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.

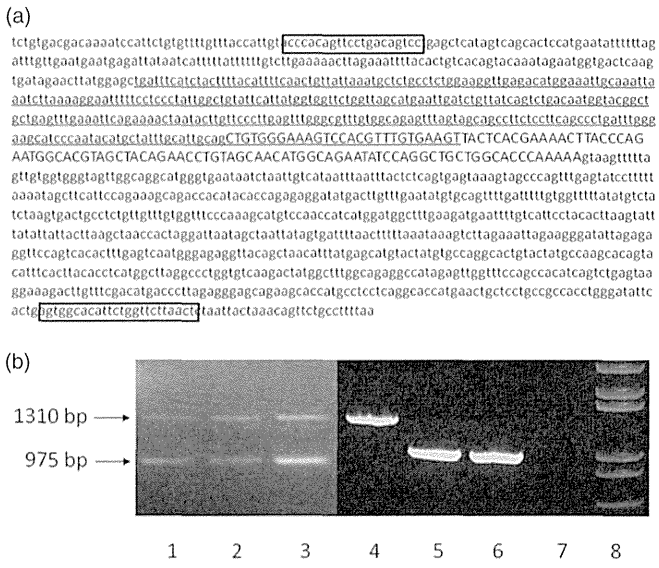

Fig. 1 Genomic sequence determination of 335 bp deoxyguanosine kinase (*DGUOK*) deletion in the family of patients 1 and 2. (a) Capitalization, sequence of exon 2; two rectangles, long-range polymerase chain reaction (PCR) primer sets; underline, 335 bp deletion. The large 335 bp deletion encompassing 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

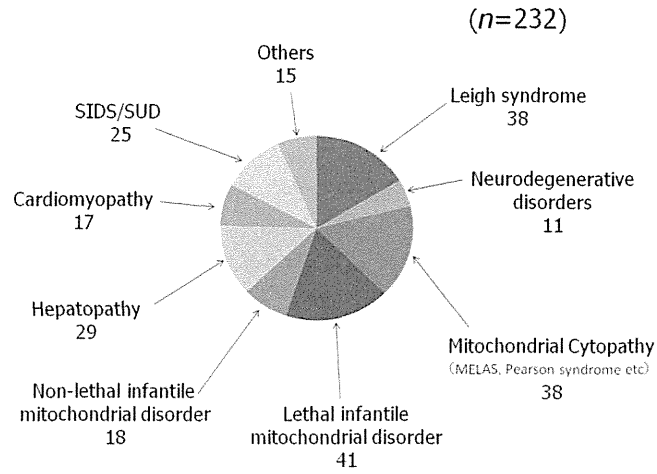

Fig. 2 Clinical diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan. Neurodegenerative disorders, neurodegenerative disorders unclassified to specific diseases. Patients with non-lethal infantile mitochondrial disorder started with symptoms such as lethal infantile mitochondrial disorder 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

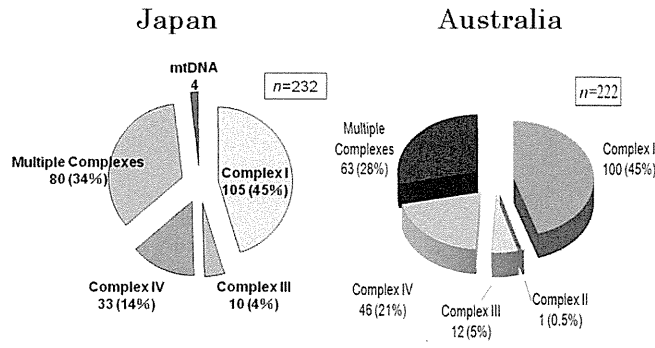

Fig. 3 Percentage distribution of enzymatic diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan and those reported previously in Australia. The enzymatic diagnosis of MRCD showed similar trends in prevalence between the Japanese and Australian patients,[17] with respiratory chain complex 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.

diagnosis of a complex II abnormality. Similarly, according to the Australian data, the most common abnormality was in complex I (45%), followed by abnormalities in multiple complexes (28%), complex IV (21%), and complex III (5%); only one patient had a complex II abnormality.

