zevnik B, Anastassiadis K et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 1998;95:379–391.

6 Seki T, Yuasa S, Oda M et al. Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. Cell Stem Cell 2010;7:11–14.

7 Ban H, Nishishita N, Fusaki N et al. Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors. Proc Natl Acad Sci USA 2011;108:14234–14239.

8 Ebert AD, Yu J, Rose FF, Jr. et al. Induced pluripotent stem cells from a spinal muscular atrophy patient. Nature 2009;457:277–280.

9 Marchetto MC, Carromeu C, Acab A et al. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell 2010;143:527–539.

10 Liu GH, Suzuki K, Qu J et al. Targeted gene correction of laminopathy-associated LMNA mutations in patient-specific iPSCs. Cell Stem Cell 2011;8:688–694.
11 Yusa K, Rashid ST, Strick-Marchand H et al. Targeted gene correction of alpha1-antitrypsin deficiency in induced pluripotent stem cells. Nature 2011;478:391–394.
12 Tanaka A, Woltjen K, Miyake K et al. Efficient and reproducible myogenic differentiation from human iPS cells: Prospects for modeling Miyoshi Myopathy in vitro. PLoS One 2013;8:e61540.
13 Tulpule A, Kelley JM, Lensch MW et al. Pluripotent stem cell models of Shwachman-Diamond syndrome reveal a common mechanism for pancreatic and hematopoietic dysfunction. Cell Stem Cell 2013;12:727–736.
14 Hamasaki M, Hashizume Y, Yamada Y et al. Pathogenic mutation of ALK2 inhibits induced pluripotent stem cell reprogramming and maintenance: Mechanisms of reprogramming and strategy for drug identification. Stem Cells 2012;30:2437–2449.
15 Kondo T, Asai M, Tsukita K et al. Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular Abeta and differential drug responsiveness. Cell Stem Cell 2013;12:487–496.
16 Carstea ED, Morris JA, Coleman KG et al. Niemann-Pick C1 disease gene: Homology to mediators of cholesterol homeostasis. Science 1997;277:228–231.
17 Naureckiene S, Sleat DE, Lackland H et al. Identification of HE1 as the second gene of Niemann-Pick C disease. Science 2000;290:2298–2301.
18 Vanier MT. Niemann-Pick disease type C. Orphanet J Rare Dis 2010;5:16.
19 Heron B, Valayannopoulos V, Baruteau J et al. Miglustat therapy in the French cohort of paediatric patients with Niemann-Pick disease type C. Orphanet J Rare Dis 2012;7:36.
20 Fusaki N, Ban H, Nishiyama A et al. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci 2009;85:348–362.
21 Tada S, Era T, Furusawa C et al. Characterization of mesendoderm: A diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. Development 2005;132:4363–4374.
22 Yasunaga M, Tada S, Torikai-Nishikawa S et al. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. Nat Biotechnol 2005;23:1542–1550.
23 Macho A, Decaudin D, Castedo M et al. Chloromethyl-X-Rosamine is an aldehyde-fixable potential-sensitive fluorochrome for the detection of early apoptosis. Cytometry 1996;25:333–340.
24 Smiley ST, ReersM, Mottola-Hartshorn C et al. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. Proc Natl Acad Sci USA 1991;88:3671–3675.
25 Lieberman AP, Puertollano R, Raben N et al. Autophagy in lysosomal storage disorders. Autophagy 2012;8:719–730.
26 Kabeya Y, Mizushima N, Ueno T et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000;19:5720–5728.
27 Komatsu M, Waguri S, Koike M et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 2007;131:1149–1163.
28 Camargo F, Erickson RP, Garver WS et al. Cyclodextrins in the treatment of a mouse model of Niemann-Pick C disease. Life Sci 2001;70:131–142.
29 Group N-CGW, Wraith JE, Baumgartner MR et al. Recommendations on the diagnosis and management of Niemann-Pick disease type C. Mol Genet Metab 2009;98:152–165.
30 Patterson MC, Vecchio D, Prady H et al. Miglustat for treatment of Niemann-Pick C disease: A randomised controlled study. Lancet Neurol 2007;6:765–772.
31 Platt FM, Neises GR, Dwek RA et al. N-Butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis. J Biol Chem 1994;269:8362–8365.
32 Loftus SK, Morris JA, Carstea ED et al. Murine model of Niemann-Pick C disease: Mutation in a cholesterol homeostasis gene. Science 1997;277:232–235.
33 Yu W, Gong JS, Ko M et al. Altered cholesterol metabolism in Niemann-Pick type C1 mouse brains affects mitochondrial function. J Biol Chem 2005;280:11731–11739.
34 Cluzeau CV, Watkins-Chow DE, Fu R et al. Microarray expression analysis and identification of serum biomarkers for Niemann-Pick disease, type C1. Hum Mol Genet 2012;21:3632–3646.
35 Pacheco CD, Elrick MJ, Lieberman AP. Tau deletion exacerbates the phenotype of Niemann-Pick type C mice and implicates autophagy in pathogenesis. Hum Mol Genet 2009;18:956–965.
36 Pacheco CD, Kunkel R, Lieberman AP. Autophagy in Niemann-Pick C disease is dependent upon Beclin-1 and responsive to lipid trafficking defects. Hum Mol Genet 2007;16:1495–1503.
37 Liao G, Wen Z, Irizarry K et al. Abnormal gene expression in cerebellum of Npc1-/- mice during postnatal development. Brain Res 2010;1325:128–140.
38 Davidson CD, Ali NF, Micsenyi MC et al. Chronic cyclodextrin treatment of murine Niemann-Pick C disease ameliorates neuronal cholesterol and glycosphingolipid storage and disease progression. PLoS One 2009;4:e6951.
39 Liu B, Li H, Repa JJ et al. Genetic variations and treatments that affect the lifespan of the NPC1 mouse. J Lipid Res 2008;49:663–669.
40 Rosenbaum AI, Zhang G, Warren JD et al. Endocytosis of beta-cyclodextrins is responsible for cholesterol reduction in Niemann-Pick type C mutant cells. Proc Natl Acad Sci USA 2010;107:5477–5482.
41 Pontikis CC, Davidson CD, Walkley SU et al. Cyclodextrin alleviates neuronal storage of cholesterol in Niemann-Pick C disease without evidence of detectable blood-brain barrier permeability. J Inher Metab Dis 2013;36:491–498.
42 Uekama K, Hirayama F, Irie T. Cyclodextrin drug carrier systems. Chem Rev 1998;98:2045–2076.
43 Irie T, Uekama K. Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation. J Pharm Sci 1997;86:147–162.
44 Ramirez CM, Liu B, Taylor AM et al. Weekly cyclodextrin administration normalizes cholesterol metabolism in nearly every organ of the Niemann-Pick type C1 mouse and markedly prolongs life. Pediatr Res 2010;68:309–315.
45 Liu B, Turley SD, Burns DK et al. Reversal of defective lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the npc1-/- mouse. Proc Natl Acad Sci USA 2009;106:2377–2382.
46 Frank DW, Gray JE, Weaver RN. Cyclodextrin nephrosis in the rat. Am J Pathol 1976;83:367–382.
47 Schmid G. Preparation and application of γ-cyclodextrin. In: Duchene D, ed. New Trends in Cyclodextrins and Derivatives. Paris: Editions de Sante, 1991:27–54.
48 Arima H, Motoyama K, Irie T. Recent findings on safety profiles of cyclodextrins, cyclodextrin conjugates and polypseudorotaxanes. In: Bilensoy E, ed. Cyclodextrins in Pharmaceutics, Cosmetics and Biomedicine: Current and Future Industrial Applications. John Wiley & Sons, USA 2011:91–122.


See www.StemCells.com for supporting information available online.

PEDIATRICS INTERNATIONAL Official Journal of the Japan Pediatric Society

Pediatrics International (2015) **57**, 37–40 doi: 10.1111/ped.12550

Review Article

Diagnosis and treatment of hereditary tyrosinemia in Japan

Kimitoshi Nakamura,[1] Shirou Matsumoto,[1] Hiroshi Mitsubuchi[2] and Fumio Endo[1]

Departments of [1]Pediatrics and [2]Neonatology, Kumamoto University Hospital, Kumamoto, Japan

Abstract Hereditary tyrosinemia is an autosomal recessive inherited disease that manifests as three types (types I–III). We conducted a nationwide survey of this disease in Japan, and here review the results in relation to prevalence, clinical characteristics, and treatment and diagnosis. A definitive diagnosis of tyrosinemia type I is difficult to obtain based only on blood tyrosine level. Detection of succinylacetone using dried blood spots or urinary organic acid analysis, however, is useful for diagnosis. In tyrosinemia type I, dietary therapy and nitisinone (Orfandin®) are effective. Prognosis is greatly affected by the complications of liver cancer and hypophosphatemic rickets; even patients that are treated early with nitisinone may develop liver cancer. Long-term survival can be expected in type I if nitisinone therapy is effective. Prognosis in types II and III is relatively good.

Key words liver cancer, liver transplantation, nitisinone, succinylacetone, tyrosine.

Tyrosine is an amino acid contained in food and is also obtained internally as a metabolite of phenylalanine. In the body, phenylalanine transforms into tyrosine via phenylalanine hydroxylase. Tyrosine breaks down into 4-OH-phenylpyruvate via tyrosine aminotransferase (TAT), then to homogentisate via 4-OH-phenylpyruvate dioxygenase (HPD), which breaks down to maleylacetoacetate via homogentisate dioxygenase and then to fumarylacetoacetate hydrolase (FAH) via maleylacetoacetase, and finally to fumarate and acetoacetate via FAH (Fig. 1).[1] Tyrosinemia results from elevated tyrosine in the blood, and although all hereditary forms show autosomal recessive inheritance, the causes are variable, and hereditary tyrosinemia is therefore genetically and clinically differentiated into three types: type I, type II, and type III (Table 1).[1,2] These diseases differ genetically and enzymatically, and clinical manifestations occur via different mechanisms.

Hereditary tyrosinemia type I (MIM276700) occurs from a defect of FAH (EC 3.7.1.2), type II (MIM276600) occurs from a defect of cytosol TAT (EC2.6.1.5), and type III (MIM276710) occurs from a defect of HPD (EC1.13.11.27). Hawkinsinuria is an autosomal dominant hereditary disease that also occurs due to HPD abnormality.[3] In the hawkinsinuria patient, the residual activity of HPD forms 1,2-epoxyphenyl acetic acid, which reacts with glutathione to produce hawkinsin (2-L-cystein-S-yl-1,4-dihydroxy-cyclohex-5-en-1-yl acetic acid).

We performed a nationwide survey of hereditary tyrosinemia in Japan. In the survey, we found that it is difficult to make a definitive diagnosis of tyrosinemia type I based only on blood tyrosine level. Detection of succinylacetone using dried blood spots or urinary organic acid analysis, however, is useful for diagnosis. Dietary therapy and nitisinone (Orfandin®) are effective therapies for tyrosinemia type I.

This study was approved by the ethics committee of the Faculty of Life Science, Kumamoto University. Briefly, we sent a questionnaire to 928 Japanese institutions, including the departments of pediatrics, endocrinology and metabolism, neonatology, genetics, and transplant surgery, asking doctors if they had diagnosed or provided medical care to tyrosinemia patients. Each institution was the medical center for a locality and had ≥300. Of the 928 institutions, 659 (71%) responded. Of these 659 institutions, 15 had treated patients with tyrosinemia.

