Mutations in *GTPBP3* Cause a Mitochondrial Translation Defect Associated with Hypertrophic Cardiomyopathy, Lactic Acidosis, and Encephalopathy

Robert Kopajtich, Thomas J. Nicholls, Joanna Rorbach, Metodi D. Metodiev, Peter Freisinger, Hanna Mandel, Arnaud Vanlander, Daniele Ghezzi, Rosalba Carrozzo, Robert W. Taylor, Klaus Marquard, Kei Murayama, Thomas Wieland, Thomas Schwarzmayr, Johannes A. Mayr, Sarah F. Pearce, Christopher A. Powell, Ann Saada, Akira Ohtake, Federica Invernizzi, Eleonora Lamantea, Ewen W. Sommerville, Angela Pyle, Patrick F. Chinnery, Ellen Crushell, Yasushi Okazaki, Masakazu Kohda, Yoshihito Kishita, Yoshimi Tokuzawa, Zahra Assouline, Marlène Rio, François Feillet, Bénédict Mousson de Camaret, Dominique Chretien, Arnold Munnich, Björn Menten, Tom Sante, JoélSmet, Luc Régal, Abraham Lorber, Asaad Khoury, Massimo Zeviani, Tim M. Strom, Thomas Meitinger, Enrico S. Bertini, Rudy Van Coster, Thomas Klopstock, Agnès Rötig, Tobias B. Haack, Michal Minczuk, and Holger Prokisch
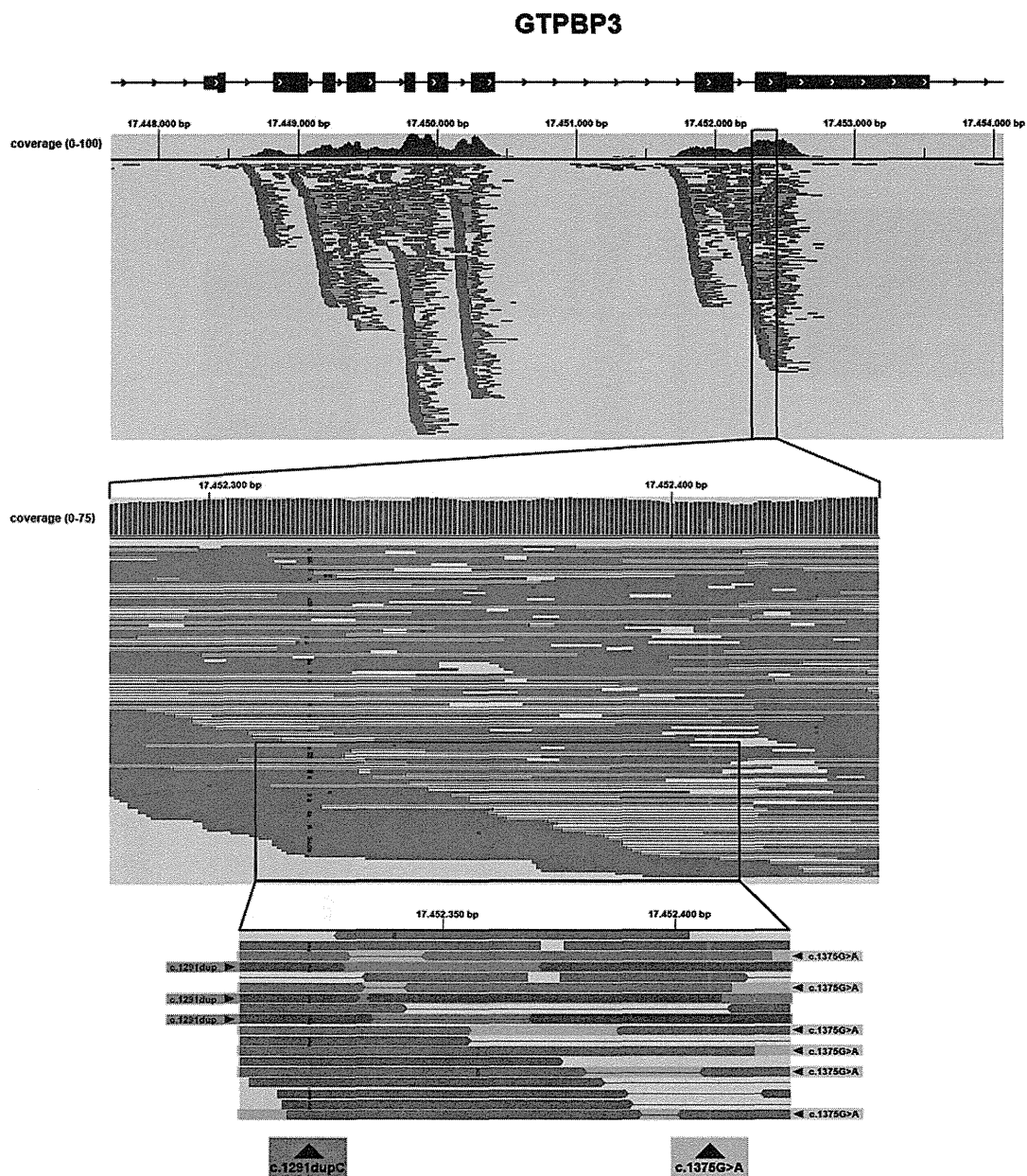
Figure S1

GTPBP3

Figure S1) Segregation analysis in family F1 in WES data

The two mutations identified in family F1 (c.1291dupC and c.1375G>A) are only separated by 97 bp which allowed analysis of both alleles despite the lack of parental material. 13 paired sequence reads were identified which covered the region of both variants. All reads contained either of the two mutations demonstrating a compound heterozygous status of the two variants. Figure S1 shows three sequence reads containing the c.1291dupC variant and five reads containing the c.1375G>A variant.
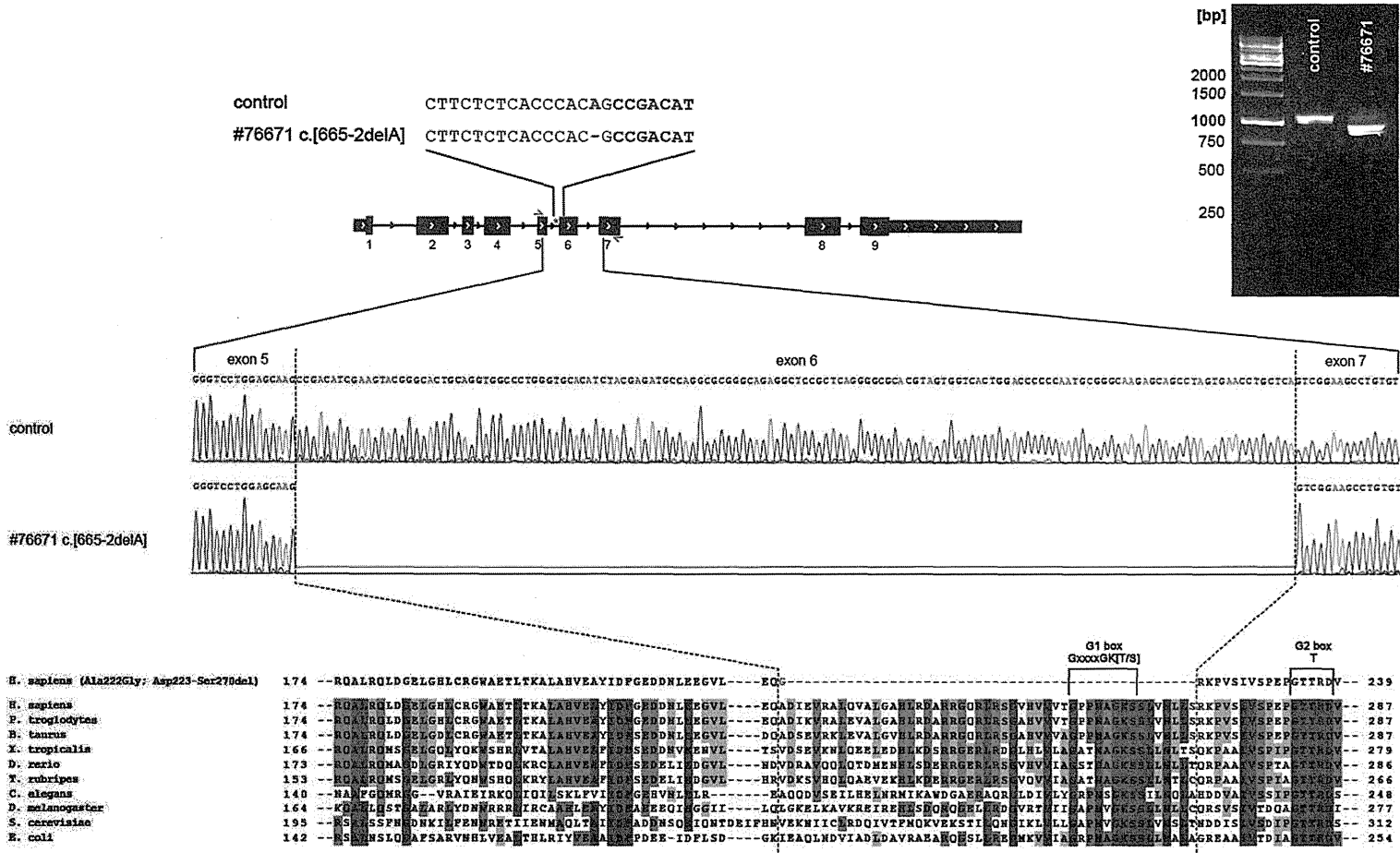
Figure S2