Manifestations, genetic diagnoses, and prognoses of MTDPS

A qPCR-based diagnosis of MTDPS was made for 16 of the 80 patients with an enzymatic diagnosis of a multiple complex abnormality, and for seven of the 105 patients with an enzymatic diagnosis of a respiratory chain complex I abnormality. Three of these 23 patients died due to sudden infant death syndrome and thus had no available records of clinical findings; the clinical findings from the remaining 20 patients were further analyzed.

The disease types among these 20 patients were compared with those reported by Sarzi *et al.*[4] (Fig. 4). Among the Japanese patients, 13 (65%) had acute hepatocerebral MTDPS, two (10%) had Alpers-like syndrome (delayed-onset hepatocerebral MTDPS), and five (25%) had encephalomyopathic MTDPS. This distribution is similar to that reported by Sarzi *et al.* We must note here that "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS. This is because no true case of Alpers syndrome has yet been identified in Japan. The results of analyses of the three main genes responsible for MTDPS are shown in Figure 5. Causative genetic anomalies were identified in six of the 20 Japanese patients (30%). No abnormality was identified in the three genes of the remaining 14 patients (70%). The responsible genes were *DGUOK* in three patients whose clinical reports are described in the previous section, *MPV17* in two patients,[7] and *POLG* in one patient whose clinical report will be published elsewhere. The individual genetic abnormalities are listed with the clinical findings in Table 1. Although three of the patients

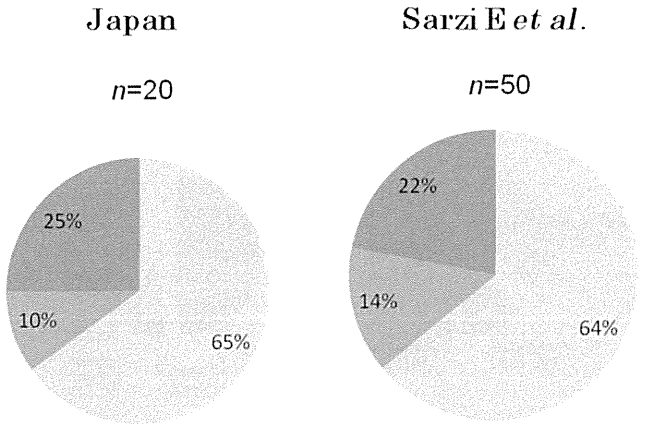

Fig. 4 Percentage distribution of disease types of mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS, because no true case of Alpers syndrome has yet been identified in Japan. The distribution of disease types in the present study did not differ from that reported by Sarzi *et al.*[4]. (▒) Hepatocerebral, (▒) Alpers–like syndrome, (▒) Encephalomyopathic.

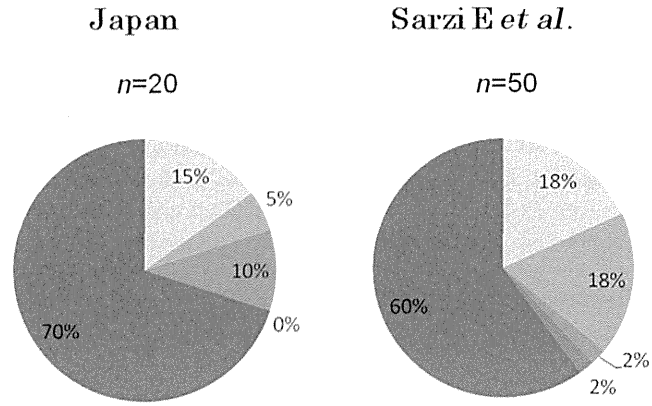

Fig. 5 Percentage distribution of responsible genes for mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* The causative gene was not identified in the majority of patients in each population. Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the causative genetic abnormalities identified by Sarzi *et al.*,[4] while three genes, *DGUOK*, *POLG*, and *MPV17*, contained 30% of the abnormalities found in the Japanese patients. (▒) *DGUOK*, (▒) *POLG*, (▒) *MPV17*, (▒) *TK2*, (▒) *unknown*. DGUOK, deoxyguanosine kinase; POLG, DNA polymerase γ.