Pathophysiology and epidemiology

In tyrosinemia type I, several pathologies occur due to the toxicity of fumarylacetoacetate, which builds up in cells due to the enzyme defect of FAH.[4–7] The defect results in abnormalities of gene expression, inhibited enzyme activity, apoptosis, chromosomal instability, and carcinogenesis in hepatocytes. In particular, decreased gene expression in patients with tyrosinemia type I can result in conditions such as hypoglycemia, metabolic disorders of amino acids, and reduction in coagulation factors. Chromosomal instability leads to a high incidence of juvenile liver cancer, and hepatocyte death due to apoptosis can cause progression to liver failure. Cellular damage also appears in the proximal renal tubules, causing Fanconi syndrome, including aminoaciduria, glycosuria, and metabolic acidosis, which ultimately result in hypophosphatemic rickets.

In tyrosinemia type II, clinical manifestations occur due to high concentrations of tyrosine in the body fluids.[8] Some cases are linked to the low solubility of tyrosine. The temperature of the skin and cornea can decrease more readily than in other areas, which could result in tyrosine crystal deposition leading to cellular damage. Delayed cognitive development is also often seen in cases of tyrosinemia type II. Although this is thought to be

Correspondence: Kimitoshi Nakamura, MD PhD, Department of Pediatrics, Kumamoto University Hospital, Honjo 1-1-1, Chuo-ku, Kumamoto 860-8556, Japan. Email: nakamura@kumamoto-u.ac.jp

Received 27 October 2014; accepted 19 November 2014.

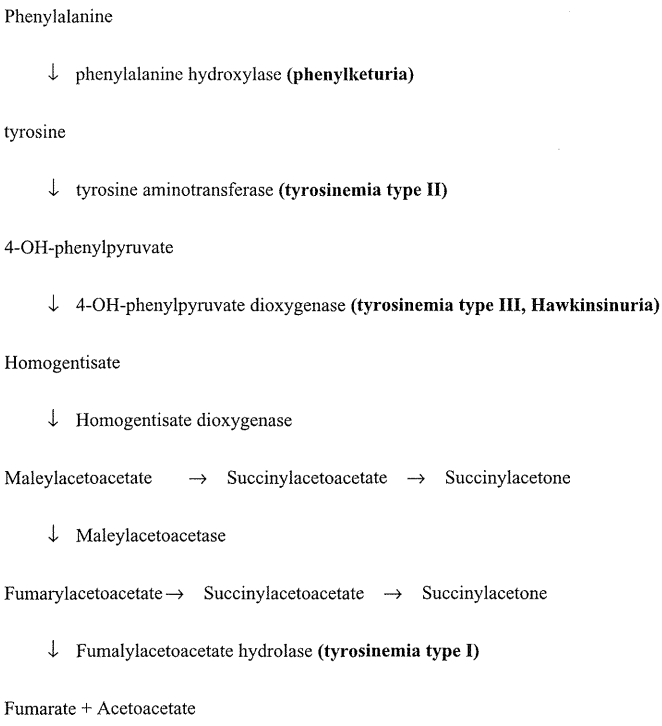

Fig. 1 Tyrosine metabolism: the metabolic pathway from phenylalanine through tyrosine to fumarate and acetoacetate. Tyrosinemia type I occurs due to a deficit of fumarylacetoacetate hydrolase, type II occurs from a deficit of tyrosine aminotransferase, and type III occurs from a deficit of 4-OH-phenylpyruvate dioxygenase.

linked to elevated tyrosine in the blood, the detailed mechanism has not been clarified. Moreover, large amounts of 4-OH-phenylpyruvate, the product of tyrosine transamination, and its oxides are excreted in urine.[9] This is due to the aspartate aminotransferase converting tyrosine to 4-OH-ohenylpyruvate when it is present at high concentration.

In tyrosinemia type III, the levels of tyrosine and 4-OH-phenylpyruvate, the α-keto acid of tyrosine, are increased. In addition, 4-OH-phenylpyruvate and its oxides excreted in the urine are markedly elevated.

We conducted a nationwide survey of hereditary tyrosinemia (71% response rate) in Japan, and received responses regarding five cases of type I, two cases of type II, and one case of type III. Responses were also received regarding 10 cases of tyrosinemia due to an unknown cause, which may be due to transient neonatal hepatitis.[10] Given that some hereditary tyrosinemia type III patients can be asymptomatic, we believe that there are more patients with tyrosinemia type III than were discovered through the survey. The prevalence of hereditary tyrosinemia type I in Europe is reported to be 1 in 125 000 people;[8] therefore, the incidence in Japan is clearly lower. Elevated tyrosine in the blood can be detected using tandem mass spectrometry, but because blood tyrosine can be high in newborns for a variety of reasons, it is difficult to identify true cases of hereditary tyrosinemia. For this reason, tyrosinemia is currently not a component of routine newborn screening. Screening of newborns for tyrosinemia, however, could be possible by measuring succinylacetone in the blood.[11] Nonetheless, there is a wide variety of genetic and biochemical disease groups that present with tyrosinemia. In the present survey, elevated tyrosine (>440 μmol/L; >8 mg/dL) was observed in 0.85% of newborns. In Europe, 0.5–1.8% of newborns have high tyrosine, indicating that it is a relatively frequent abnormality,[1] but, because hereditary tyrosinemia is only very rarely encountered, the diagnosis must be made on the basis of its characteristic clinical manifestations and specific tests.

Clinical manifestations

Tyrosinemia type I is clinically characterized by progressive liver dysfunction and renal tubule damage.[1,2] There are three subtypes of liver dysfunction: acute, subacute, and chronic. The acute type begins several weeks after birth with hepatomegaly, poor development, diarrhea, vomiting, and jaundice. Severe cases of dysfunction progress to liver failure; if left untreated, death occurs 2–3 months after birth. The subacute type manifests as liver dysfunction from several months to approximately 1 year after birth. In the chronic type, liver dysfunction progresses slowly, but can eventually result in cirrhosis or liver failure. Liver cancer occurs in many cases, and multiple tumors have been reported in such cases. With respect to the kidneys, renal tubular dysfunction can result in diseases such as hypophosphatemic rickets and

Table 1 Classification of tyrosinemia

Disease	Inheritance	Serum tyrosine elevation	Enzyme	Clinical manifestations
Type I	AR	Mild, >4 mg/dL (220 μmol/L)	Fumarylacetoacetate hydrolase	Liver failure, hypoglycemia, renal tubular disorder, galactosemia, neuropathy, hepatocellular carcinoma
Type II	AR	Very high, >20 mg/dL (1100 μmol/L)	Tyrosine aminotransferase	Mental retardation, abnormal keratinization, corneal ulcer
Type III	AR	Moderate, >10 mg/dL (550 μmol/L)	4-OH-phenylpyruvate dioxygenase	Ataxia, convulsion, mental retardation (mild)
Hawkinsinuria	AD	Transient	4-OH-phenylpyruvate dioxygenase	Growth retardation, appetite loss
Secondary tyrosinemia	–	Variable		Dependent on symptoms of the primary disease
Neonatal transient tyrosinemia	–	Variable		Asymptomatic

AD, autosomal dominant; AR, autosomal recessive.

vitamin D-resistant rickets. Moreover, succinylacetone inhibits aminolevulinate dehydratase, which causes bouts of abdominal pain, polyneuropathy, and other manifestations resembling acute intermittent porphyria.

Tyrosinemia type II involves higher blood tyrosine level than types I and III, but does not exhibit the liver and kidney dysfunction found in type I.[1,8] The skin lesions that appear in type II are due to deposition of needle-like tyrosine crystals, which cause excess keratinization and erosion that is limited to the palms and soles. Deposits of tyrosine crystals also appear on the cornea, leading to corneal erosion and ulceration. Corneal changes appear several months after birth, earlier than the skin manifestations, although in some cases clear manifestations do not appear until adolescence or later. Delayed mental development is observed in some cases of particularly high blood tyrosine concentration.

Manifestations in tyrosinemia type III are milder than those in types I and II, and some cases are asymptomatic.[1,3] Manifestations including ataxia, spasms, and mildly delayed cognitive development have been reported. These do not appear in types I or II, and may be linked to an increase in 4-OH-phenylpyruvate in body fluids. Many cases are diagnosed when these manifestations appear, although it is thought that some cases are asymptomatic and are likely undiagnosed.

Laboratory studies

Besides tyrosinemia types I, II, and III, high blood tyrosine can occur due to other causes, making differentiation and accurate diagnosis necessary.[1,10] The liver dysfunction associated with type I is particularly important for differential diagnosis. For differentiating between types II and III, transient tyrosinemia in newborns can be problematic. Blood amino acid analysis and tandem mass spectrometry are useful tools for monitoring blood tyrosine.

The presence or absence of liver dysfunction is important for diagnosing hereditary tyrosinemia type I. Liver dysfunction causes serum transaminase to rise, coagulation factor synthesis to decrease, and a reduction in albumin and cholinesterase.[1,8] Renal tubular dysfunction can cause hypophosphatemia, glycosuria, and proteinuria. An increase in α-fetoprotein is also a characteristic marker of type I. Blood amino acid analysis commonly indicates elevated levels of many amino acids, including tyrosine (>220 μmol/L, 4 mg/dL), methionine (>130 μmol/L, 2 mg/dL), and threonine (>170 μmol/L, 2 mg/dL). In urinary amino acid analysis, there is increased excretion of tyrosine and many other amino acids. Disorders of porphyrin metabolism result in an increase in δ-aminolevulinic acid in the urine. Hepatomegaly, cirrhosis, and fatty liver are common observations on imaging. Findings including abnormal liver tissue structure, abnormal hepatocyte morphology, and fatty liver are seen on liver biopsy, but these are non-specific, and are therefore not useful to make a definitive diagnosis. A definitive diagnosis requires the confirmation of increased succinylacetone in an analysis of urinary organic acids (Fig. 2). Elevated levels of the tyrosine metabolites 4-OH-phenylpyruvate, 4-OH-phenylacetate, and 4-OH-phenyllactate are also observed in urinary organic acid analysis. Enzyme diagnosis can be done by measuring hydrolase activity in samples of hepatocytes and cultured skin fibroblasts.

Skin and eye findings can be used to suggest hereditary tyrosinemia type II.[8] Tyrosine is extremely high in blood amino acid analysis, at ≥1100 μmol/L (20 mg/dL). Further, large amounts of 4-OH-phenylpyruvate, 4-OH-phenylacetate, and

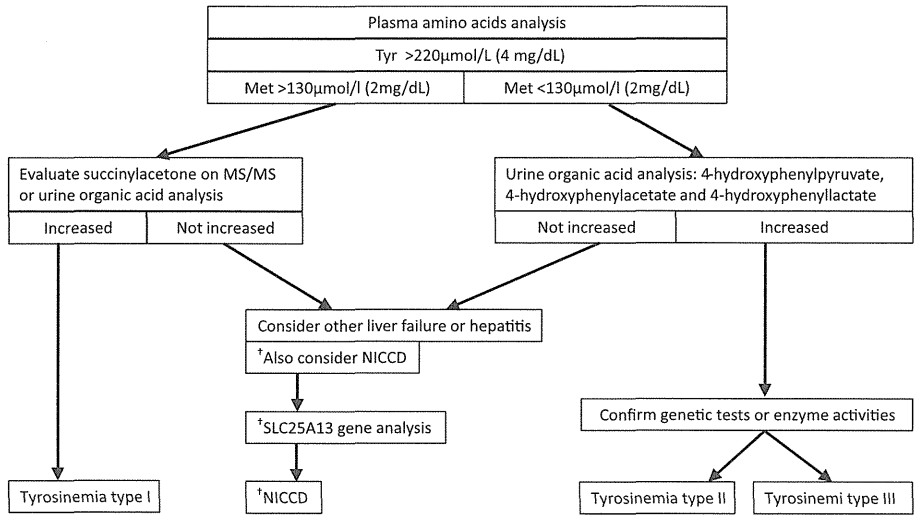

Fig. 2 Diagnosis of hereditary tyrosinemia. This algorithm can be used for differentiating among types of tyrosinemia. Increased succinylacetone in the blood or urine is used to diagnose tyrosinemia type I. Types II and III are diagnosed from elevated 4-OH-phenylpyruvate, 4-OH-phenylacetate, and 4-OH-phenyllactate, which are not accompanied by elevated succinylacetone on urinary organic acid analysis. It is generally easy to differentiate types II and III based on their clinical manifestations. When distinguishing between them is difficult, enzyme activity should be measured or genetic analysis should be performed. †If the disease is thought to be something other than hereditary tyrosinemia, it needs to be differentiated from neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). NICCD is diagnosed by analyzing *SLC25A*. MS/MS, tandem mass spectroscopy.