Figure S2) Splice site mutation in individual #76671 causes skipping of exon 6

Analysis of cDNA derived from fibroblasts of individual #76671 yielded a smaller than expected PCR product, indicating alternative splicing. Sanger sequencing revealed that the c.665-2delA mutation affects the conserved splice acceptor site. The splice acceptor upstream of exon 7 is alternatively used, yielding a mature mRNA that lacks exon 6. The resulting protein product is predicted to contain a 1 amino acid exchange followed by a 48 amino acid deletion (p.Ala322Gly; Asp223-270del).
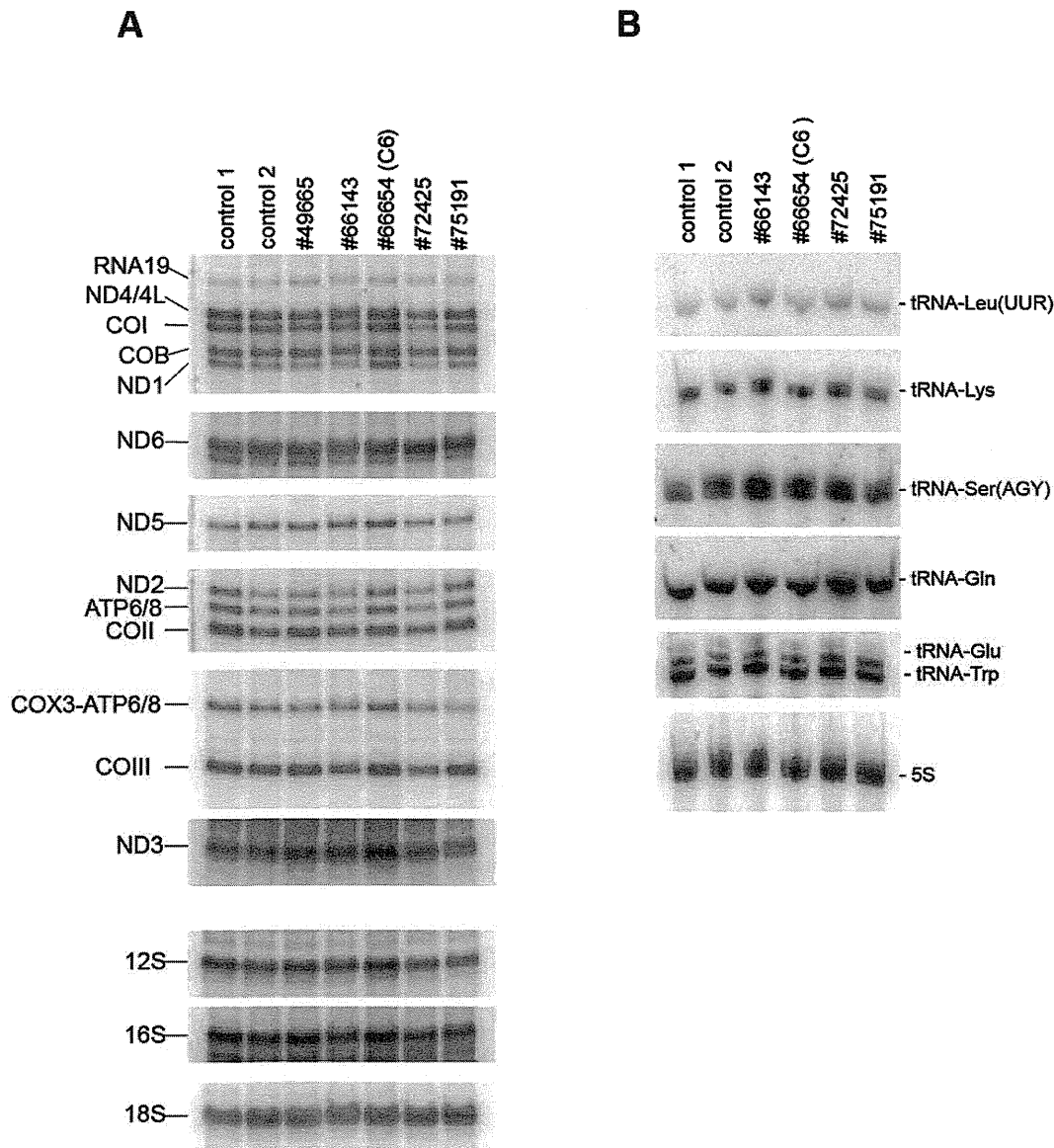
Figure S3

Figure S3) Northern blot analysis of the steady-state levels of mitochondrial transcripts in GTPBP3 patient fibroblasts.

A) Northern blot analysis of total RNA isolated from the *GTPBP3* patient or control primary fibroblasts. The blots were probed with the mt-mRNA- and mt-rRNA-specific probes as indicated. The cytosolic 18S rRNA was used as a loading control.

B)) High-resolution Northern blot analysis of total RNA isolated from the *GTPBP3* patient or control primary fibroblasts. The blots were probed with the mt-tRNA -specific probes as indicated. The cytosolic 5S rRNA was used as a loading control.
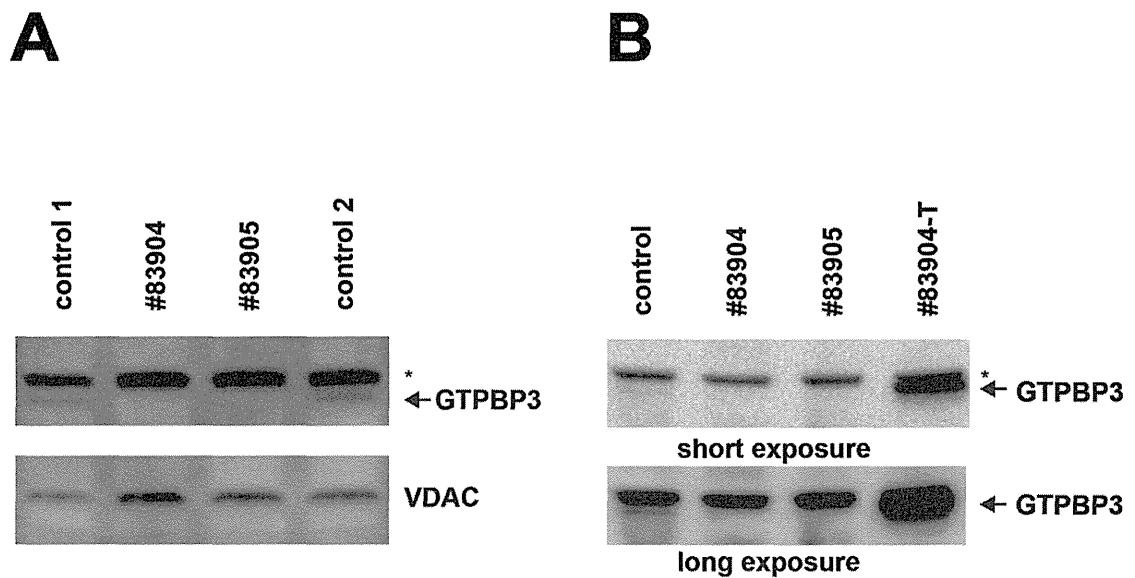
Figure S4

A

B

Figure S4) Analysis of GTPBP3 protein levels in patient fibroblasts

A) Immunoblot analysis of GTPBP3 protein levels in fibroblasts from affected individuals #83904 and #83905 from family F9. VDAC served as a mitochondrial loading control. (Asterics indicates a non-specific band.)

B) Comparison of the electrophoretic migration of GTPBP3 in un-transfected cells (lane control) and cells derived from one of the affected individuals transfected with a plasmid (pIRES2-EGFP) for *GTPBP3* cDNA expression (lane #83904-T). (Asterics indicates a non-specific band.)
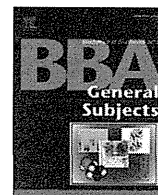
Diagnosis and molecular basis of mitochondrial respiratory chain disorders: Exome sequencing for disease gene identification☆,☆☆

CrossMark

A. Ohtake [a,*], K. Murayama [b], M. Mori [c], H. Harashima [a], T. Yamazaki [a], S. Tamaru [d], Y. Yamashita [d], Y. Kishita [d], Y. Nakachi [d], M. Kohda [d], Y. Tokuzawa [d], Y. Mizuno [d], Y. Moriyama [d], H. Kato [d], Y. Okazaki [d]

[a] Department of Pediatrics, Faculty of Medicine, Saitama Medical University, Saitama 350-0495, Japan
[b] Department of Metabolism, Chiba Children's Hospital, Chiba 266-0007, Japan
[c] Department of Pediatrics, Jichi Medical University, Tochigi 329-0498, Japan
[d] Research Center for Genomic Medicine, Saitama Medical University, Saitama 350-0495, Japan

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ABSTRACT

Mitochondrial disorders have the highest incidence among congenital metabolic diseases, and are thought to occur at a rate of 1 in 5000 births. About 25% of the diseases diagnosed as mitochondrial disorders in the field of pediatrics have mitochondrial DNA abnormalities, while the rest occur due to defects in genes encoded in the nucleus. The most important function of the mitochondria is biosynthesis of ATP. Mitochondrial disorders are nearly synonymous with mitochondrial respiratory chain disorder, as respiratory chain complexes serve a central role in ATP biosynthesis. By next-generation sequencing of the exome, we analyzed 104 patients with mitochondrial respiratory chain disorders. The results of analysis to date were 18 patients with novel variants in genes previously reported to be disease-causing, and 27 patients with mutations in genes suggested to be associated in some way with mitochondria, and it is likely that they are new disease-causing genes in mitochondrial disorders. This article is part of a Special Issue entitled Frontiers of Mitochondrial Research.

1. Introduction

1.1. Mitochondrial disorders

Mitochondrial disorders have the highest incidence among congenital metabolic disorders, and are thought to occur at a rate of 1 in 5000 births [1]. The common view of mitochondrial disorders is that they include mitochondrial encephalopathy and myopathy, with onset due to mitochondrial DNA defects inherited through the maternal line. In fact, however, only about 25% of the diseases diagnosed as mitochondrial disorders in the field of pediatrics have mitochondrial DNA abnormalities [2,3], while the rest occur due to defects in genes encoded in the nucleus. Most cases are sporadic (do not have a clear genetic association), and a majority of cases resulting from nuclear gene abnormalities

are autosomal recessive. Mitochondrial DNA has a circular structure with a length of 16.6 kbp, and encodes only 13 proteins [4]. These 13 proteins are part of the structural composition of complex I (7 proteins), complex III (1 protein), complex IV (3 proteins) and complex V (2 proteins) in the respiratory chain. They do not include any complex II structural proteins. The remaining genes encoded in mitochondrial DNA are 22 tRNAs and two ribosomal RNAs, and mitochondrial disorders due to defects in these RNAs have also been reported. Meanwhile, a certain amount of the gene products encoded in the nucleus exists in the mitochondria, and roughly 1500 are thought to serve important roles in mitochondrial function [5]. In this analysis, we focused on mitochondrial disorders thought to occur due to defects in genes encoded in the nucleus. Mitochondria have many functions, one of the most important being biosynthesis of energy (ATP), and we assume for the following discussion that mitochondrial disorders are nearly synonymous with mitochondrial respiratory chain disorders (MRCD), as respiratory chain complexes [6] serve a central role in ATP biosynthesis.