underwent liver transplantation during infancy, five of them died before 2 years of age. Patient 5 lived longer than the others because of dietary and pharmaceutical treatment targeting the mitochondrial respiratory chain complex II.[7]

The *DGUOK*-related patients were two sisters, with a homozygous 335 bp deletion (Fig. 1a; g.11692_12026del335; encompassing 308 bp of intron 1 and 27 bp at the start of exon 2), and a compound heterozygote patient, genetically unrelated to these sisters, with the same deletion and a c.743T>C (p.L248P) missense mutation. The large 335 bp deletion encompassing from intron 1 to exon 2 causes the complete skipping of exon 2, and the resultant mRNA has a premature termination codon (p.A48fsX90). Each parent and healthy sister is heterozygous for this mutation (Fig. 1b). The p.L248P variation is not listed as a polymorphism in the ensembl_mart_47 database (martdb.ensembl.org) and has not been reported as a disease-causing mutation. Moreover, the alignment shows that Leu248 is absolutely conserved in all species (Fig. 6).[18]

The *MPV17* patients were previously reported compound heterozygote siblings.[7] The *POLG* patient was a compound heterozygote. The genetic mutations noted in these six patients were confirmed to be absent in DNA of 100 healthy Japanese controls (data not shown).

Like Sarzi *et al.*, who did not find the responsible gene or genes in 60% of the patients, we were unable to identify the responsible gene or genes in a majority of the cases. We sequenced the whole exome of all the MTDPS patients to identify the underlying nuclear disease genes using next-generation sequencing system (data not shown). This did not identify pathogenic mutations in any of the known genes associated with MTDPS (*TK2, SUCLA2, RRM2B, SUCLG1, MGME1, C10orf2, TYMP, and AGK*) in the present MTDPS patients.

Table 1 Clinical and molecular characteristics for Japanese hepatocerebral MTDPS patients

Patient	Sex	Initial symptoms (age)	Outcome (age)	Clinical diagnosis	Complications	Liver transplantation	Blood lactate/ pyruvate (mmol/L)	%mtDNA in liver	Identified mutations	Ref
1	F	Failure to thrive (3 months)	Dead (1 year)	Hereditary tyrosinemia	Developmental delay	Done	Not available	3	*DGUOK* (g.11692_12026del335 (p.A48fsX90) homozygote)	
2	F	Tachypnea (2 days)	Dead (9 months)	Mitochondrial hepatopathy	Hypoglycemia	Not done	20.9/0.27	6	*DGUOK* (g.11692_12026del335 (p.A48fsX90) homozygote)	
3	F	Feeding difficulty (8 days)	Dead (1 year)	Mitochondrial hepatopathy	Developmental delay, failure to thrive	Done	2.9/0.14	2	*DGUOK* (g.11692_12026del335 (p.A48fsX90) / c.743T>C (p.L248P))	
4	M	Failure to thrive, acholic stool (3 months)	Dead (1 year)	Hepatic failure	Developmental delay	Done	Not available	8	*MPV17* (c.451insC (p.L151fsX189)/ c.509C>T (p.S170F))	7
5	M	Failure to thrive, vomiting (8 months)	Dead (6 years)	Hepatic failure	Developmental delay, gastroesophageal reflux, respiratory failure	Done (at 6 years)	Normal	7	*MPV17* (c.451insC (p.L151fsX189)/ c.509C>T (p.S170F))	7
6	F	Failure to thrive (4 months)	Dead (7 months)	Hepatic failure	Hypotonia	Not done	1.76/0.1	3	*POLG* (c.2869G>C (p.A957V) / c.3354T>C (p.I1185T))	