4-OH-phenyllactate are found on urinary organic acid analysis. Liver biopsy is required to measure specific enzyme activity. In addition, there are two forms of TAT: sTAT and mTAT, the former existing in the soluble fraction, and the latter localized to the mitochondria. Patients with type II tyrosinemia lack sTAT; therefore, these enzymes need to be measured separately during enzyme diagnosis.

Hereditary tyrosinemia type III does not have characteristic clinical manifestations.[1,3] For blood amino acids, tyrosine rises to 550–1100 μmol/L (10–20 mg/dL), and large amounts of 4-hydroxyphenylpyruvate and its oxides are detected in the urine. Liver enzymes can be measured for a definitive diagnosis. Hawkinsinuria, a mild form of type III, is diagnosed by detecting hawkinsin in the urine.

Treatment

When a patient has high tyrosine, it should be determined whether it is type I, type II, type III, or whether the tyrosinemia is due to some other cause, while simultaneously administering symptomatic treatment. In the absence of organ damage, newborns with high tyrosine are usually placed under observation. It is important to act quickly to prevent liver dysfunction from progressing in type I, for which the HPD inhibitor nitisinone (Orfandin®; 1 mg/kg/day) is used along with dietary therapy (formula without phenylalanine and tyrosine).[12] If nitisinone is effective, succinylacetone will decline to below the measurement sensitivity level, but tyrosine will rise. Thus, dietary therapy should aim to keep tyrosine at or below 500 μmol/L (9 mg/dL).[13] A formula without phenylalanine and tyrosine is available to help reduce the blood tyrosine level. Approximately 90% of patients are reported to respond to nitisinone if treatment is initiated early. The therapeutic effect is evaluated on liver function tests and by measuring serum α-fetoprotein. A positive prognosis can be expected if serum α-fetoprotein is maintained within the normal range (<10 ng/dL). Liver failure occurs in many patients who do not receive nitisinone, necessitating liver transplantation. Yet, in some cases, patients who are given nitisinone can nonetheless develop liver cancer or require a liver transplantation.

In type II, the aim of treatment is to reduce tyrosine in the blood, which will improve the skin and eye manifestations. To achieve this, patients are placed on a diet low in phenylalanine and tyrosine to maintain blood tyrosine <550 μmol/L (10 mg/dL). Formula without phenylalanine and tyrosine is recommended to reduce the blood tyrosine level. Similarly, type III patients are placed on a diet low in phenylalanine and tyrosine.

Complications and prognosis

In type I, prognosis is greatly affected by complicating liver cancer and hypophosphatemic rickets, which may result even in cases of early nitisinone treatment. Liver cancer was reported to occur within 10 years in 5% of children who began treatment at or before 2 years old.[13] Thus, regular imaging and other examinations should be performed during treatment so that liver cancer can be discovered early. Long-term survival can be expected for patients with type I if nitisinone treatment is effective. Types II and III have relatively good prognoses.

Conclusion

Although there are many causes of tyrosinemia, hereditary tyrosinemia can be diagnosed from clinical symptoms and biochemical analysis. Medical treatment requires nitisinone for type I, and formula without tyrosine and phenylalanine for types I, II and III. Early diagnosis is necessary for better prognosis.

Acknowledgments

The work was supported by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan. We thank Dr Torayuki Okuyama and Dr Mureo Kawahara of the National Center for Child Health and Development, Dr Michinori Ito of Kagawa National Children's Hospital, and Dr Go Tajima of Hiroshima University for useful discussions and information. The authors declare no conflicts of interest.

References

1 Mitchell GA, Grompe M, Lambert M *et al.* Hypertyrosinemia. In: Scriver CR, Beaudet AL, Sly WS *et al* (eds). *The Metabolic and Molecular Basis of Inherited Metabolic Disease*, 8th edn. McGraw Hill, New York, 2001; 1777–805.
2 Nakamura K, Mitsubuchi H, Endo F *et al.* Animal model of tyrosinemia. *J. Nutrition* 2007; **137**: 1573S–5S.
3 Tomoeda K, Awata H, Matsuura T *et al.* Mutations in the 4-hydroxyphenylpyruvic acid dioxygenase gene are responsible for tyrosinemia type III and hawkinsinuria. *Mol. Genet. Metab.* 2000; **71**: 506–10.
4 Kelsey G, Ruppert S, Beermann F *et al.* Rescue of mice homozygous for lethal albino deletions: Implications for an animal model for the human liver disease tyrosinemia type I. *Genes Dev.* 1993; **7**: 2285–97.
5 Grompe M, Al-Dhalimy M, Finegold M *et al.* Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. *Genes Dev.* 1993; **7**: 2298–307.
6 Endo F, Kubo S, Awata H *et al.* Complete rescue of lethal albino c14Cos mice by null mutation of 4-hydroxyphenylpyruvate dioxygenase and induction of apoptosis of hepatocytes in these mice by in vivo retrieval of the tyrosine catabolic pathway. *J. Biol. Chem.* 1997; **272**: 24426–32.
7 Kubo S, Sun M, Miyahara M *et al.* Hepatocyte injury in tyrosinemia type I is induced by fumarylacetoacetate and is inhibited by caspase inhibitors. *Proc. Natl Acad. Sci. USA* 1998; **95**: 9552–7.
8 Meissner T, Betz RC, Pasternack SM *et al.* Richner–Hanhart syndrome detected by expanded newborn screening. *Pediatr. Dermatol.* 2008; **25**: 378–80.
9 Fois A, Borgogni P, Cioni M *et al.* Presentation of the data of the Italian registry for oculocutaneous tyrosinaemia. *J. Inherit. Metab. Dis.* 1986; **9**: 262–4.
10 Rice DN, Houston IB, Lyon IC *et al.* Transient neonatal tyrosinaemia. *J. Inherit. Metab. Dis.* 1989; **12**: 13–22.
11 Mayorandan S, Meyer U, Gokcay G *et al.* Cross-sectional study of 168 patients with hepatorenal tyrosinaemia and implications for clinical practice. *Orphanet J. Rare Dis.* 2014; **9**: 107.
12 Lindstedt S, Holme E, Loch EA *et al.* Treatment of hereditary tyrosinemia type I by inhibition of 4-hydroxyphenylpyruvate dioxygenase. *Lancet* 1992; **340**: 813–17.
13 Scott CR. The genetic tyrosinemias. *Am. J. Med. Genet. C* 2006; **142C**: 121–6.

INTERNAL MEDICINE

□ CASE REPORT □

A Sporadic Case of Fabry Disease Involving Repeated Fever, Psychiatric Symptoms, Headache, and Ischemic Stroke in an Adult Japanese Woman

Jun Sawada[1], Takayuki Katayama[1], Kohei Kano[1], Asuka Asanome[1], Kae Takahashi[1], Tsukasa Saito[1], Junko Chinda[1], Naoki Nakagawa[1], Nobuyuki Sato[1], Takashi Kimura[2], Osamu Yahara[2], Ken Momosaki[3], Kimitoshi Nakamura[3] and Naoyuki Hasebe[1]

Abstract

Fabry disease can cause various neurological manifestations. We describe the case of a Japanese woman with Fabry disease who presented with ischemic stroke, aseptic meningitis, and psychiatric symptoms. The patient had a mutation in intron 4 of her α-galactosidase A gene, which was not detected in her family. This case suggests that Fabry disease should be considered in young patients who exhibit central nervous system symptoms such as ischemic stroke, even if there is no family history of the condition. The episodes of aseptic meningitis and stroke experienced by our patient suggest that persistent inflammation might be the mechanism underlying Fabry disease.

Key words: Fabry disease, ischemic stroke, aseptic meningitis, psychiatric symptom, chronic inflammation, sporadic

(Intern Med 54: 3069-3074, 2015)
(DOI: 10.2169/internalmedicine.54.4719)

Introduction

Fabry disease is an X-linked multisystem lysosomal storage disorder caused by mutations in the α-galactosidase A (GAL) gene, which is located at Xq22 (1). It results in the progressive accumulation of neutral glycosphingolipids, mainly globotriaosylceramide, in various organs. The clinical symptoms of Fabry disease include renal dysfunction, cardiac disease (especially left ventricular hypertrophy), cutaneous angiokeratomas, corneal dystrophy, hypohidrosis, gastrointestinal conditions, neuropathic pain, and neurological disorders (2). Symptoms of the disease are seen in both hemizygous males and heterozygous females (3, 4). We herein report a sporadic case of Fabry disease involving aseptic meningitis, ischemic stroke, and psychiatric symptoms in an adult Japanese woman.

Case Report

The patient was a Japanese woman who had been aware of limb pain from the ages of 9 to 15 and who had experienced a hallucinatory/delusional state after the delivery of her daughter at the age of 27. Occasional fevers of unknown origin subsequently emerged. She was admitted to a psychiatric hospital after exhibiting delusional and violent behavior at the age of 29. She received psychological treatment, but her delusions persisted. She was re-admitted to another psychiatric hospital due to the exacerbation of her psychiatric symptoms at the age of 39. Her psychiatric symptoms improved after electroconvulsive therapy, but she subsequently developed a high fever, a throbbing headache, and dizziness. No nausea, vomiting, or nuchal stiffness was observed. Her Mini-Mental State Examination score was 20 (date: -1,

[1]Division of Cardiology, Nephrology, Pulmonology, and Neurology, Department of Internal Medicine, Asahikawa Medical University, Japan, [2]Department of Neurology, National Hospital Organization Asahikawa Medical Center, Japan and [3]Department of Pediatrics, Graduate School of Medical Science, Kumamoto University, Japan
Received for publication December 17, 2014; Accepted for publication April 5, 2015
Correspondence to Dr. Jun Sawada, sawajun@asahikawa-med.ac.jp

Table. Laboratory Findings.