1.2. Mitochondrial disorders of nuclear origin

As stated above, of the approximately 1500 genes encoded in the nucleus that are thought to be involved in biosynthesis and mitochondrial function, more than 100 have been reported to be causes of mitochondrial disorders [7–9] (Table 1). Among these, about 90% of genes have an autosomal recessive inheritance pattern, and only a small portion

Table 1
The genetic basis of MRCD.

mtDNA mutations: 35/37 genes	
tRNAs, subunits, rRNAs, and deletions & duplications	
Nuclear mutations: 117 genes	
Nuclear-encoded subunits: 27/~80 genes	**mtDNA replication: 5 genes**
Complex I: *NDUFV1, 2, NDUFB3, 9*	*POLG, POLG2, C10orf2, MPV17, AGK*
NDUFA1, 2, 9, 10, 11, 12, NDUFS1, 2, 3, 4, 6, 7, 8	**mtDNA expression: 24 genes**
Complex II: *SDHA, SDHB, SDHC, SDHD*	*LRPPRC, TACO1, MTPAP, MRPS16, MRPS22,MRPL3,*
Complex III: *UQCRB, UQCRQ*	*GFM1,TSFM,TUFM,TRMU,C12orf65,MTFMT, DARS2,*
Complex IV: *COX6B1, COX4I2, COX7B*	*RARS2,YARS2,SARS2,AARS2,HARS2,MARS2,EARS2,*
Complex V: *ATP5E*	*RMND1, MTO1, FARS2, GFM2*
Import, processing, assembly: 38 genes	**Nucleotide transport, synthesis: 9 genes**
Complex I: *C8orf38, C20orf7, NDUFAF1, F2, F3, F4,*	*SLC 25A4, SLC25A3, TYMP, DGUOK, TK2, PUS1,*
FOXRED1, NUBPL, ACAD9 , AIFM1	*SUCLA2, SUCLG1, RRM 2B*
Complex II:*SDHAF1, SDHAF2*	**Membrane composition: 14 genes**
Complex III:*BCS1L, HCCS , TTC19*	*COQ2, COQ6, COQ9, PDSS1, PDSS2, CABC1,*
Complex IV:*SURF1, SCO2, SCO1 , COX10, COX15,*	*SERAC1, MPC1, NMT, TAZ , CYCS, OPA1, MFN2, DNM1L*
ETHE1, FASTKD2, C2orf64, C12orf62	
Complex V:*ATPAF2, TMEM70*	
Multiple:*TIMM8A , SPG7, HSP D1 , AFG3L2 , DNAJC19, GFER*	
Iron/FeS: *FXN, ISCU, GLRX5, ABCB7, NFU1, BOLA3*	
117 nuclear gene defects	Categories are based on D.R Thorburn's paper[7]

95: autosomal recessive·
10: autosomal dominant·
5: recessive *or* dominant·
7: X-linked·

have a dominant inheritance pattern [10]. There have also been seven reported cases of mitochondrial disorders from defects in genes encoded by the X chromosome. By function, these include genes involved in the structural composition of the complexes and mitochondrial biosynthesis, genes involved in membrane composition, genes involved in the synthesis and transport of nucleic acids, genes involved in regulating the expression of mitochondrial DNA, and genes involved in mitochondrial DNA replication.

We have actively analyzed the exomes of patients with MRCD in order to identify the cause. Here, we briefly describe our project and discuss the results of exome analyses performed to date, touching on some of the problems that have been encountered.

2. Outline of exome analysis project for MRCD patients

Fig. 1 outlines our current project. It is supported by the Ministry of Education, Culture, Sports, Science and Technology's Research Program of Innovative Cell Biology by Innovative Technology (Cell Innovation) (http://www.cell-innovation.org/english/html/program/theme_010_okazaki.html). First, analyses of enzyme activity [11], quantity and size were performed using fibroblasts from patient skin or biopsy specimens from diseased organs of patients suspected of having MRCD in clinical practice [12]. Quantity and size were analyzed using blue native polyacrylamide gel electrophoresis (BN-PAGE) [13]. Next, among patients in whom decreased enzyme activity or complex formation abnormalities were seen biochemically, whole exome analysis was performed in those with no known mitochondrial DNA abnormalities, and the obtained candidate causal genes were confirmed at the cellular level by rescue experiment or other methods, such as siRNA experiment. Many patients with mitochondrial disorders have primary symptoms in the central nervous system, but brain biopsy in these patients is untenable. Therefore, induced pluripotent stem (iPS) cells were created using fibroblasts from the skin of patients from whom informed consent was obtained. These iPS cells were then differentiated into neurons and glia cells to reproduce the pathology of mitochondrial dysfunction that occurs specifically in the nervous system, based on the notion that this may lead to treatment at the cellular level and ultimately to treatment in humans.

3. Clinical diagnosis of MRCD

Mitochondria exist in all tissues, and symptoms are presented in various organs and/or pathological entities. In pediatric MRCD, symptoms are broadly divided into: (1) encephalomyopathy symptoms; (2) gastrointestinal/hepatic symptoms; and (3) myocardial symptoms [14]. So-called "mitochondrial encephalomyopathy," which has traditionally been considered the main form of mitochondrial disease, belongs among the relatively mild mitochondrial diseases and occurs mostly in older people. Fig. 2 shows a breakdown of clinical diagnoses of mitochondrial disorders in our institute as of January 2013 [15]. Patients with the traditionally described nerve and muscle symptoms numbered 111 in total, including 50 with Leigh syndrome, 11 with neurodegenerative disorders for which no clear cause could be identified, and 50 with so-called "mitochondrial encephalomyopathy." These 111 patients accounted for 40% of the total of 275 patients. Conversely, other forms accounted for two-thirds of cases, among which were 49 cases of lethal infantile mitochondrial disease (LIMD). Together with non-lethal infantile mitochondrial disease (NLIMD), which follows the same course but in which patients survive beyond 1 year of age, the number reached 71, and was by far the most common clinical diagnosis. LIMD encompasses hyperlactacidemia occurring in the neonatal period together with multiple organ failure. Most cases have poor outcomes, and it is thought that most of these patients died with the cause remaining unknown and no diagnosis established. Next were mitochondrial disorders showing single organ dysfunction only, such as mitochondrial hepatopathy (12%) and cardiomyopathy (7%).

4. Exome analysis of MRCD patients

As most mitochondrial diseases occur sporadically with only a few cases discovered in one family line, linkage analysis using a large pedigree cannot be applied, thus suggesting that we cannot use information on chromosomal localization for causal gene identification. When identifying disease-causing genes using bioinformatics analysis for exome data, knowledge of the inheritance patterns is very important [16]. As approximately 90% of MRCD-causing genes show a recessive mode of
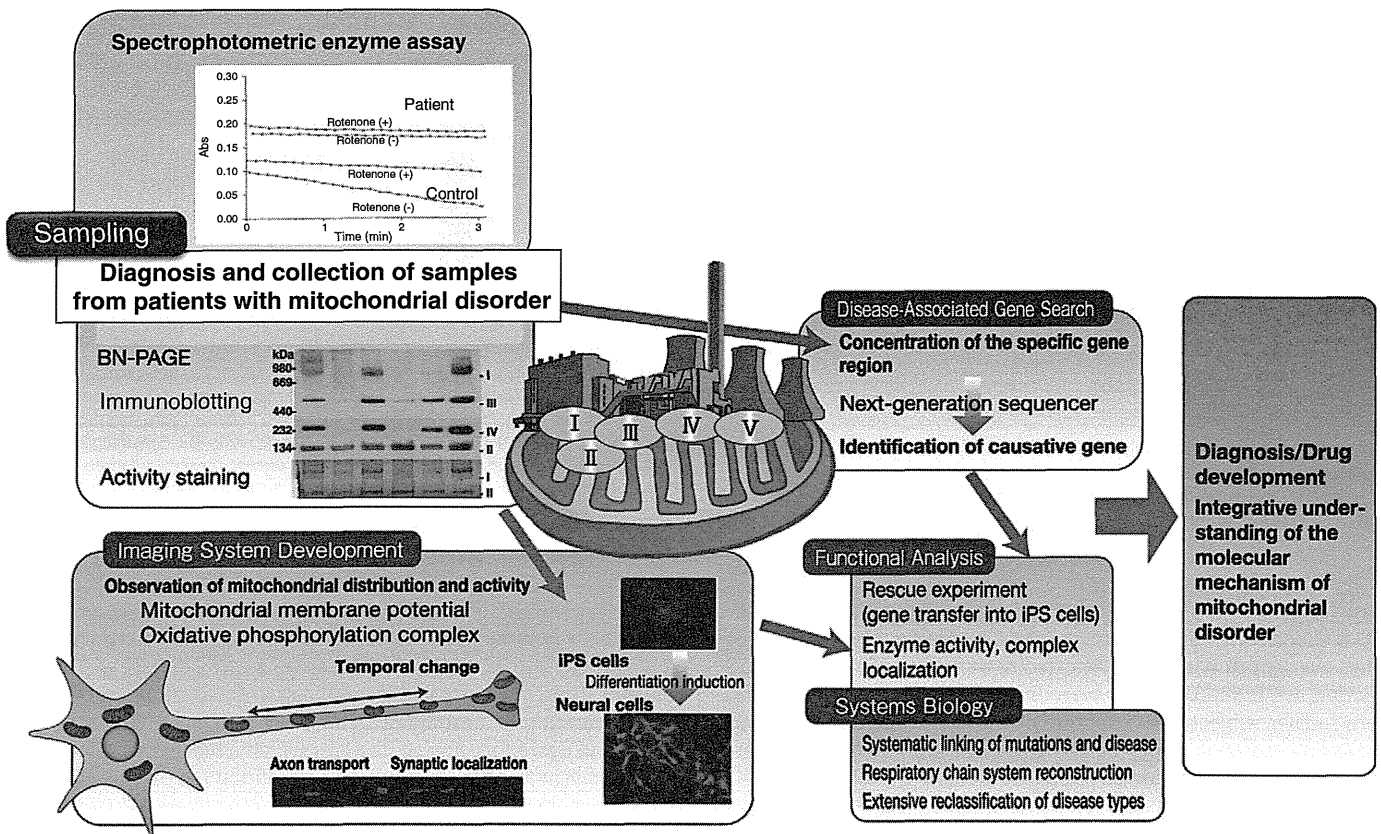
Fig. 1. Outline of exome analysis project for MRCD patients. The first step is 'Sampling', which refers to diagnosis and collection of samples from patients with mitochondrial disorders using both spectrophotometric enzyme assay [11] and BN-PAGE [13]. The next step is 'Disease-Associated Gene Search' using exome analysis. In 'Functional Analysis' and 'System Biology', candidate causal genes are confirmed at the cellular level by rescue experiment or other means. In 'Imaging System Development', induced pluripotent stem cells are created using fibroblasts and differentiated into neurons and glia cells to reproduce the pathology of mitochondrial dysfunction. The final purpose of our project is integrative understanding of the molecular mechanisms of mitochondrial disorders.