Shaded columns, two pairs of siblings. MTDPS, mitochondrial DNA depletion syndrome.

```
Human        241  -----ALMNIPVLVLDV--NDDFSEEVTKQEDLMREVNTFVKNL------  277
Pan Trog     241  -----ALMNIPVLVLDV--NDDFSEEVTKQEDLMREVNTFVKNL------  277
Canis        241  -----ALLNIPVLVLDV--NDDFSEEVTKQEELMKKVNIFVKNL------  277
Bos          241  -----ALLNIPVLVLDV--NDDFSEEVTIQEELMRRVNTFVKNL------  277
Mus          241  -----ALQHVPVLVLDV--TEDFSENAARQEELMGQVNTFMRNL------  277
Rat          241  -----ALRHVPVLVLNI--SEDFSENAAKQEELMGQVNTFMRNL------  277
Danio        233  -----QLMKVPVLVLDA--EVAFEQNPEVQDCLLSKVRDFLSQL------  269
Arabidopsis  483  NHMHSSIQKVPALVLDCEPNIDFSRDIEAKTQYARQVAEFFEFVKKKQET  532
Oryza        408  DHMHSSIQKVPALVLDCEHDIDFNKDIEAKRQ------------------  439
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Fig. 6 ClustalW multiple sequence alignment of deoxyguanosine kinase (*DGUOK*) orthologs. The alignment shows that amino acid 248Leu mutated in the patient is absolutely conserved in all species. URLs: HomoloGene, http://www.ncbi.nlm.nih.gov/homologene (for the DGUOK ortholog amino acid sequences of human [accession no. NP_550438.1], Pan [accession no. XP_001153473.1], Canis [accession no. XP_533001.2], Bos [accession no. NP_001014888.2], Mus [accession no. XP_001107072.1], Rat [accession no. NP_001100072.1], Danio [accession no. XP_001093561.1], Arabidopsis [accession no. NP_565032.2], Oryza [accession no. NP_001044956.1]). ClustalW, http://www.ebi.ac.uk/Tools/clustalw/.[18]

Of the genetic mutations identified, *POLG* mutations were less prevalent than in Caucasian subjects. Only one of the present 15 cases of Alpers syndrome or hepatocerebral MTDPS were caused by recessive *POLG* mutations, compared with eight of 39 such cases diagnosed in France.

Sixteen of the 20 Japanese MTDPS patients were deceased. Sarzi *et al.* reported that 29 of the 50 MTDPS patients they analyzed were deceased. The data of the deceased patients were plotted to obtain curves of the ages of death (in months) in the two groups for comparison (Fig. 7). MTDPS patients had a short life in both study groups; many died during or before reaching early childhood. On log-rank test and Gehan-Breslow-Wilcoxon test no significant differences were seen between the survival data.

Discussion

We started an enzyme diagnosis referral service for children suspected of MRCD in 2007 and have diagnosed MRCD in 30–40 patients from around Japan annually since then. In the last year we have made >100 new MRCD diagnoses. Approximately half of the diagnoses are for neonates. There are approximately one million births in Japan annually. Under the assumption that the patients referred for enzyme diagnosis represent approximately half of all Japanese MRCD patients, the prevalence of neonatal-onset MRCD becomes $50 \times 2/1\,000\,000 = 1/10\,000$. When patients with juvenile-onset and adult-onset mitochondrial disease are factored in, the prevalence of these diseases in Japan becomes one in several thousand, which is comparable to the prevalence in Western countries.[1]

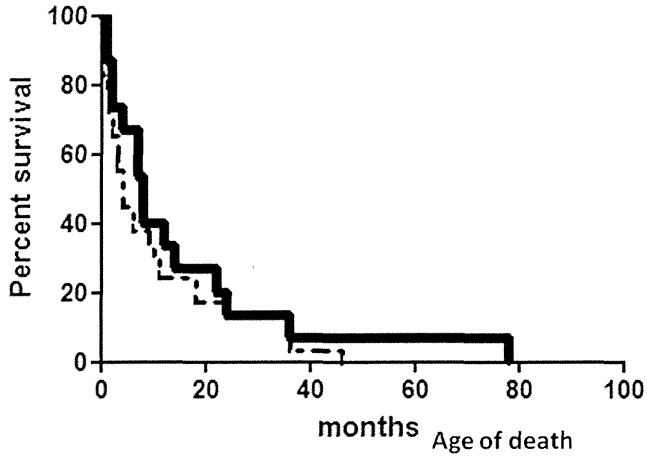

Fig. 7 Comparison of the ages of death (in months) in the two studies. A commonality between the Japanese patients and the Sarzi *et al.* patients[4] was observed. No significant difference in disease severity was identified (log-rank test, $P = 0.3637$; Gehan-Breslow-Wilcoxon test, $P = 0.2667$). (▬) Japanese, $n = 16/20$; (-•-) Sarzi *et al.*, $n = 29/50$.

It is noteworthy that >10% of the patients lacked lactic acidemia, which many physicians still regard as synonymous with mitochondrial disease. Hence, mitochondrial disease must also be considered in lactic acidemia-free patients with unexplained signs and symptoms in multiple organs.