Age at time of the assessment		39	42
Urinalysis			
Protein		negative	negative
Occult blood		negative	negative
Peripheral blood			
White blood cells	(/μL)	8,100	6,390
Red blood cells	($\times10^4$/μL)	281	427
Hemoglobin	(g/dL)	8.2	12.6
Platelet count	($\times10^4$/μL)	48.4	39.6
Blood chemistry			
Total protein	(g/dL)	7.1	8.6
Albumin	(g/dL)	3.6	4.6
Urea nitrogen	(mg/dL)	11.1	11.0
Creatinine	(mg/dL)	0.7	0.47
C-reactive protein	(mg/dL)	1.5	0.20
Total bilirubin	(mg/dL)	0.3	0.5
HDL cholesterol	(mg/dL)	59.4	54.2
LDL cholesterol	(mg/dL)	76.0	139.4
Triglycerides	(mg/dL)	59.0	87.0
Fasting glucose	(mg/dL)	102.0	92.0
Hemoglobin A1c (JDS)	(%)	4.4	5.2
Immunoglobulin G	(mg/dL)	1,733.0	2,078.7
Immunoglobulin M	(mg/dL)	113.0	125.9
Immunoglobulin A	(mg/dL)	289.0	235.8
Cerebrospinal fluid			
Appearance		translucent	n.d.
Pressure	mmH_2O	150	n.d.
Cells	/μL	10	n.d.
Neutrophils	(%)	7	n.d.
Lymphocytes	(%)	93	n.d.
Protein	(mg/dL)	64.2	n.d.
Immunoglobulin G	(mg/dL)	9.2	n.d.
Immunoglobulin G index		0.657	n.d.
Glucose	(mg/dL)	49.0	n.d.
(Serum glucose)	(mg/dL)	(102.0)	n.d.

HDL: high-density lipoprotein, LDL: low-density lipoprotein, JDS: Japan Diabetes Society, n.d.: not determined

place: -2, calculation: -5, recall of name: -1, and composition: -1). A laboratory examination revealed increased serum levels of C-reactive protein (1.5 mg/dL) and immunoglobulin G (IgG) (1,733.0 mg/dL), anemia (red blood cells: 281×10^4/μL, hemoglobin: 8.2 g/dL), and an elevated platelet count (48.4×10^4/μL) (Table). A cerebrospinal fluid (CSF) analysis detected mild mononuclear-dominant pleocytosis (10 cells/μL) and an elevated protein level (64.2 mg/dL) (Table). Bacterial, fungal, and mycobacterial cultures of the patient's CSF were all negative. In addition, polymerase chain reaction-based tests for the herpes simplex and herpes zoster viruses were negative, and no malignant cells were detected during a cytological examination. Brain magnetic resonance imaging (MRI) revealed mild frontal lobe atrophication, but did not detect any focal lesions. ^{99m}TC-ECD single-photon emission computed tomography revealed mild bilateral reductions in cerebral blood flow (Fig. 1). Electroencephalography showed a basic α wave rhythm, accompanied by a moderate number of θ waves (Fig. 2). The administration of acyclovir and globulin did not markedly improve the patient's symptoms. About a year later, her fever improved spontaneously. However, at 42 years of age she became aware of numbness in her left upper and lower extremities and was admitted to our hospital. A neurological examination demonstrated left hemisensory hypesthesia and dysesthesia. No other neurological symptoms were observed. Diffusion-weighted images of the brain obtained on admission showed a hyperintense lesion in the right thalamus, which was suggestive of a recent cerebral infarction (Fig. 3). Fluid attenuated inversion recovery (FLAIR) images of the brain showed mild atrophication of the bilateral hippocampus and frontal lobe. Magnetic resonance angiography of the brain did not detect any abnormalities in the intracranial arteries (Fig. 3). A blood cell count found that the patient's platelet count was slightly elevated (39.6×10^4/μL), and a blood biochemical analysis detected slightly increased serum levels of low-density lipoprotein cholesterol (139.4 mg/dL) and IgG (2,078.7 mg/dL). No renal dysfunction or proteinuria was observed (Table). An electrocardiogram revealed left ventricular hypertrophy (Fig. 4A), and echocardiography detected diffuse hypertrophy of the left ventricle and akinesis of the inferior basal wall (Fig. 4B). The administration of edaravone and clopidogrel was initiated, but the patient's sensory disturbances continued. A leukocyte enzyme assay detected reduced α-GAL activity (11.9 Agal U, cut-off value: <20 Agal U), and a gene analysis revealed the presence of a heterozygous mutation in intron 4 of the α-GAL gene (IVS4-1G>A). However, genetic analyses of the patient's parents and daughter did not detect this mutation. The patient was therefore diagnosed with sporadic Fabry disease. No cutaneous angiokeratomas, corneal dystrophy, hypohidrosis, or gastrointestinal disorders were observed. The patient was initially treated with agalsidase-α, but this was subsequently stopped due to fatigue. She currently takes clopidogrel and bisoprolol, and no new neurological manifestations, such as strokes, or a worsening of her psychiatric symptoms have occurred to date.

Discussion

Fabry disease affects both the peripheral nervous system and the central nervous system (CNS). CNS involvement is considered to be a major complication of Fabry disease, as are renal and cardiac involvement. The clinical CNS symptoms of Fabry disease include ischemic strokes, hearing problems, psychiatric disorders, and cognitive impairment (5). Strokes are one of the major complications of classical Fabry disease. A study of patients aged 18-55 years who had suffered cryptogenic strokes detected a high prevalence of Fabry disease (5% of males and 3% of females) (6). In patients with Fabry disease, strokes tend to occur more frequently in the vertebrobasilar system than in the carotid circulation (6). In Fabry disease, dolichoectasia of the large arteries is considered to be associated with hypertension and the formation of intravascular deposits of globotriaosylceramide in the endothelial cells, which leads to the progressive occlusion of the long perforating arteries in the vertebrobasilar territory. In addition, cardioembolic infarctions have been reported to occur in combination with hypertrophic cardiomyopathy, valvular disease, and arrhythmia, and atherosclerosis related to end-stage renal failure is

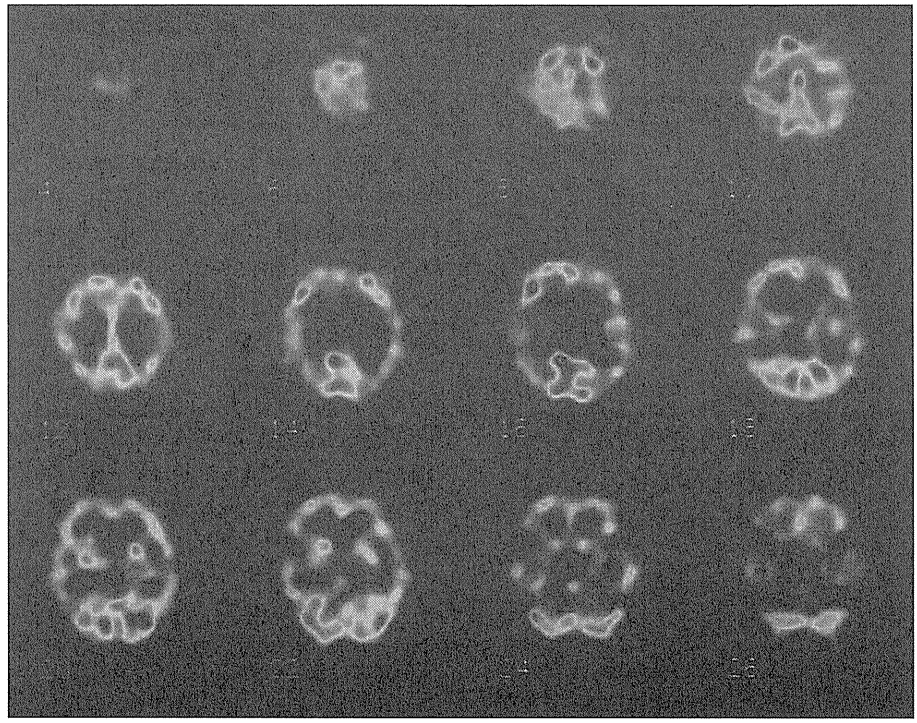

Figure 1. ^{99m}**Tc ECD single- photon emission computed tomography revealed mild bilateral reductions in cerebral blood flow.**

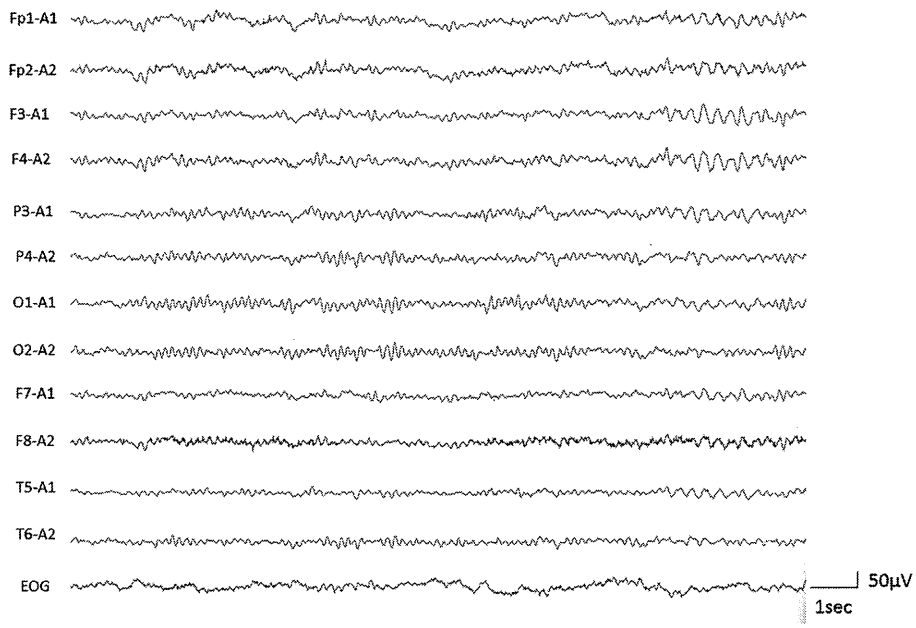

Figure 2. Electroencephalography detected a basic α wave rhythm, accompanied by a moderate number of θ waves.

also seen in Fabry disease (7).

The development of aseptic meningitis in a patient with Fabry disease has been reported in a number of cases (7-12). In most of these cases, the patients suffered multiple or recurrent strokes, which exhibited a vertebrobasilar predominance. The high frequency of strokes in this subgroup suggests that aseptic meningitis and stroke are related in Fabry disease (13, 14).