inheritance (as shown in Table 1), we prioritized such genes as harboring rare variants in a homozygous or compound heterozygous fashion. Low priority is given to the analysis of genes showing mutation in only one allele because patients and healthy control individuals
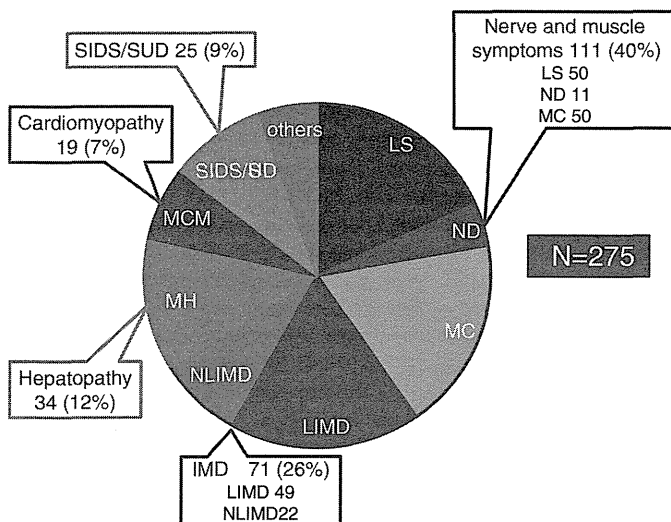
Fig. 2. Breakdown of clinical diagnoses of mitochondrial disorders in our institute as of January 2013. LS, Leigh syndrome; ND, neurodegenerative disorder; MC, mitochondrial cytopathy; IMD, infantile mitochondrial disease (lethal and non-lethal); MH, mitochondrial hepatopathy; MCM, mitochondrial cardiomyopathy; SIDS, sudden infant death syndrome; SUD, sudden unexpected death.

harbored a comparable number of rare heterozygous alleles; we were unable to prioritize dominant-acting genes.

Our current bioinformatics analysis pipeline is as follows: read alignment was performed with a Burrows–Wheeler Aligner (BWA, version 0.7.0) [17] using the 1000 Genomes project phase II reference genome (hs37d5.fa). PCR duplicate reads were removed using Picard (version 1.89) (http://picard.sourceforge.net) and non-mappable reads were removed using SAMtools (version 0.1.19) [18]. After filtering out these reads, the Genome Analysis Toolkit (GATK) version 2.4-9-nightly-2013-04-12-g3fc5478 [19] was used to realign insertions and deletions, and for quality recalibration and variant calling (UnifiedGenotyper). Detected variants were annotated using ANNOVAR (version 2013Feb21) [20] and custom ruby scripts. The effect of the mutations on protein function was assessed by SIFT and GERP using dbNSFP [21]. The positions of mutations were based on RefSeq transcript sequences. Variants were assessed by comparing allele frequencies in the dbSNP135, Exome Sequencing Project (ESP5400) data set, and 1000 Genomes Projects (based on phase 1 release v3 called from 20101123 alignments). As mitochondrial disorders are rare, we excluded variants present in dbSNP with a frequency > 0.1%. After filtering out these variants, the VAAST program [22] was used to create a candidate gene list in each patient showing recessive characteristics.

As stated above, because mitochondrial disease patients have very high heterogeneity, the number of patients sharing the same gene mutation is quite low. Hence, attention should be directed towards removing these mutations from the disease candidates when the same amino acid substitutions are shared among multiple patients in our study, because these variants are highly likely to be SNPs unique to the Japanese population. Using these criteria, we are able to narrow down the number of variants to a mean of several genes for each patient. After listing

Annotate variants — 118,700 variants/sample

Variant filtering

Convert to gene-level list — 23 genes/sample (Min. 11, Max 49)

Genes previously reported to cause MRCD (knowledge driven) — 0 ~ 1 genes/sample

Genes suggested to have some type of relationship with mitochondria (knowledge driven) — 0 ~ 3 genes/sample
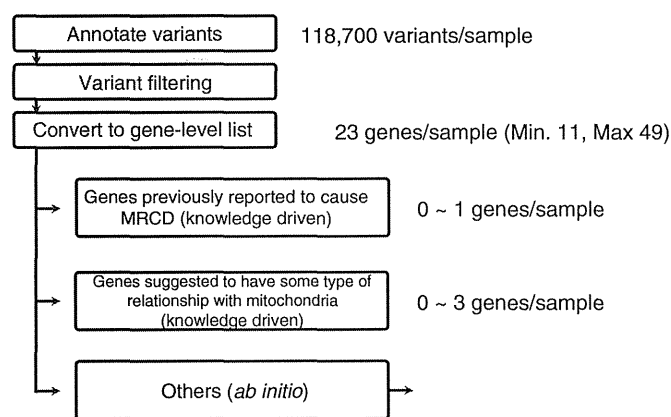
Others (ab initio)

Fig. 3. Narrowing down of gene mutations discovered by exome analysis. After filtering out variants with the methods described in the 'Exome analysis of MRCD patients' section, genes were divided into three categories: (1) those that have previously been reported to cause MRCD; (2) those for which some relationship with mitochondria has been suggested; and (3) others (ab initio).

(N = 104)

■ Reported genes responsible for MRCD
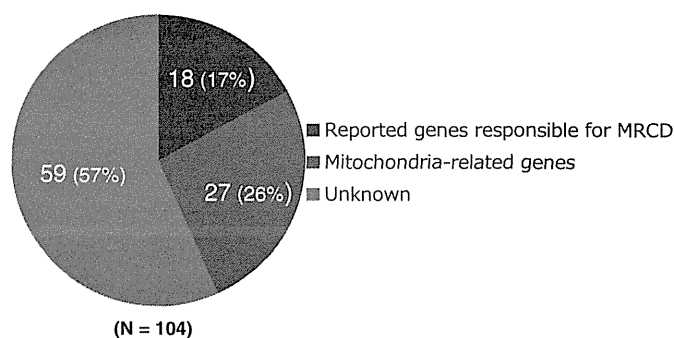■ Mitochondria-related genes
■ Unknown

Fig. 4. Candidate genes with exome analysis for MRCD patients. Results of analysis for 104 patients to date (as of January 2013) are shown. Eighteen patients (17%) had variants previously reported to be disease-causing. Twenty-seven patients (26%) had mutations in genes suggested to be associated somehow with mitochondria. The remaining 59 patients (57%) are highly likely to contain completely novel disease-causing mutations for which no data have been obtained to date.

these candidate variants, we further investigated whether these variants are located within genes related to mitochondrial function. When genes overlapped with those reported to be related to mitochondrial function, we found that they were likely to be causative genes and were further subjected to experimental analysis such as haplotype phasing or functional assay including rescue experiments. To prepare a list of genes reported to be related to mitochondria, we included genes annotated as somehow related to mitochondria in the UniProt (http://www.uniprot.org/) [23] database, as well as the MitoCarta database (http://www.broadinstitute.org/pubs/MitoCarta/index.html) [24], which includes approximately 1000 gene products listed with the use of shotgun proteomics and mitochondrial localization analysis.

We also investigated whether there is Long Contiguous Stretch of Homozygosity (LCSH) using Affymetrix SNP arrays in a majority of patients. Although no cases of consanguineous marriage were reported in the interviews with the primary physician, about 5% of cases harbor LCSH proven by SNP arrays. When homozygous mutations are localized in these LCSH regions, the mutations are highly likely to be causative of disease.

5. Results of exome analysis for MRCD patients

The variants (mutations) found in the process of narrowing down the gene mutations discovered to date are shown in Fig. 3. These genes were narrowed down to the final candidate genes and divided into three categories: (1) those that have previously been reported to cause MRCD; (2) those for which some relationship with mitochondria has been suggested; and (3) others (ab initio). The results of analysis of 104 patients to date (as of January 2013) are shown in Fig. 4. Eighteen patients (17%) had variants previously reported to be disease-causing. Among these 18 patients, one had a homozygote of a previously reported mutation and two had a compound heterozygote of a reported and a novel mutation (data not shown). All other mutations found in this study were new. Twenty-seven patients (26%) had mutations in genes suggested to be associated somehow with mitochondria, and it is likely that they are novel disease-causing genes in mitochondrial disorders. Table 2 lists the functions of the genes in these 27 cases. For the remaining 59 cases, each patient has about 20 gene variants that are unique to each patient, and it is necessary to confirm whether any of these mutations can actually cause the disease. These 59 patients are highly likely to contain completely novel disease-causing mutations for which no clues have been obtained to date. The biggest issue we currently face is how to confirm the disease-causing gene from these 20 gene variants for each patient.