The enzymatic diagnosis of MRCD showed similar trends in prevalence between Japanese and Australian patients, 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 in both populations.

Twenty percent of the patients with multiple respiratory chain disorders in the present study and 50% of the patients in the Sarzi *et al.* study[4] had MTDPS. Although MTDPS was the leading cause of MRCD in both groups, MTDPS represented a smaller proportion of the MRCD in Japan. According to the Online Mendelian Inheritance in Man database, MTDPS can be classified as encephalomyopathic, hepatocerebral, or specific (a classification that includes mitochondrial neurogastrointestinal encephalopathy [MNGIE] and Sengers syndrome). Encephalomyopathic MTDPS features respiratory failure and myopathy. Hepatocerebral MTDPS is characterized by liver disorders, growth disorders, and hypoglycemia. The distribution of the disease type classifications of the Japanese patients did not differ from the distribution reported by Sarzi *et al.*

Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the causative genetic abnormalities in the Sarzi *et al.* study, while three genes, *DGUOK*, *POLG*, and *MPV17*, contained 30% of the abnormalities found in the Japanese patients. The causative gene, however, was not identified in the majority of patients in each study. The six Japanese hepatocerebral MTDPS patients in whom the responsible gene was identified are listed in Table 1. The serious nature of this disease is evident, given that all six experienced onset as neonates or infants and died during or before reaching early childhood.

Deoxyguanosine kinase deficiency was originally described as the cause of infantile-onset hepatocerebral mitochondrial disease, typically featuring hepatic failure, nystagmus and hypotonia.[19] Recently it has been shown that patients with *DGUOK* mutation may present with neonatal hemochromatosis[20] or adult-onset myopathy and mitochondrial DNA multiple deletions, with or without liver involvement.[21,22] We found two novel *DGUOK* mutations in two apparently unrelated Japanese families. Three patients in two families had typical signs and symptoms of hepatocerebral MTDPS, and both parents in each family were

heterozygous for these mutations. A 335 bp deletion in *DGUOK* was found in both families, and may therefore be a common mutation in the Japanese population.

The present analysis of MTDPS patients concludes with a comparison of the ages of death (in months) in the two groups (Fig. 7). A commonality between the Japanese patients and the Sarzi *et al.* patients was the early age of death: most patients died during or before reaching early childhood. *DGUOK* deficiency was most serious in both studies. Likewise, many patients in each study experienced onset as neonates or infants. No significant difference in disease severity was identified between the two studies.

The present results indicate a lower prevalence of *POLG* mutations in the Japanese population, which is likely attributable to several common mutations found in Caucasian people that appear to be ancient European founder mutations (p.A467T, p.G848S, and p.W748S).[23] In children with recessive *POLG* mutations, these three mutations represented seven of 16 mutant alleles reported by Sarzi *et al.*[4] A recent study collated the prevalence of these three mutations in 10 studies reporting a total of 249 POLG patients and found that they represented 49% of mutant alleles in predominantly Caucasian patients.[24] Most Caucasian POLG patients will thus have at least one allele carrying one of these three founder mutations, and Hakonen *et al.* suggested that they may have been spread during Viking times.[23] The carrier frequency of these mutations is as high as 2% in some European countries. Their expected absence in Asian patients likely explains a lower prevalence of recessive POLG disease in Asian populations.