CSF pleocytosis in Fabry disease is assumed to be caused by the abnormal accumulation of sphingolipids within the pia, arachnoid membranes, and choroidal stroma (15). A recent study detected significant associations between cerebral lesions and genotypic polymorphisms affecting interleukin-6, endothelial nitric oxide synthase, factor V, or protein Z, which are involved in inflammation, vascular wall biology, and the clotting mechanism (16). These findings suggest that

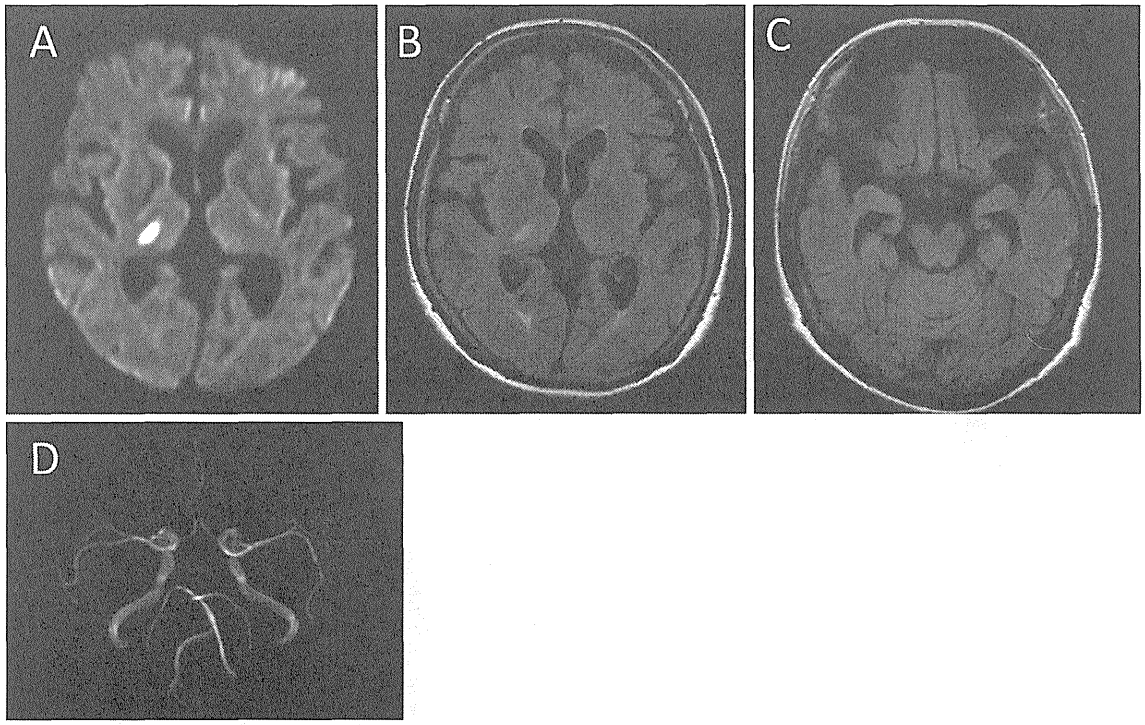

Figure 3. Magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA) findings of the patient's brain on admission. A: Diffusion-weighted images showing a hyperintense lesion in the right thalamus. B, C: Fluid attenuated inversion recovery images showing mild atrophication of the bilateral hippocampus and frontal lobe. D: MRA did not detect any intracranial artery abnormalities.

the above mentioned proteins influence the cerebral vasculopathy seen in Fabry disease.

Another important feature of Fabry disease is its associated psychiatric symptoms. A single case series of 33 men with Fabry disease showed that 18% of them had psychiatric disorders, with depression being the most common condition (17). Fabry disease has been reported to produce a confused state and psychotic syndromes in rare cases (17-20). One of the latter reports suggested that the co-occurrence of Fabry disease and schizophrenia-like psychiatric symptoms was coincidental (20). However, in the present case, the patient's psychiatric symptoms and fever emerged simultaneously; suggesting that aseptic meningoencephalitis caused by Fabry disease might have induced the patient's psychiatric symptoms. In a previous study (19), it was suggested that thalamic lesions might be related to the pathogenesis of psychotic symptoms in Fabry disease. However, our patient experienced psychiatric symptoms before she suffered a thalamic infarction. Thus, psychiatric symptoms can emerge in Fabry disease in the presence or absence of thalamic lesions. When a younger patient presents with psychiatric symptoms accompanied by a fever of unknown origin, left ventricular hypertrophy, renal dysfunction, minor stroke and/or sensory disturbance, then Fabry disease should be considered in the differential diagnosis.

To date, more than 600 mutations in the human α-GAL gene have been found to cause Fabry disease. About 70% of them are missense or nonsense mutations, whereas the remainder consist of splicing regulatory, or small or gross insertion/deletion mutations (21). The first report of a Fabry disease-associated mutation in intron 4 of the α-GAL gene (IVS4-1G>A), as was detected in our case, was published in 2006 (22). Such mutations are usually inherited, and cases involving *de novo* mutations are rare (23). However, cases of Fabry disease involving *de novo* mutations have been reported in various countries (21, 23-32). In an analysis of Japanese pedigrees associated with reduced α-GAL activity, 5 out of 93 pedigrees (5.6%) were found to possess *de novo* mutations (33). In addition to the present study, there have been two detailed case reports on sporadic Fabry disease in Japanese patients. One case involved a 16-year-old man who presented with angiokeratoma, limb neuropathic pain, anhidrosis, and mild corneal opacity and who exhibited a mutation in intron 5 of the α-GAL gene (IVS5+1 A>G) (34). Another case involved a 42-year old woman who displayed proteinuria and mild hypohidrosis and possessed a Pro210 Ser mutation (35). To the best of our knowledge, our report is the first to describe a case of sporadic Fabry disease in a patient who presented with aseptic meningitis, ischemic stroke, and psychiatric symptoms.

The mutation in intron 4 of the α-GAL gene (IVS4-1G>A) that was detected in the present case resulted in the classical Fabry disease phenotype (22). However, the patient's CNS symptoms were more marked than the symptoms associated with other organs. Heterozygous female patients with Fabry disease can present with various clinical manifesta-

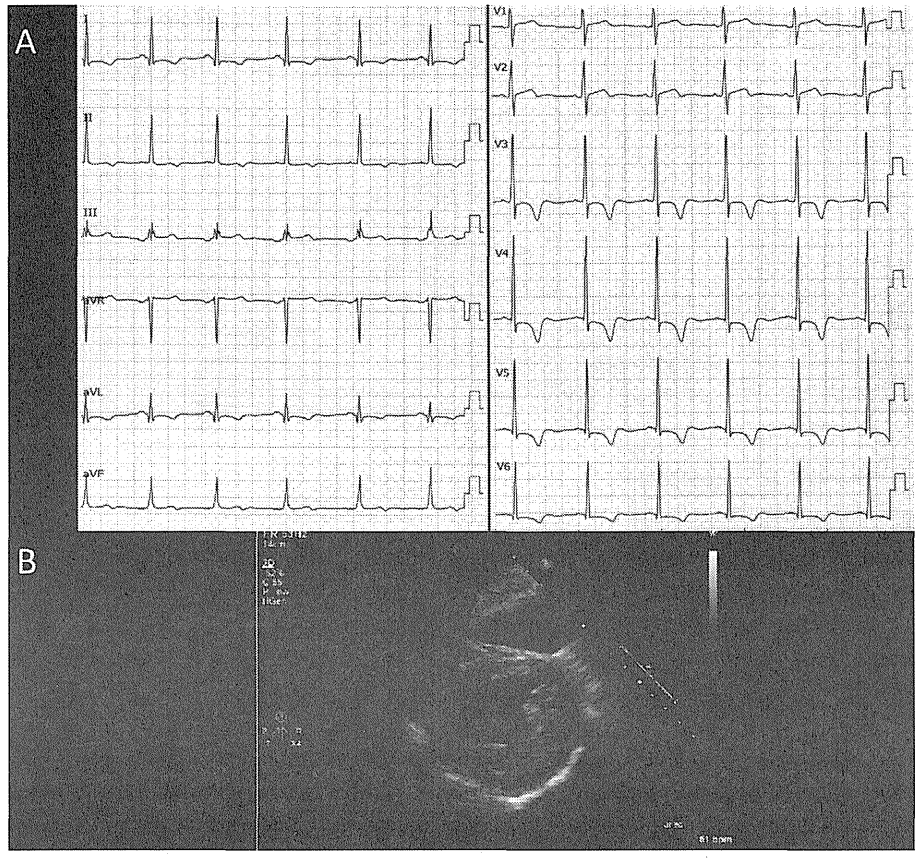

Figure 4. Twelve-lead electrocardiogram (ECG) (A) and transthoracic echocardiography (TTE) (B) obtained on admission. A: The twelve-lead ECG revealed a normal sinus rhythm and left ventricular hypertrophy. ST-T changes that were indicative of left ventricular strain were also noted. B: On the short-axis view, TTE showed left ventricular hypertrophy, especially in the posterolateral lesion.

tions (36), and it is important to keep in mind that atypical clinical manifestations can be observed in such patients. The pathogenic mechanism of the CNS symptoms seen in Fabry disease might be elucidated by examining the relationship between α-GAL gene mutations and CNS symptoms.

In conclusion, we reported a case of sporadic Fabry disease involving a patient that presented with psychiatric symptoms, aseptic meningitis, and ischemic stroke. Fabry disease can present with various neurological manifestations. This case suggests that Fabry disease should be considered during the differential diagnosis of young patients who suffer cryptogenic strokes or display CNS symptoms, including aseptic meningitis or psychosis, and experience fevers of unknown origin, even if they have no family history of Fabry disease.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

The authors thank Dr. K. Tabata of Asahikawa Keisenkai Hospital for providing the patient's psychiatric treatment and Professor Y. Makita of Asahikawa Medical University for providing genetic counseling to the patient and her family.

References

1. Spada M, Pagliardini S, Yasuda M, et al. High incidence of later-onset fabry disease revealed by newborn screening. Am J Hum Genet **79**: 31-40, 2006.
2. Mehta A, Ricci R, Widmer U, et al. Fabry disease defined: baseline clinical manifestations of 366 patients in the Fabry Outcome Survey. Eur J Clin Invest **34**: 236-242, 2004.
3. Wilcox WR, Oliveira JP, Hopkin RJ, et al. Females with Fabry disease frequently have major organ involvement: lessons from the Fabry Registry. Mol Genet Metab **93**: 112-128, 2008.
4. MacDermot KD, Holmes A, Miners AH. Anderson-Fabry disease: clinical manifestations and impact of disease in a cohort of 98 hemizygous males. J Med Genet **38**: 750-760, 2001.
5. Fellgiebel A, Muller MJ, Ginsberg L. CNS manifestations of Fabry's disease. Lancet Neurol **5**: 791-795, 2006.
6. Rolfs A, Böttcher T, Zschiesche M, et al. Prevalence of Fabry disease in patients with cryptogenic stroke: a prospective study. The Lancet **366**: 1794-1796, 2005.
7. Lidove O, Chauveheid MP, Caillaud C, et al. Aseptic meningitis and ischaemic stroke in Fabry disease. Int J Clin Pract **63**: 1663-1667, 2009.
8. Dubost JJ, Viallard IL, Sauvezie B. Chronic meningitis in Fabry's disease. J Neurol Neurosurg Psychiatry **48**: 714-715, 1985.
9. Uyama E, Ueno N, Uchino M, et al. Headache associated with aseptic meningeal reaction as clinical onset of Fabry's disease. Headache **35**: 498-501, 1995.
10. Perrot X, Nighoghossian N, Derex L, et al. [Vertebro-basilar

ischemic strokes and aseptic meningitis, late complications of Fabry's disease]. Rev Neurol (Paris) **158**: 596-598, 2002 (in French, Abstract in English).

11. Schreiber W, Udvardi A, Kristoferitsch W. Chronic meningitis and lacunar stroke in Fabry disease. J Neurol **254**: 1447-1449, 2007.

12. Lidove O, Chauveheid MP, Benoist L, Alexandra JF, Klein I, Papo T. Chronic meningitis and thalamic involvement in a woman: Fabry disease expanding phenotype. J Neurol Neurosurg Psychiatry **78**: 1007, 2007.

13. Eng CM, Fletcher J, Wilcox WR, et al. Fabry disease: baseline medical characteristics of a cohort of 1765 males and females in the Fabry Registry. J Inherit Metab Dis **30**: 184-192, 2007.

14. Linhart A, Kampmann C, Zamorano JL, et al. Cardiac manifestations of Anderson-Fabry disease: results from the international Fabry outcome survey. Eur Heart J **28**: 1228-1235, 2007.

15. Lou HO, Reske-Nielsen E. the central nervous system in Fabry's disease. A clinical, pathological, and biochemical investigation. Arch Neurol **25**: 351-359, 1971.