6. Conclusion and future prospects

The above describes the progress we have made in exome analysis of neonatal or infantile MRCD patients. While we have identified many candidate genes, the causes of MRCD are extremely diverse and heterogeneous. Thus, in many cases, it is difficult to demonstrate conclusively that a mutation in a candidate gene is the true cause. We have performed analyses focusing on cases in which a biochemical diagnosis was established at the cellular level in addition to clinical symptoms such as enzyme activity and complex formation abnormalities. Nonetheless, confirmation of the causal genes with rescue experiments or other means is difficult. In the future, it will be necessary to increase the case number or search for patients with similar symptoms and similar gene mutations in collaboration with researchers throughout the world. We are currently conducting analyses of pediatric patients with a focus on MRCD, and gene mutations (amino acid substitutions) harbored by patients of the childhood onset type are probably variants conferring major damage on enzyme activity or protein function. Onset is also thought to occur in adulthood rather than in childhood in some cases of milder (hypomorphic: partial loss of function) variants with the same gene defect. As these are thought to include nerve diseases,

Table 2
Functions of new disease-causing candidate genes for MRCD.

MtoX#1	Non-receptor tyrosine kinase
MtoX#2	Acyl-CoA thioesterase
MtoX#3	Fatty acid β oxidation
MtoX#4	tRNA synthetase
MtoX#5	ABC transporter superfamily
MtoX#6	ATR-dependent AMP-binding enzyme family
MtoX#7	Heme biosynthesis
MtoX#8	AAA ATPase family
MtoX#9	Pre-mRNA splicing factor
MtoX#10	Creatine kinase
MtoX#11	Synaptic transmission
MtoX#12	Synthesis of Coenzyme Q
MtoX#13	Heme biosynthetic process
MtoX#14	Citrate synthase family.
MtoX#15	Cholesterol metabolism
MtoX#16	Mitochondrial fission
MtoX#17	Muscle organ development
MtoX#18	Cholesterol biosynthetic process
MtoX#19	Ribosomal protein
MtoX#20	Tumor suppressor
MtoX#21	A component of complex I
MtoX#22	A protease, located in inner membrane
MtoX#23	Regulation of PDH
MtoX#24	Mitochondrial translation
MtoX#25	Queuosine biosynthetic process
MtoX#26	Mitochondrial carrier family
MtoX#27	Methyltransferase superfamilya

mental disorders, and diabetes or other metabolic diseases of unknown cause, we plan to conduct research based on the assumption that such cases include those caused by abnormalities in genes identified in MRCD patients.

Acknowledgements

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SHORT COMMUNICATION

Homoplasmy of a mitochondrial 3697G>A mutation causes Leigh syndrome

Yutaka Negishi[1], Ayako Hattori[1], Eri Takeshita[2], Chika Sakai[2], Naoki Ando[1], Tetsuya Ito[1], Yu-ichi Goto[2] and Shinji Saitoh[1]

Herein we report on three siblings with Leigh syndrome (LS) harboring a homoplasmic m.3697G>A mutation (G131S) in the MT-ND1 gene. The siblings' phenotypically normal mother had the same, albeit heteroplasmic, mutation. Complex I deficiency (8% of average control values) was demonstrated in a biceps brachii muscle from one of the patients. Heteroplasmic m.3697G>A has been reported in patients with Leber's hereditary optic neuropathy, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes and Stüve–Wiedemann syndrome. Because all three patients in this series carried m.3697G>A in a homoplasmic manner and had LS, we suggest that homoplasmy of m.3697G>A may cause the LS phenotype.
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INTRODUCTION

Leigh syndrome (LS) is a subacute necrotizing encephalomyelopathy characterized by bilateral symmetric necrotic lesions of gray matter nuclei in the basal ganglia, diencephalon, cerebellum, or brainstem.[1] Mutations in both nuclear- and mitochondrial-encoded genes involved in energy metabolism cause LS,[1] with an increasing number of new mutations reported in recent years. The heteroplasmic mitochondrial mutation m.3697G>A has been identified in patients with Leber's hereditary optic neuropathy, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes and Stüve–Wiedemann syndrome,[2–5] indicating the pathogenicity of the mutation in mitochondrial disorders. Herein we report on three siblings with LS who harbored a homoplasmic m.3697G>A mutation, extending the significance of this mutation to the pathogenesis of LS.

CASE REPORTS

The present study was approved by the Institutional Review Board of the National Center of Neurology and Psychiatry, and the parents of the three children provided written informed consent.

The three siblings in the present study were born from healthy parents and had no other siblings. Patient 1, a 9-year-old girl, presented with progressive gait disturbance from 1 year and 6 months of age. Brain magnetic resonance imaging at 2 years of age showed high signal intensity areas in the bilateral putamen and caudate nucleus on T_2-weighted images (Figure 1a). Patient 1 was given a hearing aid when she was 3 years old. At the time of writing, Patient 1 exhibited dystonia of the upper and lower limbs but was able to walk slowly with support and was able to speak in sentences.

Patient 2, a 7-year-old boy, started walking at 1 year of age; however, rigidity of the upper and lower limbs became prominent at 2 years of age. Brain magnetic resonance imaging revealed high signal intensity areas in the bilateral putamen, left globus pallidus, caudate nucleus and thalamus on T_2-weighted images (Figure 1b). Currently, Patient 2 is showing progression of dystonia and cannot stand without support or speak in sentences.

Patient 3, a 5-year-old girl, started walking at 1 year and 3 months of age; however, she lost the ability to walk at 1 year and 6 months of age. Brain magnetic resonance imaging revealed high signal intensity areas in the bilateral putamen, caudate nucleus, globus pallidus and brainstem on T_2-weighted images (Figure 1c). At 2 years of age, Patient 3 suddenly developed respiratory failure and muscle spasticity of the limbs and was started on artificial respiration. A tracheostomy and gastrostomy were performed, and Patient 3 is currently bedridden and being managed with artificial respiration at home. In all three siblings, the lactic acid concentration in the cerebrospinal fluid and blood was not elevated compared with the normal range (Supplementary Table).

Biochemical studies

The enzymatic activity of individual respiratory chain complexes was measured in a biceps brachii muscle specimen from Patient 1 and equivalent specimens from normal controls (n = 5) according to the methods of Trounce et al.[6] The activity of Complexes I–V is expressed relative to that of citrate synthase.[7] Only the activity of Complex I was significantly reduced compared with control values (8% of average control values; Table 1).

[1]Department of Pediatrics and Neonatology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan and [2]National Center of Neurology and Psychiatry, Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, Kodaira, Japan
Correspondence: Dr S Saitoh, Department of Pediatrics and Neonatology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-Cho, Mizuho-Ku, Nagoya 467-8601, Japan.
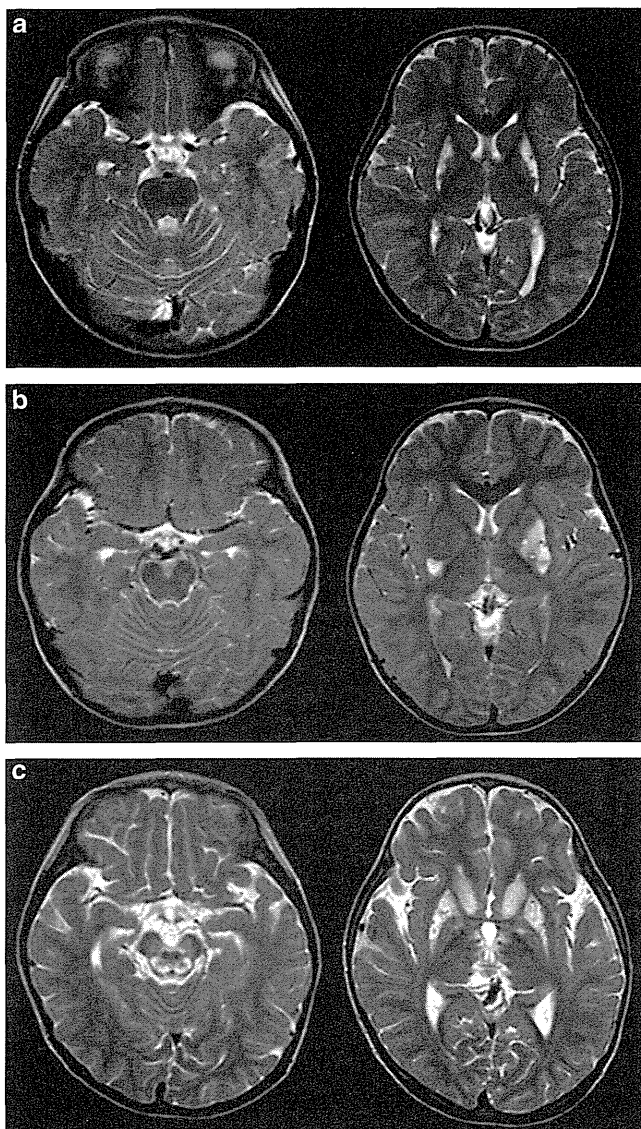E-mail: ss11@med.nagoya-cu.ac.jp

Figure 1 Magnetic resonance imaging findings in (a) Patient 1, (b) Patient 2 and (c) Patient 3. Axial T₂-weighted images (TR 4600, TE 100) show high signal intensity areas in the bilateral putamen and caudate nucleus of Patient 1 (a), bilateral putamen, left globus pallidus, caudate nucleus and thalamus of Patient 2 (b), and bilateral putamen, caudate nucleus, globus pallidus and brainstem of Patient 3 (c).

Table 1 Enzymatic activity of mitochondrial respiratory complexes

	Patient 1	Control (n= 5); range (mean ± s.d.)
Complex I/CS	0.16	0.58–4.7 (1.9 ± 1.6)
Complex II/CS	1.7	1.2–3.9 (2.4 ± 1.1)
Complex III/CS	1.5	1.9–3.7 (2.3 ± 0.8)
Complex IV/CS	1.5	1.2–3.2 (1.9 ± 0.8)
Complex V/CS	3.5	2.7–3.5 (3.0 ± 0.33)

Abbreviation: CS, citrate synthase.

Mitochondrial DNA (mtDNA) analyses

We carried out entire mtDNA sequencing after PCR amplification (primer sequences are available on request) as described elsewhere.[8] Amplified fragment were directly sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and were run on an ABI 3700 automated sequencer (Applied Biosystems). Entire mtDNA sequencing of a biceps brachii muscle specimen from Patient 1 revealed that this patient harbored a homoplasmic m.3697G>A substitution (G131S). Subsequently, this mutation was confirmed using blood samples from all three siblings and their parents. All three siblings had a homoplasmic m.3697G>A mutation, whereas their mother had a heteroplasmic m.3697G>A mutation; the mutation was not present in their father (Figure 2a).