Conclusion

Mitochondrial DNA depletion syndrome and other mitochondrial respiratory chain disorders are common, but serious, diseases that occur across all races.

Acknowledgments

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RESEARCH PAPER

New *MT-ND6* and *NDUFA1* mutations in mitochondrial respiratory chain disorders

Natsumi Uehara[1,2], Masato Mori[3], Yoshimi Tokuzawa[1], Yosuke Mizuno[1], Shunsuke Tamaru[1], Masakazu Kohda[1,4], Yohsuke Moriyama[1], Yutaka Nakachi[1,4], Nana Matoba[4], Tetsuro Sakai[5], Taro Yamazaki[5], Hiroko Harashima[5], Kei Murayama[6], Keisuke Hattori[7], Jun-Ichi Hayashi[7], Takanori Yamagata[3], Yasunori Fujita[8], Masafumi Ito[8], Masashi Tanaka[9], Ken-ichi Nibu[2], Akira Ohtake[5] & Yasushi Okazaki[1,4]

[1]Division of Functional Genomics & Systems Medicine, Research Center for Genomic Medicine, Saitama Medical University, Hidaka, Japan
[2]Department of Otolaryngology-Head and Neck Surgery, Kobe University Graduate School of Medicine, Kobe, Japan
[3]Department of Pediatrics, Jichi Medical University, Shimotsuke, Japan
[4]Division of Translational Research, Research Center for Genomic Medicine, Saitama Medical University, Hidaka, Japan
[5]Department of Pediatrics, Faculty of Medicine, Saitama Medical University, Moroyama-machi, Japan
[6]Department of Metabolism, Chiba Children's Hospital, Chiba, Japan
[7]Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan
[8]Research Team for Mechanism of Aging, Tokyo Metropolitan Institute of Gerontology, Itabashi, Japan
[9]Department of Genomics for Longevity and Health, Tokyo Metropolitan Institute of Gerontology, Itabashi, Japan

Correspondence
Yasushi Okazaki, Division of Functional Genomics & Systems Medicine, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka, Saitama 350-1241, Japan. Tel: +81-42-984-0448; Fax: +81-42-984-0449; E-mail: okazaki@saitama-med.ac.jp
Akira Ohtake, Department of Pediatrics, Faculty of Medicine, Saitama Medical University, 38 Morohongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan. Tel: +81-49-276-1220; Fax: +81-49-276-1790; E-mail: akira_oh@saitama-med.ac.jp

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Abstract

Objective: Mitochondrial respiratory chain disorder (MRCD) is an intractable disease of infants with variable clinical symptoms. Our goal was to identify the causative mutations in MRCD patients. **Methods**: The subjects were 90 children diagnosed with MRCD by enzyme assay. We analyzed whole mitochondrial DNA (mtDNA) sequences. A cybrid study was performed in two patients. Whole exome sequencing was performed for one of these two patients whose mtDNA variant was confirmed as non-pathogenic. **Results**: Whole mtDNA sequences identified 29 mtDNA variants in 29 patients (13 were previously reported, the other 13 variants and three deletions were novel). The remaining 61 patients had no pathogenic mutations in their mtDNA. Of the 13 patients harboring unreported mtDNA variants, we excluded seven variants by manual curation. Of the remaining six variants, we selected two Leigh syndrome patients whose mitochondrial enzyme activity was decreased in their