16. Altarescu G, Moore DF, Schiffmann R. Effect of genetic modifiers on cerebral lesions in Fabry disease. Neurology **64**: 2148-2150, 2005.

17. Grewal RP. Psychiatric disorders in patients with Fabry's disease. Int J Psychiatry Med **23**: 307-312, 1993.

18. Liston EH, Levine MD, Philippart M. Psychosis in Fabry disease and treatment with phenoxybenzamine. Arch Gen Psychiatry **29**: 402-403, 1973.

19. Shen YC, Haw-Ming L, Lin CC, Chen CH. Psychosis in a patient with Fabry's disease and treatment with aripiprazole. Prog Neuropsychopharmacol Biol Psychiatry **31**: 779-780, 2007.

20. Gairing S, Wiest R, Metzler S, Theodoridou A, Hoff P. Fabry's disease and psychosis: causality or coincidence? Psychopathology **44**: 201-204, 2011.

21. Li H, Zhou FS, Wang ZX, et al. Novel G144D mutation of the GLA gene in a Chinese patient with Fabry disease. J Dermatol **41**: 456-457, 2014.

22. Shabbeer J, Yasuda M, Benson SD, Desnick RJ. Fabry disease: identification of 50 novel alpha-galactosidase A mutations causing the classic phenotype and three-dimensional structural analysis of 29 missense mutations. Hum Genomics **2**: 297-309, 2006.

23. Iemolo F, Pizzo F, Albeggiani G, et al. De novo mutation in a male patient with Fabry disease: a case report. BMC Res Notes **7**: 11, 2014.

24. Bernstein HS, Bishop DF, Astrin KH, et al. Fabry disease: six gene rearrangements and an exonic point mutation in the alpha-galactosidase gene. J Clin Invest **83**: 1390-1399, 1989.

25. Eng CM, Niehaus DJ, Enriquez AL, Burgert TS, Ludman MD, Desnick RJ. Fabry disease: twenty-three mutations including sense and antisense CpG alterations and identification of a deletional hot-spot in the alpha-galactosidase A gene. Hum Mol Genet **3**: 1795-1799, 1994.

26. Madsen KM, Hasholt L, Sorensen SA, Fermer ML, Dahl N. Two novel mutations (L32P) and (G85N) among five different missense mutations in six Danish families with Fabry's disease. Hum Mutat **5**: 277-278, 1995.

27. Redonnet-Vernhet I, Ploos van Amstel JK, Jansen RP, Wevers RA, Salvayre R, Levade T. Uneven X inactivation in a female monozygotic twin pair with Fabry disease and discordant expression of a novel mutation in the alpha-galactosidase A gene. J Med Genet **33**: 682-688, 1996.

28. Davies JP, Eng CM, Hill JA, et al. Fabry disease: fourteen alpha-galactosidase A mutations in unrelated families from the United Kingdom and other European countries. Eur J Hum Genet **4**: 219-224, 1996.

29. Rodriguez-Mari A, Coll MJ, Chabas A. Molecular analysis in Fabry disease in Spain: fifteen novel GLA mutations and identification of a homozygous female. Hum Mutat **22**: 258, 2003.

30. Brokalaki EI, Hentschke M, Grabbe S, Jansen T. Fabry disease in a female patient due to a de novo point mutation at position 691 of exon 5. Eur J Med Res **11**: 306-308, 2006.

31. Paull LS, Lipinski MJ, Wilson WG, Lipinski SE. Female with Fabry disease unknowingly donates affected kidney to sister: a call for pre-transplant genetic testing. JIMD Rep **4**: 1-4, 2012.

32. Cai ZY, Zhang YK, Wang SX, Fang QY, Chen YQ. Diffuse thin glomerular basement membrane in association with Fabry disease in a Chinese female patient. Nephrol Dial Transplant **26**: 3813-3816, 2011.

33. Kobayashi M, Ohashi T, Eto Y, Ida H. [A gene analysis of Fabry disease: the pitfall]. The Lipid **24**: 302-303, 2013 (in Japanese).

34. Kashihara T, Uchida D, Oeda T, et al. [A novel gene mutation: A sporadic case of classical Fabry disease with an abnormality of splice acceptor site in intron 5]. Nihon Naibunpigakkai Zasshi (Folia Endocrinologica Japonica) **74**: 197, 1998 (in Japanese).

35. Takahashi N, Kasuno K, Tukimura T, et al. [A sporadic heterozygous female case of Fabry disease with a novel mutation discovered by health examination]. Nihon Jinzou Gakkaishi (The Japanese journal of Nephrology) **55**: 1194, 2013 (in Japanese).

36. Kobayashi M, Ohashi T, Sakuma M, Ida H, Eto Y. Clinical manifestations and natural history of Japanese heterozygous females with Fabry disease. J Inherit Metab Dis **31**(Suppl 3): S483-S487, 2008.

PLOS | GENETICS

RESEARCH ARTICLE

A Comprehensive Genomic Analysis Reveals the Genetic Landscape of Mitochondrial Respiratory Chain Complex Deficiencies

Masakazu Kohda[1☯], Yoshimi Tokuzawa[2☯], Yoshihito Kishita[2☯], Hiromi Nyuzuki[2], Yohsuke Moriyama[2,3], Yosuke Mizuno[2], Tomoko Hirata[1], Yukiko Yatsuka[2], Yzumi Yamashita-Sugahara[2], Yutaka Nakachi[1], Hidemasa Kato[1,3], Akihiko Okuda[3], Shunsuke Tamaru[2], Nurun Nahar Borna[2], Kengo Banshoya[2,4], Toshiro Aigaki[5], Yukiko Sato-Miyata[5], Kohei Ohnuma[5], Tsutomu Suzuki[6], Asuteka Nagao[6], Hazuki Maehata[6], Fumihiko Matsuda[7], Koichiro Higasa[7], Masao Nagasaki[8,9,10], Jun Yasuda[8,9], Masayuki Yamamoto[8,9], Takuya Fushimi[11], Masaru Shimura[11], Keiko Kaiho-Ichimoto[11], Hiroko Harashima[12], Taro Yamazaki[12], Masato Mori[13], Kei Murayama[11], Akira Ohtake[12]*, Yasushi Okazaki[1,2]*

1 Division of Translational Research, Research Center for Genomic Medicine, Saitama Medical University, Hidaka-shi, Saitama, Japan, **2** Division of Functional Genomics & Systems Medicine, Research Center for Genomic Medicine, Saitama Medical University, Hidaka-shi, Saitama, Japan, **3** Division of Developmental Biology, Research Center for Genomic Medicine, Saitama Medical University, Hidaka-shi, Saitama, Japan, **4** Chemicals Assessment and Research Center, Chemicals Evaluation and Research Institute, Japan (CERI), Sugito-machi, Kitakatsushika-gun, Saitama, Japan, **5** Department of Biological Sciences, Tokyo Metropolitan University, Hachioji, Tokyo, Japan, **6** Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, Bunkyo-ku, Tokyo, Japan, **7** Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto, Japan, **8** Department of Integrative Genomics, Tohoku Medical Megabank Organization, Tohoku University, Aoba-ku, Sendai, Miyagi, Japan, **9** Graduate School of Medicine, Tohoku University, Aoba-ku, Sendai, Miyagi, Japan, **10** Graduate School of Information Sciences, Tohoku University, Sendai, Miyagi, Japan, **11** Department of Metabolism, Chiba Children's Hospital, Midori, Chiba, Japan, **12** Department of Pediatrics, Saitama Medical University, Moroyama-machi, Iruma-gun, Saitama, Japan, **13** Department of Pediatrics, Matsudo City Hospital, Matsudo-shi, Chiba, Japan

☯ These authors contributed equally to this work.
* akira_oh@saitama-med.ac.jp (AOh); okazaki@saitama-med.ac.jp (YO)

OPEN ACCESS

Citation: Kohda M, Tokuzawa Y, Kishita Y, Nyuzuki H, Moriyama Y, Mizuno Y, et al. (2016) A Comprehensive Genomic Analysis Reveals the Genetic Landscape of Mitochondrial Respiratory Chain Complex Deficiencies. PLoS Genet 12(1): e1005679. doi:10.1371/journal.pgen.1005679

Editor: Gregory S. Barsh, Stanford University School of Medicine, UNITED STATES

Received: August 7, 2015

Accepted: October 27, 2015

Published: January 7, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by a grant of the Innovative Cell Biology by Innovative Technology (Cell Innovation Program) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan to YO (http://cell-innovation.nig.ac.jp/mext-life/english/index.html); the Support Project and a grant of Strategic Research Center in Private Universities from the MEXT, Japan to Saitama Medical University Research Center for Genomic Medicine (http://www.mext.go.jp/a_menu/koutou/

Abstract

Mitochondrial disorders have the highest incidence among congenital metabolic disorders characterized by biochemical respiratory chain complex deficiencies. It occurs at a rate of 1 in 5,000 births, and has phenotypic and genetic heterogeneity. Mutations in about 1,500 nuclear encoded mitochondrial proteins may cause mitochondrial dysfunction of energy production and mitochondrial disorders. More than 250 genes that cause mitochondrial disorders have been reported to date. However exact genetic diagnosis for patients still remained largely unknown. To reveal this heterogeneity, we performed comprehensive genomic analyses for 142 patients with childhood-onset mitochondrial respiratory chain complex deficiencies. The approach includes whole mtDNA and exome analyses using high-throughput sequencing, and chromosomal aberration analyses using high-density oligonucleotide arrays. We identified 37 novel mutations in known mitochondrial disease genes and 3 mitochondria-related genes (*MRPS23*, *QRSL1*, and *PNPLA4*) as novel

shinkou/07021403/002/002/1218299.htm). This work was also supported by Grants-in-Aid of the Research on Intractable Diseases (Mitochondrial Disorder) from the Ministry of Health, Labor and Welfare of Japan, special research grant from Takeda Science Foundation to YO. This work was also supported by Takeda Science Foundation to YO (http://www.takeda-sci.or.jp/). This work was supported in part by the Practical Research Project for Rare/Intractable Diseases from Japan Agency for Medical Research and Development, AMED to KM (http://www.amed.go.jp/en/). This work was also supported in part by the Project Promoting Clinical Trials for Development of New Drugs and Medical Devices, Japan Medical Association, from Japan Agency for Medical Research and Development, AMED to AO (http://www.jmacct.med.or.jp/en/what-we-do/investigator.html). This work was also supported in part by the Japan Society for the Promotion of Science (JSPS) KAKENHI 24689044 (Grant-in-Aid for Young Scientists A) to MK (https://kaken.nii.ac.jp/d/p/24689044.en.html) and by the Japan Society for the Promotion of Science (JSPS) KAKENHI 20634398 (Grant-in-Aid for Young Scientists B) to YK (https://kaken.nii.ac.jp/d/p/26870545.en.html). This work was also supported in part by Tohoku Medical Megabank Project (Special Account for reconstruction from the Great East Japan Earthquake) to MY (http://www.megabank.tohoku.ac.jp/english/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

causative genes. We also identified 2 genes known to cause monogenic diseases (*MECP2* and *TNNI3*) and 3 chromosomal aberrations (6q24.3-q25.1, 17p12, and 22q11.21) as causes in this cohort. Our approaches enhance the ability to identify pathogenic gene mutations in patients with biochemically defined mitochondrial respiratory chain complex deficiencies in clinical settings. They also underscore clinical and genetic heterogeneity and will improve patient care of this complex disorder.