DISCUSSION

The m.3697G>A mutation is located within the protein-coding region of the gene encoding ND1, a subunit of electron transport chain enzyme Complex I.[2] Complex I is the first and largest enzyme of the respiratory chain, coupling electron transfer between NADH and ubiquinone to the translocation of four protons across the membrane.[9] Complex I consists of 44 subunits.[10] Many mutations in the subunits of Complex I have been associated with human neurodegenerative diseases.[11,12] The m.3697G>A mutation changes the 131st glycine into serine. This glycine is a highly conserved residue (Figure 2b) and is located in the loop between transmembrane domains C and D.[2]

Heteroplasmic m.3697G>A mutations have been reported in patients with Leber's hereditary optic neuropathy, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes and Stüve–Wiedemann syndrome[2–5] (Supplementary Table). The m.3697G>A mutation has been reported previously in a patient with LS, but detailed clinical information or the proportion of mutant mtDNA was not available.[13] Because all three patients in our series carried homoplasmic m.3697G>A mutations and had LS, it is suggested that homoplasmy of m.3697G>A may cause the LS phenotype. Heteroplasmy of m.3697G>A may cause various mitochondrial disorders that presumably depend on the level of heteroplasmy. A correlation between mutation load and disease severity has been clearly demonstrated for the m.8993T>G and m.8993T>C mutations, in which case homoplasmy or a high mutation load causes LS, whereas lower mutation loads result in a less severe phenotype.[14] Three other mutations in the gene encoding the ND1 subunit have been reported to be associated with LS, but these mutations are present in a heteroplasmic fashion.[13] Therefore, of the MT-ND1 gene mutations identified to date, m.3697G>A is unique in causing LS only with an extremely high mutation load.

In mitochondrial disorders, lactic acid concentrations in the cerebrospinal fluid and blood are often increased;[15] in the present study, lactic acid concentrations in the cerebrospinal fluid and blood were only marginally increased in all three siblings. However, this is similar to findings in other patients with mitochondrial disease due to m.3697G>A (Supplementary Table).

The mechanism whereby the three siblings developed homoplasmy, whereas their mother carried the mutation in a heteroplasmic manner, could be explained by the bottleneck theory.[16] Amounts of mtDNA significantly decrease in early development of oocytes, and thus random selection of the mutated mtDNA could result in extensive skewing. Investigation of the ovary tissue could uncover the theory, but it is not possible to prove this theory in the pedigree in the present study because of lack of access to the ovarian tissue from the mother. Nonetheless, the bottleneck theory should be taken into account in the case of genetic counseling for mitochondrial disorders.
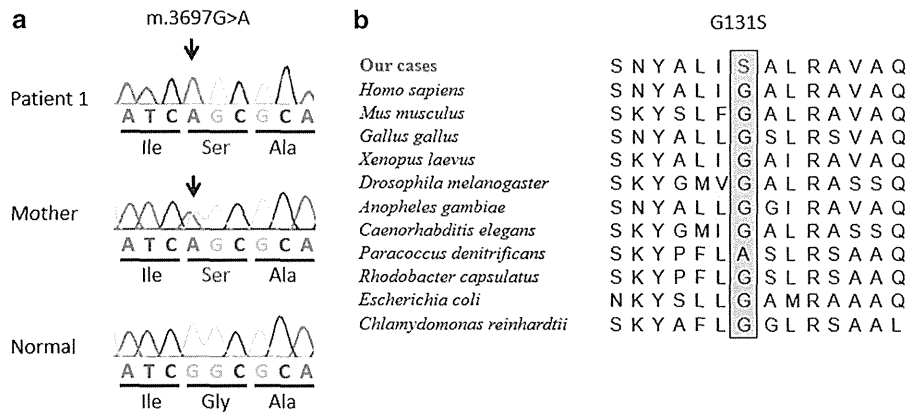
Figure 2 Genetic analysis in the pedigree. (a) Sequence analysis of mitochondrial (mt) DNA from Patient 1 and her mother revealed that the m.3697G>A substitution (arrow) was homoplasmic in Patient 1 and heteroplasmic in her mother. (b) Phylogenetic conservation of the mutated amino-acid residue.

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Case report

Leigh syndrome with Fukuyama congenital muscular dystrophy: A case report

Hidehito Kondo [a,b,*], Koichi Tanda [a], Chihiro Tabata [a], Kohei Hayashi [a], Minako Kihara [a], Zenro Kizaki [a], Mariko Taniguchi-Ikeda [c], Masato Mori [d], Kei Murayama [e], Akira Ohtake [f]

[a] Department of Pediatrics and Neonatology, Japanese Red Cross Kyoto Daiichi Hospital, Japan
[b] Department of Pediatrics, Osaka University Graduate School of Medicine, Japan
[c] Division of Pediatrics, Kobe University Graduate School of Medicine, Japan
[d] Department of Pediatrics, Jichi Medical University, Japan
[e] Department of Metabolism, Chiba Children's Hospital, Japan
[f] Department of Pediatrics, Saitama Medical University Hospital, Japan

Abstract

We report the first case of Leigh syndrome (LS) with Fukuyama congenital muscular dystrophy (FCMD). A neonate suffered from lactic acidosis and subsequently presented with poor feeding, muscle weakness, hypotonia, cardiopulmonary dysfunction, and hydrocephalus. He died at 17 months. The findings of brain magnetic resonance imaging indicated some specific features of both LS and FCMD, and FCMD gene mutation was detected. Decreased mitochondrial respiratory complex I and II activity was noted. Mitochondrial DNA sequencing showed no pathogenic mutation. A case with complex I + II deficiency has rarely been reported, suggesting a nuclear gene mutation.
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Keywords: Leigh syndrome; FCMD; Mitochondria; Complex I + II deficiency

1. Introduction

Fukuyama congenital muscular dystrophy (FCMD), one of the most common autosomal recessive disorders in the Japanese population, is characterized by congenital muscular dystrophy with cortical dysgenesis. The gene responsible for FCMD is located on 9q31. Most FCMD-bearing chromosomes (87%) have a 3-kb retrotransposal insertion in the 3′-untranslated region of the gene [1].

Leigh syndrome (LS) is a progressive neurodegenerative disorder with psychomotor retardation, signs and symptoms of brain stem and/or basal ganglia involvement, and raised lactate levels in blood and/or cerebrospinal fluid (CSF). In majority of the cases, dysfunction of the mitochondrial respiratory chain is responsible for the disease. LS is caused by either mitochondrial or nuclear gene mutations with large genetic heterogeneity [2]. Here, we report the first case of LS with FCMD.

2. Case report

2.1. Index case

A Japanese boy was born at term as the third child to non-consanguineous healthy parents. His serum creatine

* Corresponding author at: Department of Pediatrics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan. Tel.: +81 6 6879 3932; fax: +81 6 6879 3939.
E-mail address: hkondo@ped.med.osaka-u.ac.jp (H. Kondo).

kinase concentration was extremely high (45149 IU/L) on the day of birth without any anomaly. Serum lactate level, plasma amino acid profiles, and carnitine profiles were normal. Urinary organic acid profiles showed no specific abnormalities. The patient suddenly suffered from severe lactic acidosis, hyperglycemia, and acute heart failure at day 17. Levels of lactate and pyruvate in the CSF were 4.9 mM and 0.21 mM. A mitochondrial disorder was suspected and treatment was started with carnitine, ubiquinone, and other vitamins in addition to cardiotonics and insulin. The infant's condition improved, but he subsequently presented with poor feeding, muscle weakness, and hypotonia at 1 month. Hypertrophic cardiomyopathy occurred at 3 months and cardiopulmonary function worsened after repeated lactic acidosis, and he required mechanical ventilation from the age of 6 months. He presented with an enlarged head circumference and a tense anterior fontanelle at 12 months, and died of pneumonia at 17 months.

Magnetic resonance imaging (MRI)at 2 months revealed cerebellar cysts, pachygyria, and T2-hyperintense lesions in white matter and the brainstem, but basal ganglia were normal (Fig. 1A). A follow-up investigation at 4 months indicated extended T2-hyperintense lesions (Fig. 1B). A brain computed tomography (CT) scan at 14 months showed severe hydrocephalus and extensive cerebral atrophy (Fig. 1C).

Cerebellar cysts and pachygyria are characteristic of FCMD, genetic testing for *FCMD* was performed. We examined retrotransposal insertion into the 3′-untranslated region (UTR) of the *FCMD* gene using a polymerase chain reaction (PCR)-based diagnostic method involving peripheral blood leukocytes of this case and his parents [1]. A homozygous mutation of this case and heterozygous mutation of his parents were detected. Repeated lactic acidosis and brain stem lesions led us to suspect LS. A skin biopsy was performed for mitochondrial analysis at 1 month. Activities of mitochondrial respiratory chain complex (Co) I, II, III, and IV were assayed from skin fibroblasts, as described previously [3]. The activities were also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker and to Co II activity, and evaluated according to the diagnostic criteria [4]. Respiratory chain complex I and II activities were very low, but CS, Co III, and Co IV activities were normal (Table 1). Expression of the mitochondrial respiratory chain CoI, II, III, and IV proteins was concurrently examined by Western blotting
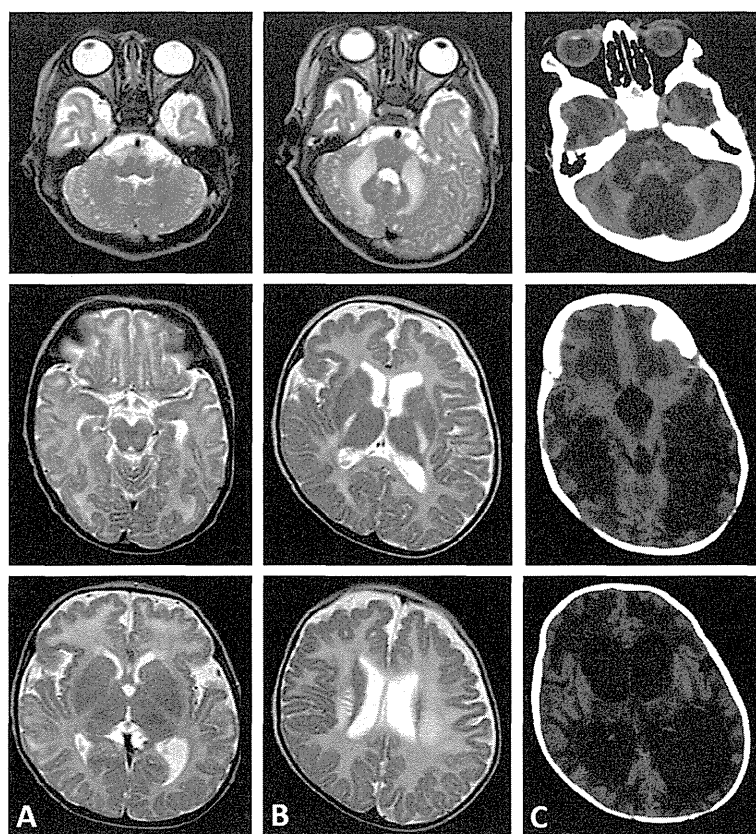
Fig. 1. Magnetic resonance image (MRI) at the age of 2 months (A) shows cerebellar cysts (A, top), bilateral symmetrical lesions in the brainstem (A, middle), pachygyria, and T2-hyperintensity in white matter, predominantly in the frontal lobes (A, bottom). An MRI at 4 months of age indicated T2-hyperintensity extending into the middle cerebellar peduncles, posterior limb of the internal capsule, and the corona radiata (B). A brain computed tomography scan at 14 months of age showed severe hydrocephalus, widespread hypodensity of white matter, and extensive cerebral atrophy (C).