fibroblasts and performed a cybrid study. We confirmed that m.14439G>A (*MT-ND6*) was pathogenic, while m.1356A>G (*mitochondrial 12S rRNA*) was shown to be a non-pathogenic polymorphism. Exome sequencing and a complementation study of the latter patient identified a novel c.55C>T hemizygous missense mutation in the nuclear-encoded gene *NDUFA1*. **Interpretation**: Our results demonstrate that it is important to perform whole mtDNA sequencing rather than only typing reported mutations. Cybrid assays are also useful to diagnose the pathogenicity of mtDNA variants, and whole exome sequencing is a powerful tool to diagnose nuclear gene mutations as molecular diagnosis can provide a lead to appropriate genetic counseling.

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Introduction

The mitochondrial respiratory chain (RC) is a pathway for vital energy generation in which ATP is generated as a form of energy by the substrates generated from glycolysis and β-oxidation. The pathway is composed of five multi-enzyme complexes (complexes I–V), two electron carriers, a quinone (coenzyme Q), and a small hem-containing protein (cytochrome c) that are located in the inner mitochondrial membrane. These RC complexes are formed from subunits encoded by both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA), with the exception of complex II, which is entirely encoded by nDNA.

mtDNA is a circular double-stranded DNA molecule ~16 kb in length that encodes 37 genes comprising 13 proteins, 22 mitochondrial tRNAs, and 2 rRNAs.[1,2] Defects in mitochondrial function are associated with numerous neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease, and, in particular with mitochondrial respiratory chain disorder (MRCD). MRCD is genetically, clinically, and biochemically heterogeneous, and it can give rise to any symptoms, in any organs or tissues, at any age and with any mode of inheritance.[3] One in 5000 births is a conservative realistic estimate for the minimum birth prevalence of MRCD.[4] Especially in children, MRCD is an intractable disease and can be regarded as the most common group of inborn errors of metabolism.[5,6]

Some MRCD patients have typical clinical findings that are caused by specific point mutations or large deletions of mtDNA. Typical clinical features include mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), myoclonus epilepsy associated with ragged-red fibers (MERRF), Leber's hereditary optic neuropathy (LHON), and chronic progressive external ophthalmoplegia (CPEO).[2] Although mtDNA mutations or deletions are usually found in adults showing typical clinical findings, they account for only a minority of children with MRCD. Therefore, the diagnosis of MRCD in children by screening known mtDNA mutations is rather difficult.[7] Hence, a combination of general biochemical study, histological study, and genetic analysis is essential for the diagnosis of MRCD, especially in children.[6]

In this study, we performed whole mtDNA sequencing for 90 children diagnosed with MRCD by RC enzyme assay with the aim of identifying causative mtDNA mutations.

Subjects, Materials, and Methods

Patients

Ninety Japanese pediatric patients diagnosed with MRCD and without characteristic clinical syndromes were studied. The primary diagnosis for these patients was definite or probable MRCD based on the criteria of Bernier et al.,[8] and a mitochondrial RC residual enzyme activity of <20% in a tissue, <30% in a fibroblast cell line, or <30% in two or more tissues (Data S1). Informed consent was obtained from the patients and their families before participation in the study.