Author Summary

Mitochondria play a crucial role in ATP biosynthesis and comprise proteins encoded in both the nuclear and mitochondrial genomes. Although more than 250 mitochondrial disease-causing genes have been reported, the exact genetic causes in patients remain largely unknown. Here, we aimed to provide further insights into the pathogenic mechanisms of mitochondrial disorders. We investigated the genes encoded in the nuclear and mitochondrial genomes using comprehensive genomic analysis in 142 patients with mitochondrial respiratory chain complex deficiencies. We identified 3 novel disease-causing mitochondria-related genes (*MRPS23*, *QRSL1*, and *PNPLA4*) as well as other disease-causing genes and novel pathogenic mutations in known mitochondrial disease-causing genes. All pathogenic mutations in this study are validated by genetic and/or functional evidence. Our findings, including the achievement of firm genetic diagnoses for 49 of 142 patients (34.5%), were higher than the general diagnosis rate of approximately 25% and demonstrated the value of comprehensive genomic analysis. Accordingly, we have shed light on the genetic heterogeneity underlying mitochondrial disorders.

Introduction

Human oxidative phosphorylation (OXPHOS) disease has the highest incidence among congenital metabolic disorders characterized by a biochemical respiratory chain complex deficiencies and is thought to occur at a rate of 1 in 5,000 births[1]. No more than 15–30% of pediatric diseases diagnosed as mitochondrial disorders show mitochondrial DNA (mtDNA) abnormalities[2,3]; the remaining cases occur because of defects in genes encoded in the nucleus. A certain amount of nuclear-encoded gene products are present in the mitochondria, and roughly 1,500 are thought to play important roles in mitochondrial function[4,5].

It is particularly difficult to diagnose patients with OXPHOS disease at the molecular level because of the massive numbers of potentially involved nuclear genes and genes not yet linked to human disease. Therefore, identification of the causative genes and an understanding of the pathogenic mechanisms of OXPHOS disease remain unsolved challenges.

Recent studies[6,7] have shown that heterogeneous genetic backgrounds as well as genes previously not linked to mitochondrial functions or localization are associated with this disease. However, because of phenotypic and locus heterogeneity, only a fraction of patients has been identified to date. Limitations in target resequencing have motivated us to apply a comprehensive genomic analysis for more accurate molecular diagnosis and for the identification of novel causative genes.

Here, we aimed to determine whether a comprehensive genomic analysis approach could be used to reveal the broad spectrum of genetic background of the disease[8]. One hundred and forty-two unrelated individuals with displayed childhood-onset mitochondrial respiratory

chain complex deficiencies were selected. We applied long-range polymerase chain reaction (PCR)-based whole mtDNA sequencing, whole exome sequencing (WES), and high-density oligonucleotide arrays to identify single-nucleotide variants (SNVs), small insertions or deletions (indels), and chromosomal aberrations for comprehensive genomic analyses.

Results

Comprehensive genomic analysis in 142 patients

In this study, 142 patients with childhood-onset mitochondrial respiratory chain complex deficiencies were enrolled and subjected to comprehensive genomic analyses (detailed clinical characteristics are described in S1 Table). A schematic workflow of these analyses is shown in Fig 1. Comprehensive genomic analyses included three approaches: (i) amplicon-based whole mtDNA sequencing for pathogenic mutations and large duplications/deletions, (ii) WES for pathogenic mutations in nuclear DNA, and (iii) high-density oligonucleotide arrays for chromosomal aberrations. The prioritized variants derived from each approach are described below.

Prioritized variants in 142 patients

After comprehensive genomic analysis shown in Fig 1, rare variants were filtered out and prioritized on the basis of the strategy described below. For mtDNA variants, we targeted variants confirmed and reported in MITOMAP[9]. Exome sequencing covered 89% (ranged: from

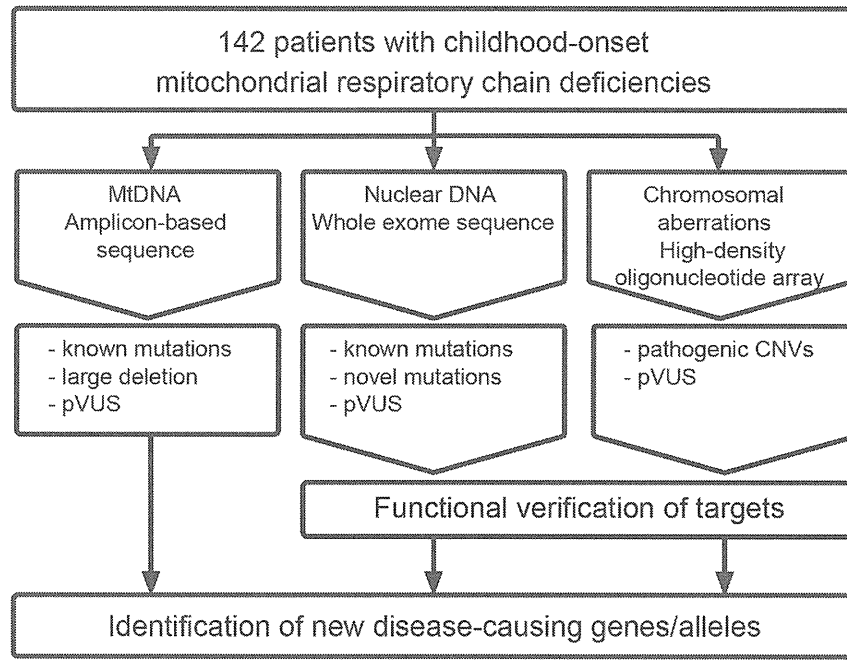

Fig 1. Schematic of comprehensive genomic analysis of 142 patients. All 142 patients were subjected to mtDNA amplicon-based sequencing, WES, and high-density oligonucleotide array analysis in parallel. Variants were filtered on the basis of their rarity in public databases and population-matched datasets. For each analysis, candidate variants were prioritized on the basis of the type of variant. Candidate variants were validated by Sanger sequencing and tested for segregation within the family if DNA was available. mtDNA, mitochondrial DNA; pVUS, prioritized variant of unknown significance; CNV, copy number variation

doi:10.1371/journal.pgen.1005679.g001

70%–to 98%) of the targeted bases, with more than 20-fold coverage. Detailed sequence statistics is shown in S2 Table.

The precise strategy for WES variant prioritization is shown in S2 Fig. We evaluated our prioritization pipeline to validate whether it could feasibly enrich known OXPHOS disease-causing genes or mitochondria-related genes (S3 Fig). Known OXPHOS disease-causing genes were clearly enriched in disease cases, whereas no prioritized genes were detected in healthy controls. Compared with healthy controls, mitochondria-related genes also exhibited a 1.64-fold enrichment. No enrichment was observed in randomly selected genes. These results suggest that mitochondria-related gene enrichment is caused by unidentified causative genes.

To analyze chromosomal aberrations, we focused on rather large (>100 Kb) deletions or duplications. For prioritizing candidate aberrations, we filtered out deleted or duplicated regions found in the 524 in-house controls and manually curated the pathogenicity of the aberrations by referring to the OMIM, DGV, and DECIPHER databases.

A breakdown of the 142 patients according to prioritized variants is shown in Fig 2. Of the 142 patients with mitochondrial respiratory chain complex deficiencies, 102 (71.8%) harbored at least 1 prioritized mtDNA mutation, nuclear gene mutation, or chromosomal abnormality. Ten (7.0%) patients harbored mtDNA mutations, including one large deletion (S4 Fig). In 29 patients (20.4%), firm molecular diagnoses were made in 20 genes previously linked to mitochondrial disorders. We newly confirmed 3 mitochondria-related genes (*MRPS23*, *QRSL1*, and *PNPLA4*) as causative genes of mitochondrial respiratory chain complex deficiencies. Three patients (2.1%) harbored mutations in genes known to cause monogenic diseases (*MECP2* and *TNNI3*). Intriguingly, 4 patients (2.8%) had pathogenic chromosomal deletions previously linked to other disorders (6q24.3-q25.1, 22q11.21, and 17p12) but not linked to mitochondrial respiratory chain complex deficiencies.

In 53 (37.3%) patients, we identified and designated candidate genes or loci as prioritized variants of unknown significance (pVUS) because these variants have possibilities to be pathogenic but have insufficient evidence to support a disease linage. The current lack of functional validation for linking these genes with mitochondrial disorders led us to classify these variants as inconclusive with respect to pathogenicity (S3, S4 and S5 Tables). The remaining 40 (28.2%) patients lacked prioritized nuclear variants, mtDNA variants, and chromosomal abnormalities.

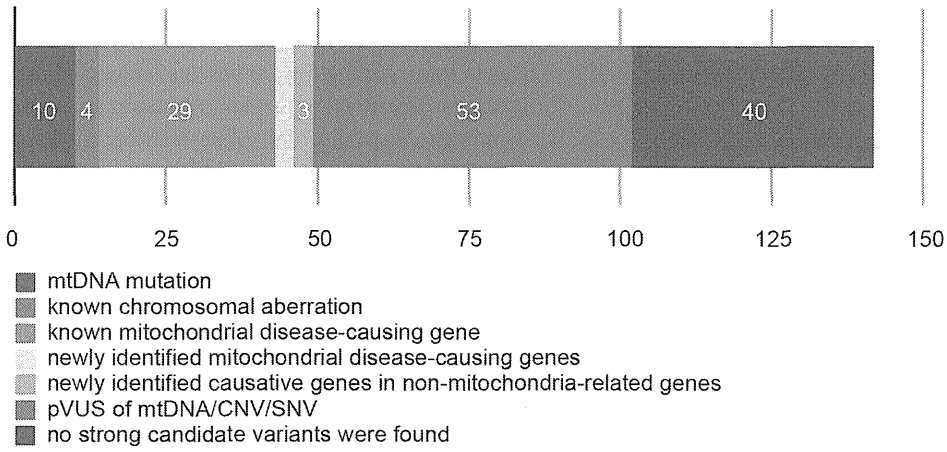

Fig 2. Breakdown of 142 patients categorized by the type of mutations/variants in comprehensive genomic analysis. Numerals in each colored box indicate the number of patients. Patients harboring multiple variants were assigned to each box on the basis of the highest priority variant in all cases.

doi:10.1371/journal.pgen.1005679.g002

New genetic diagnoses for cases with previously established nuclear disease genes

Twenty-two genes were prioritized in 31 patients (Table 1). Of these, 29 patients harbored 41 disease-causing mutations in 20 genes known to cause OXPHOS disease: *ACAD9*, *BOLA3*, *COQ4*, *COX10*, *EARS2*, *ECHS1*, *GFM1*, *GTPBP3*, *KARS*, *MPV17*, *NDUFA10*, *NDUFAF6*, *NDUFB11*, *NDUFS4*, *RARS2*, *RRM2B*, *SCO2*, *SUCLA2*, *TAZ*, and *TUFM*. All such mutations were confirmed through Sanger sequencing and haplotype phasing. In particular, 8 patients had homozygous mutations, 19 had compound heterozygous mutations, and 2 had hemizygous mutations. Of the 41 mutations, 37 were novel and 4 were reported as pathogenic in the Human Gene Mutation Database[10] (HGMD, professional version 2013.10).