Table 1
Activities of mitochondrial respiratory chain complex (Co) I, II, III, and IV; citrate synthase (CS) from skin fibroblasts. Enzyme activities are expressed as a percentage of mean relative activity of 35 normal controls and relative to CS and Co II.

	Co I	Co II	Co II + III	Co III	Co IV	CS
Crude activity (%)	32	18	21	56	45	80
CS ratio (%)	38	21	24	65	55	
Co ll ratio (%)	177		112	312	259	
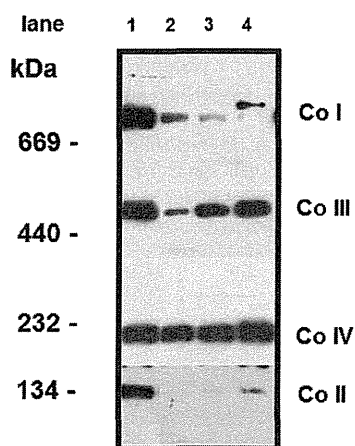
Fig. 2. Blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis of skin fibroblasts 1: control; 2: this case; 3: a double of lane 2; 4: a triple of lane 2. The bands corresponding to Co II were almost invisible and those corresponding to Co I were markedly weak, whereas the intensities of the Co III and IV bands gradually became strong.

using blue native polyacrylamide gel electrophoresis (BN-PAGE), as described previously [5]. The BN-PAGE analysis showed that the bands corresponding to Co II were almost invisible and those corresponding to Co I were markedly weak (Fig. 2). This finding was in agreement with the enzyme activity assay results. The mitoSEQr™ system (Applied Biosystems, Foster City, CA, USA) was used for the entire mitochondrial DNA analysis. Genomic DNA was extracted from skin fibroblasts. Data were analyzed with SeqScape Software v2.5 and compared with mitochondrial DNA sequences (Mitomap: www.mitomap.org). Several base substitutions were detected, but no pathogenic mutation was detected in the entire mitochondrial DNA sequence. High resolution chromosome analysis was normal.

2.2. Family history

The index case's older brother had repeated afebrile convulsions since the age of 2 months, but brain MRI findings were normal. Laboratory tests showed elevated level of lactate (2.92 mM) in CSF and a normal level of serum creatine kinase (75 IU/L). He died of sudden cardiac dysfunction at 4 months. The second child was a healthy girl with normal development.

3. Discussion

A case of FCMD and mitochondrial respiratory chain disorder (MRCD) has never been reported. The pathophysiology of FCMD and MRCD is quite different, therefore, low activities of the respiratory chain complexes in this case were probably not due to FCMD. LS is clinically characterized by a wide variety of manifestations involving multiple organs in infancy or early childhood. Thus, the early onset of his symptoms suggested that LS was the main cause.

White matter abnormalities in patients with FCMD are often detected by MRI as transient T2-hyperintensity. Kato et al. reported that the pathological origin of white matter lesions is dysmyelination and that the lesions are masked by brain development [6]. In this case, the extended signal abnormalities had different features compared with those of FCMD. Some cases of complex II deficiency with extensive T2-hyperintensities in white matter have been reported [7,8]. The white matter abnormalities in our case may have been associated with the complex II deficiency. The patient presented with progressive hydrocephalus, but he had no prior clinical signs of intraventricular hemorrhage or infection in CSF. Patients with FCMD, who are homozygotes for the insertion mutation with hydrocephalus have never been reported [9]. A few patients with LS develop cerebellar atrophy or ventricular enlargement [10].

Many cases of combined complex deficiencies have been reported, but a case with a complex I + II deficiency has rarely been reported. The entire mitochondrial DNA sequencing in this case showed no pathogenic mutation. These findings suggest that LS in this case was the result of a nuclear gene mutation.

The genes responsible for mitochondrial disease located contiguous to the FCMD gene have not been identified. The infant's older brother was suspected to have MRCD without obvious clinical signs of FCMD. Therefore, we speculated that the present case was unlikely to be a contiguous gene syndrome. We are investigating this patient's fibroblasts using next-generation sequencing to identify the causative nuclear gene mutation and the relation between the two diseases.

Potential conflict of interest report

The authors indicated no potential conflict of interest.

Acknowledgments

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Original Article

Molecular diagnosis of mitochondrial respiratory chain disorders in Japan: Focusing on mitochondrial DNA depletion syndrome

Taro Yamazaki,[1,6] Kei Murayama,[3] Alison G Compton,[6] Canny Sugiana,[6]* Hiroko Harashima,[1] Shin Amemiya,[1] Masami Ajima,[3] Tomoko Tsuruoka,[3] Ayako Fujinami,[3] Emi Kawachi,[3] Yoshiko Kurashige,[4] Kenshi Matsushita,[4] Hiroshi Wakiguchi,[4] Masato Mori,[5] Hiroyasu Iwasa,[2] Yasushi Okazaki,[2] David R Thorburn[6] and Akira Ohtake[1]

[1]Department of Pediatrics, Faculty of Medicine and [2]Translational Research Center, International Medical Center, Saitama Medical University, Saitama, [3]Department of Metabolism, Chiba Children's Hospital, Chiba, [4]Department of Pediatrics, Kochi Medical School Kochi University, Kochi, [5]Department of Pediatrics, Jichi Medical University, Tochigi, Japan and [6]Murdoch Childrens Research Institute, Royal Children's Hospital and Department of Paediatrics, University of Melbourne, Melbourne, Victoria, Australia

Abstract   **Background**: Although mitochondrial respiratory chain disorders (MRCD) are one of the most common congenital metabolic diseases, there is no cumulative data on enzymatic diagnosis and clinical manifestation for MRCD in Japan and Asia.
Methods: We evaluated 675 Japanese patients having profound lactic acidemia, or patients having symptoms or signs of multiple-organ origin simultaneously without lactic acidemia on respiratory chain enzyme activity assay and blue native polyacrylamide gel electrophoresis. Quantitative polymerase chain reaction was used to diagnose mitochondrial DNA depletion syndrome (MTDPS). Mutation analysis of several genes responsible for MTDPS was also performed.
Results: A total of 232 patients were diagnosed with a probable or definite MRCD. MRCD are common, afflicting one in every several thousand people in Japan. More than one in 10 of the patients diagnosed lacked lactic acidemia. A subsequent analysis of the causative genes of MTDPS identified novel mutations in six of the patients. A 335 bp deletion in deoxyguanosine kinase (*DGUOK*; g.11692_12026del335 (p.A48fsX90)) was noted in two unrelated families, and may therefore be a common mutation in Japanese people. The proportion of all patients with MTDPS, and particularly those with recessive *DNA polymerase γ (POLG)* mutations, appears to be lower in Japan than in other studies. This is most likely due to the relatively high prevalence of ancient European *POLG* mutations in Caucasian populations. No other significant differences were identified in a comparison of the enzymatic diagnoses, disease classifications or prognoses in Japanese and Caucasian patients with MRCD.
Conclusion: MTDPS and other MRCD are common, but serious, diseases that occur across all races.

Key words   *DGUOK* deletion mutation, enzymatic diagnosis, mitochondrial DNA depletion syndrome, mitochondrial respiratory chain disorder, racial difference.

Mitochondrial respiratory chain disorders (MRCD) are disorders of the oxidative phosphorylation system, which is responsible for ATP production. MRCD are the most common congenital metabolic diseases, afflicting at least 1 in 5000 persons.[1] Mitochondrial DNA depletion syndrome (MTDPS), in which mitochondrial DNA (mtDNA) level is lower than normal, is one of the major MRCD. A number of responsible genes of MTDPS have been identified, and the pathophysiology of this disease is partially characterized at the molecular level.[2–5] We have previ-

ously diagnosed and characterized MRCD cases in Japan using respiratory chain enzyme analysis.[6–9] Having recently analyzed the molecular diagnoses and clinical manifestations of MRCD in Japanese patients, and analyzing several genes responsible for hepatocerebral MTDPS, we herein discuss and compare the collected data to those reported for MRCD outside of Japan.

Methods

Patients and samples

The subjects consisted of patients clinically suspected of having MRCD. We measured respiratory chain enzyme activity and quantity for patients with profound lactic acidemia, or patients with symptoms or signs of multiple-organ origin simultaneously without lactic acidemia. Other metabolic disorders were excluded on plasma tandem mass spectrometry and urine organic acid analysis. Approximately half of candidates were <1 year old,

and nearly 90% were <10 years old. In total, 1051 samples from 675 patients in 657 families were analyzed. Of the samples, 479 were cultured skin fibroblast cells, 239 were liver samples, 208 were muscle samples, 84 were myocardial samples, and 41 were other samples (including 25 kidney and seven brain samples).

Respiratory chain enzyme analysis

Both an *in vitro* respiratory chain enzyme activity assay[10] and blue native polyacrylamide gel electrophoresis (BN-PAGE)[11–13] were used to quantify the activity and amount of respiratory chain enzyme complexes. A diagnosis of MRCD was made when the results from the enzyme activity or BN-PAGE raised the diagnostic criteria assessment to definite or probable for MRCD according to the diagnostic criteria of Bernier *et al.*[14]

Entire mtDNA analysis

DNA was purified according to standard methods. The mitoSEQr™ system (Applied Biosystems, Foster City, CA, USA) was used for entire mtDNA analysis in each patient diagnosed with MRCD.

Quantitative polymerase chain reaction for diagnosis of MTDPS

Quantitative polymerase chain reaction (qPCR)[15] was used to determine whether mtDNA depletion was present in patients with decreased activity level of multiple respiratory chain enzymes (the mtDNA gene *MT-ND1* was compared against a nuclear gene, *CFTR* exon 24). A diagnosis of MTDPS was made when the relative copy number of mtDNA to nuclear DNA was <35% of that in healthy control tissue using four independent experiments.

Mutation analysis of genes responsible for MTDPS

Mutation analysis was performed on the genomic DNA using primers designed to amplify the coding exons and the exon–intron boundaries of DNA polymerase γ (*POLG*; NM_002693.2), deoxyguanosine kinase (*DGUOK*; NM_080916.1 and NM_080918.1), and *MPV17* (NM_002437.4).[16] Fragments were analyzed by direct sequencing using ABI 3130XL (Applied Biosystems, Melbourne, Vic., Australia). Long-range PCR encompassing the 335 bp deletion was performed using primers shown in Figure 1(a).

DNA from healthy Japanese controls

A PSC Cell Line Purified DNA 100 set (Japan Health Sciences Foundation, Tokyo, Japan) was used as control DNA for healthy Japanese.

Statistical analysis

The log-rank test and Gehan-Breslow-Wilcoxon test were used to test for statistically significant differences.

Ethics

This study was approved by the Institutional Review Board in Saitama Medical University.

Fig. 1 Genomic sequence determination of 335 bp deoxyguanosine kinase (*DGUOK*) deletion in the family of patients 1 and 2. (a) Capitalization, sequence of exon 2; two rectangles, long-range polymerase chain reaction (PCR) primer sets; underline, 335 bp deletion. The large 335 bp deletion encompassing from the end of intron 1 to the beginning of exon 2 causes the complete skipping of exon 2, and the resultant mRNA has a premature termination codon (p.A48fsX90). (b) Lane 1, father; lane 2, mother; lane 3, middle healthy sister; lane 4, normal control; lane 5, patient 1; lane 6, patient 2; lane 7, no sample; lane 8, molecular weight marker. The 1310 bp band represents the normal sized PCR product. The 975 bp band represents the PCR product with 335 bp *DGUOK* deletion in this family.

Case reports: DGUOK deficiency in three Japanese patients

Patient 1

This Japanese girl was the first child to unrelated healthy parents and was born without any complications at 40 weeks of gestational age, weighing 2510 g. At 3 months of age, she was referred to hospital because of failure to thrive, nystagmus and incomplete head control. Laboratory tests showed mild liver dysfunction of unknown etiology. She was suspected to have hereditary tyrosinemia because her blood tyrosine level was 800 nmol/mL (cut-off, 500 nmol/mL), but urinary succinylacetone was not detected. At the age of 18 months, her liver dysfunction deteriorated to the level of liver failure with prolonged coagulation time (hepaplastin time 39%), and she underwent a liver transplantation, but died of cardiac tamponade at 19 months of age. Liver respiratory chain enzyme assay showed low activity of complexes I, III, and IV (0%, 9%, and 28% of normal control, respectively). In contrast, complex II activity was normal and citrate synthase was moderately increased (74% and 308%, respectively). On BN-PAGE analysis, the band corresponding to assembled complex I was invisible and those of complex III and IV were strikingly weak (data not shown). On qPCR, liver mtDNA was markedly decreased (3%), confirming a diagnosis of hepatocerebral MTDPS.

Patient 2

A healthy sister of patient 1 was born 2 years after her elder sister died. A third girl was born 4 years after her eldest sister died, without any complications at 40 weeks of gestation, with a weight of 2750 g. At 2 days of age, she was referred to hospital due to tachypnea, hypoglycemia, and metabolic acidosis. After that, mild liver dysfunction was found (total bilirubin, 4.2 mg/dL; direct bilirubin, 1.4 mg/dL; aspartate aminotransferase, 215 IU/L; alanine aminotransferase, 49 IU/L; γ-glutamyl transpeptidase, 842 IU/L) with hyperammonemia (180 μg/dL). Blood lactate and pyruvate were 20.9 mmol/L, and 0.27 mmol/L, respectively. Because of her eldest sister's course, she did not undergo liver transplantation and she died of liver failure at 9 months of age. The liver showed low activity of complexes I, III, and IV (0%, 6%, and 17% of normal control, respectively). In contrast, complex II activity was normal and citrate synthase was moderately increased (105% and 281%, respectively), as for the eldest sister. On qPCR, liver mtDNA was markedly decreased (6%) and she was diagnosed with hepatocerebral MTDPS.

Patient 3

A Japanese girl, unrelated to patients 1 and 2, was born as the third child to unrelated healthy parents at 37 weeks of gestational age weighing 1688 g. Symmetrical intrauterine growth retardation was noted from 30 weeks gestation. Her eldest brother died at 1 year 4 months with a hepatic disorder of unknown origin. Her elder sister was healthy. At 8 days of age, she was suffering from feeding difficulty with liver dysfunction and nystagmus. Developmental delay and failure to thrive gradually progressed. At the age of 8 months, her liver dysfunction deteriorated to the level of liver failure, and she underwent liver transplantation, but died at 18 months of age. Liver respiratory chain enzyme assay showed low activity of complexes I, III, and IV (12%, 12%, and 16% of normal control, respectively). In contrast, complex II and citrate synthase activity were normal (68% and 106%, respectively). On qPCR, liver mtDNA was markedly decreased (2%) and she was diagnosed with hepatocerebral MTDPS.

Results

Characteristics of Japanese children diagnosed with MRCD

In total, we diagnosed MRCD in 232 patients; these patients comprised 34% of the study group. The age distribution of these patients is as follows; nearly 40% before 1 month of age, three-fourths by age 1 year, and >90% by age 7 years. One hundred and twenty patients (52%) were male, and approximately half of the diagnosed patients were deceased. Diverse clinical diagnoses are shown in Figure 2. Eighty-seven patients (38%) had neurological disorders consisting of Leigh syndrome, neurodegenerative disorders, and so-called mitochondrial cytopathy. Fifty-nine (25%) had a lethal or non-lethal infantile mitochondrial disorder. Twenty-nine (13%) had mitochondrial hepatopathy, and 17 (7%) had mitochondrial cardiomyopathy. Among all MRCD, 28 patients (12%) lacked lactic acidemia, a feature that traditionally prompts suspicion of MRCD. The entire mitochondrial DNA
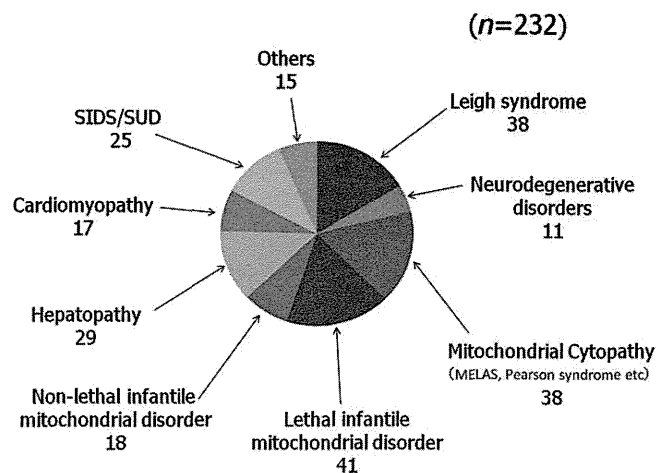
Fig. 2 Clinical diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan. Neurodegenerative disorders, neurodegenerative disorders unclassified to specific diseases. Patients with non-lethal infantile mitochondrial disorder started with symptoms such as lethal infantile mitochondrial disorder but survived beyond 1 year old. SIDS, sudden infant death syndrome; SUD, sudden unexplained death.

sequence was determined for 139 patients, but a causative genetic abnormality was found in only 34 (24%) of these patients (data not shown); indicating that, in most cases, the causative gene or genes may be present in nuclear DNA.

The enzymatic diagnoses were compared with Australian data (Fig. 3).[17] In Japanese patients, a respiratory chain complex I abnormality was most common (105 patients, 45%), followed, in decreasing order of prevalence, by respiratory chain abnormalities in multiple complexes (80 patients, 34%), a complex IV abnormality (33 patients, 13%), and a complex III abnormality (10 patients, 4%). No patient was given a probable or definitive
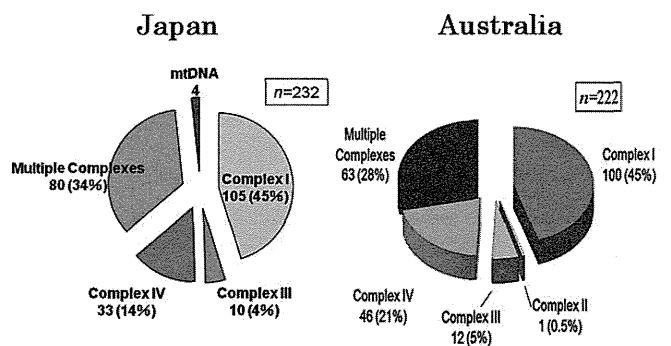

Fig. 3 Percentage distribution of enzymatic diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan and those reported previously in Australia. The enzymatic diagnosis of MRCD showed similar trends in prevalence between the Japanese and Australian patients,[17] with respiratory chain complex I being the most common type of MRCD, followed by abnormalities in multiple complexes, complex IV abnormalities, and complex III abnormalities. Complex II abnormalities were very rare among the two populations.