BOLA3, which plays an essential role in iron–sulfur cluster production, was mutated in 4 unrelated patients with severe lactic acidosis and combined respiratory chain complex deficiencies (MIM 614299). Three of these patients (Pt045, Pt268, and Pt314) exhibited multiple organ failure; Pt268 and Pt314 had hypertrophic cardiomyopathy, and Pt045 developed seizures. All 4 patients exhibited decreased complex II activity and harbored the c.287A>G (p.H96R) mutation. Pt314 and Pt286 patients showed clear long contiguous stretches of homozygosity (LCSH) (2.8 Mb, 3.2 Mb respectively) around this p.H96R mutation. Pt268 also showed a short contiguous stretch of homozygosity (0.3 Mb). This homozygous region encompassing *BOLA3* was shared between these unrelated individuals. Sanger sequencing identified the parents for these three patients as heterozygous carriers of this mutation. No p.H96R carriers were found in NHLBI GO Exome Sequencing Project (ESP6500), and 1 Japanese carrier in 1000 Genomes Project (1KG) was found. We screened for mutations that violated the Hardy–Weinberg principle and only identified the p.H96R mutation. These results suggest that p.H96R is common in the Japanese population and has originated from a single founder (S5 and S6 Figs).

NDUFAF6, which plays an important role in complex I assembly, was mutated in 4 unrelated patients: Pt101, Pt512, and Pt598 exhibited regression, whereas Pt330 exhibited muscle atrophy. All patients had complex I deficiency (MIM 256000). Pt101 shared 1 allele with Pt512 and another with Pt598. Pt330 harbored homozygous mutation c.820A>G (p.R274G) located in 1.3 Mb LCSH. Sanger sequencing identified the parents as heterozygous carriers of this mutation. Only 1 family was reported to harbor a mutation in this gene[18] (S7, S8 and S9 Figs).

NDUFB11, recently reported as causative gene for microphthalmia with linear skin defects syndrome (MIM 300952) and encoding a complex I component, was mutated in Pt067, a boy born to non-consanguineous parents under conditions of intrauterine growth restriction; this patient presented with heart failure, respiratory failure, complex I deficiency, and lethal infantile mitochondrial disorder (LIMD). He harbored a hemizygous *de novo* mutation, c.361G>A (p.E121K), and there was no NDUFB11 protein expression in his fibroblasts (S10 Fig). Because the p.E121 residue is highly conserved in this gene, we performed functional *in vivo* assays using a *dndufb11*-knockdown *Drosophila* model (S11 Fig); compared with controls, the mean lifespan was significantly reduced and the metabolic rate was lower in knockdown flies. Blue-native polyacrylamide gel electrophoresis (BN-PAGE) analysis showed a loss of complex I assembly, and lactate and pyruvate levels were increased in the knockdown flies. The *in vivo* *dndufb11*-knockdown *Drosophila* experiment further supported this conclusion. While preparing this manuscript, two girls harboring mutations in *NDUFB11* with microphthalmia with linear skin defects were reported by van Rahden et al[19]. Our patient was a male and died 55 h after birth. He presented with redundant skin but had no linear skin defects.

Pt459, a boy with lactic acidosis, developmental delays, hypertrophic cardiomyopathy, seizure, and combined complex deficiencies (I and IV), harbored the compound heterozygous

Table 1. New genetic diagnoses for cases with previously established nuclear disease genes.

ID	Clinical diagnosis	Complex type	Genetic diagnosis	Gene	Variations	Supporting evidence
Pt090	NLIMD	CI	Firm	*ACAD9* (NM_014049)	c.1150G>A:p.V384M, c.1817T>A:p.L606H	Segregation
Pt025	NLIMD	CI	Firm	*ACAD9* (NM_014049)	c.811T>G:p.C271G, c.1766-2A>G	Rescue
Pt045	LIMD	CC	Firm	*BOLA3* (NM_212552)	c.225_229del:p.K75fs, c.287A>G:p.H96R	Shared rare variant in four patients, rescue
Pt268	LIMD	CC	Firm	*BOLA3* (NM_212552)	c.287A>G:p.H96R, c.287A>G:p.H96R	Shared rare variant in four patients, rescue
Pt286	LD	CC	Firm	*BOLA3* (NM_212552)	c.287A>G:p.H96R, c.287A>G:p.H96R	Shared rare variant in four patients, rescue
Pt314	CM	CC	Firm	*BOLA3* (NM_212552)	c.287A>G:p.H96R, c.287A>G:p.H96R	Shared rare variant in four patients, rescue
Pt113	LIMD	CC	Firm	*COQ4* (NM_016035)	c.718C>T:p.R240C, c.421C>T:p.R141X	Rescue; patient is included in the study by Brea-Calvo et al[11]
Pt223	CM	CC	Firm	*COX10* (NM_001303)	c.862G>A:p.G288R, c.1259C>T:p.P420L	Rescue
Pt691	ND	CIV	Firm	*EARS2* (NM_001083614)	c.319C>T:p.R107C, c.1466G>A:p.R489Q	*De novo* (c.319C>T)
Pt346	LIMD	CI	Firm	*ECHS1* (NM_004092)	c.176A>G:p.N59S, c.476A>G:p.Q159R	Known; patient is included in the study by Haack et al[12]
Pt376	LD	CIV	Firm	*ECHS1* (NM_004092)	c.98T>C:p.F33S, c.176A>G:p.N59S	Known; patient is included in the study by Haack et al[12]
Pt112	HD	CC	Firm	*GFM1* (NM_024996)	c.170C>A:p.S57Y, c.748C>T:p.R250W	Known[13]
Pt751	LD	CC	Firm	*GTPBP3* (NM_032620)	c.8G>T:p.R3L, c.923-947del (p.E309fs)	Patient is included in the study by Kopajtich R. et al[14]
Pt459	MC	CC	Firm	*KARS* (NM_005548)	c.1343T>A:p.V448D, c.953T>C:p.I318T	Rescue
Pt339	HD (MTDPS)	CC	Firm	*MPV17* (NM_002437)	c.293C>T:p.P98L, c.376-1G>A	Known[15], mtDNA decreased to 20.5% in hepatic tissue
Pt057	NLIMD	CI	Firm	*NDUFA10* (NM_004544)	c.383_384insTAA:p.S128delinsIS, c.881T>C:p.L294P	Rescue
Pt512	LD	CI	Firm	*NDUFAF6* (NM_152416)	c.226T>C:p.S76P, c.805C>G:p.H269D	Independent rare variant in two patients (c.805 C >G), rescue
Pt598	LD	CI	Firm	*NDUFAF6* (NM_152416)	c.206A>T:p.D69V, c.371T>C:p.I124T	Independent rare variant in two patients (c.371 T >C)
Pt101	LD	CI	Firm	*NDUFAF6* (NM_152416)	c.371T>C:p.I124T, c.805C>G:p.H269D	Independent rare variants in two patients (c.371T>C, c.805C>G), segregation
Pt330	MC	CI	Firm	*NDUFAF6* (NM_152416)	c.820A>G:p.R274G, c.820A>G:p.R274G	Rescue
Pt067	LIMD	CI	Firm	*NDUFB11* (NM_001135998)	c.361G>A:p.E121K (hemizygous)	Rescue, *de novo*, functional assay
Pt711	LD	CI	Firm	*NDUFS4* (NM_002495)	c.340T>C:p.W114R, c.340T>C:p.W114R	LCSH
Pt222	NLIMD	CIV	Firm	*RARS2* (NM_020320)	c.1321C>T:p.L441F, c.1306G>T:p.D436Y	Segregation
Pt652	MC	CC	Firm	*RRM2B* (NM_015713)	c.97C>T:p.P33S, c.97C>T:p.P33S	Segregation
Pt628	LD	CC	Firm	*SCO2* (NM_001169109)	c.577G>A:p.G193S, c.773T>C:p.M258T	Known[16], segregation

(*Continued*)

Table 1. (*Continued*)

ID	Clinical diagnosis	Complex type	Genetic diagnosis	Gene	Variations	Supporting evidence
Pt105	MC	CIV	Firm	*SUCLA2* (NM_003850)	c.1048G>A:p.G350S, c.1048G>A:p.G350S	Known[17], LCSH, no deletion detected by array
Pt634	NLIMD	CC	Firm	*TAZ* (NM_000116)	c.36_57del:p.12_19del (hemizygous)	NDP
Pt559	NLIMD	CIV	Firm	*TUFM* (NM_003321)	c.440T>A:p.L147H, c.440T>A:p.L147H	Segregation
Pt622	LIMD	CC	Firm	*TUFM* (NM_003321)	c.440T>A:p.L147H, c.162delC:p.Y54X	Rescue, segregation
Pt550	LIMD (MTDPS)	CC	pVUS	*LRPPRC* (NM_133259)	c.1253A>C:p.N418T, c.2741C>A:p.P914Q	N.A.
Pt001	LIMD	CC	pVUS	*PC* (NM_022172)	c.1822G>A:p.G608R, c.2120C>T:p.T707M	N.A.

All listed variants were confirmed by Sanger sequencing of gDNA or cDNA. GERP scores of all listed variants, except for *EARS2* (c.319C>T), were >2.5. CI, complex I deficiency; CIV, complex IV deficiency; CC, combined complex deficiencies; CM, cardiomyopathy; HD, hepatic disease; LD, Leigh's disease; LIMD, lethal infantile mitochondrial disorder; MC, mitochondrial cytopathy; ND, neurodegenerative disorder; NLIMD, non-lethal infantile mitochondrial disorder; *de novo*, confirmed *de novo* variant by trio-based sequencing; known, known disease variant; NDP, no detectable protein; LCSH, long contiguous stretches of homozygosity from high-density oligonucleotide array; segregation, variant segregates with disease in family; N.A., not available.

doi:10.1371/journal.pgen.1005679.t001

mutations c.1343T>A (p.V448D) and c.953T>C (p.I318T) in *KARS*. KARS is a lysyl-transfer RNA synthetase that produces 2 proteins that localize to the cytosol and mitochondria. A cDNA complementation assay revealed that mitochondrial KARS successfully rescued the enzyme defect, but not cytosolic form (S12 Fig). Detailed information and evidential support for other known genes are described in S1 Text.

Newly identified mutations in mitochondria-related genes

Five (*MRPS23*, *C1QBP1*, *ALAS2*, *SLC25A26*, *QRSL1*) genes were identified as novel candidate genes (Tables 2 and S3). These genes were previously reported links to mitochondrial function but not mitochondrial respiratory chain complex deficiencies. Of these, we obtained pathogenic support for mutations in *MRPS23* and *QRSL1*. In addition, candidate genes that have no evidence of functional involvement in current mitochondrial biology are good targets for underlying novel mitochondrial biological functions. In one such case, we identified *PNPLA4* as a novel causative gene for mitochondrial respiratory chain complex deficiencies and proved its mitochondrial localization for the direct evidence of mitochondrial functions. The supportive evidence included (i) the identification of independent mutations in candidate genes in unrelated individuals with exquisitely similar phenotypes, (ii) rescue of patients' cellular phenotypes in a cDNA complementation assay, and (iii) identification of a *de novo* mutation in the candidate gene. Other pVUS for candidate genes are shown in S3 Table.

A component of the highly conserved mitochondrial ribosome small subunit *MRPS23*[22] was mutated in Pt276, a boy with hepatic disease and combined respiratory chain complex deficiencies. In this patient, enzyme activities in complexes I and IV were decreased by 28% and 14% of the normal fibroblastic values, respectively. The patient was born to a non-consanguineous family. However, high-density oligonucleotide array analysis identified an approximately 500 kb contiguous stretch of homozygosity encompassing *MRPS23*. No other candidate gene was prioritized in our comprehensive genomic analysis. Pt276 harbored a homozygous