Patient summaries are shown in Tables 1, 2. The details of the two patients studied in the cybrid assay are as follows: Patient (Pt) 377 is a 1-year-old girl born after a normal pregnancy to non-consanguineous parents. She has a normal brother and sister. She was hospitalized with gait difficulties at the age of 1 year. Blood lactate levels were high. Brain magnetic resonance imaging (MRI)

Table 1. Distribution of mtDNA variants and clinical features.

Characteristics		Non-pathogenic mutations	Low probability variants	New pathogenic deletions	Known variants	Total
Number of subjects		61 (100%)	13 (100%)	3 (100%)	13 (100%)	90 (100%)
No consanguinity		57 (93%)	12 (92%)	3 (100%)	11 (85%)	84 (93%)
Age at onset	≤1 y.o.	54 (89%)	10 (77%)	3 (100%)	9 (69%)	76 (84%)
Status	Alive	33 (54%)	7 (54%)	1 (33%)	11 (85%)	53 (59%)
	Dead	28 (46%)	6 (46%)	2 (67%)	2 (15%)	37 (41%)
Sex	Female	30 (49%)	3 (23%)	2 (67%)	6 (46%)	41 (46%)
	Male	31 (51%)	10 (77%)	1 (33%)	7 (54%)	49 (54%)

y.o., years old.

Table 2. Summary of unreported mutations and deletions.

Patient ID	Age at onset	Clinical diagnosis	Enzyme assay (organ)	mtDNA variation	Locus	Heteroplasmy
377	1 year	LD	1 (Fb)	m.14439G>A	*ND6*	Homo (Fb)
190	1 year 6 months	LD	1,4 (M)	m.11246G>A	*ND4*	73% (fb)
508	0 days	SIDS	1 (Hep,Car)	m.4638A>G	*ND2*	86% (Fb), 0% (Hep, Car)
004	0 months	MC	1 (Fb)	m.5537A>G[1]	*tRNATrp*	27.4% (Fb)
271	0 months	ELBW	1 (Hep)	m.10045T>C	*tRNAGly*	Homo (hep)
312[2]	5 years	LD	1 (Fb) probably	m.1356A>G	*12S rRNA*	66% (Fb)
372	2 days	LIMD	1 (Hep)	Deletion (3424 bp) nt12493-15916		65.7% (Fb), 89.9% (Hep)
336	11 months	HD	1 (Hep)	Deletion (6639 bp) nt7734-14372		9.2% (Fb), 92.6% (Hep)
390	0 days	MC	1,4 (M,Hep)	Deletion (5424 bp) nt8574-13997		44.9% (Fb), 86.4% (Hep)

LIMD, lethal infantile mitochondrial disorder; HD, hepatic disease; LD, Leigh's disease; MC, mitochondrial cytopathy; SIDS, sudden infant death syndrome; ELBW, extremely low birth weight infant; Fb, fibroblast; Hep, liver; Car, heart; M, muscle.

[1]Expected to be causative because of the other reported mutation on the same position.

[2]m.1356A>G was confirmed as non-pathogenic and nDNA mutation was identified in Pt312.

showed bilateral and symmetrical hyperintensity foci in the basal ganglia. She developed progressive motor regression and became bedridden. Pt312 is a 5-year-old boy born after 36 weeks' gestation following a normal pregnancy to non-consanguineous parents. His birth weight was 2154 g. He has a sister who is his fraternal twin. At 5 months of age, his parents noticed hypotonia and nystagmus. At 10 months of age, he had generalized epilepsy and blood lactate and his pyruvate levels were high. A brain MRI revealed symmetrical high T2 signals in the midbrain.

Whole mtDNA sequencing and detection of variants

Genomic DNA (gDNA) was extracted from skin fibroblasts (Data S1), blood, liver, and cardiac muscle using either phenol/chloroform- or column-based extraction. Whole mtDNA was first polymerase chain reaction (PCR)-amplified as two separate large amplicons (LA1 and LA2) avoiding the nonspecific amplifications from nDNA.[9] Second-round PCR was performed using 46 primer pairs (mitoSEQrTM; Applied Biosystems, Carlsbad, CA) and the LA1 and LA2 amplicon mixture from first-round PCR as a template. PCR conditions were as follows: first-round PCR was performed in a reaction mixture containing 0.2 mmol/L of each dNTP, 0.25 U of Takara Ex Taq (Takara Bio, Shiga, Japan), 1× Ex Taq Buffer, 0.3 μmol/L of each primer, and extracted gDNA in a total volume of 50 μL. Initial denaturation was performed at 94°C for 2 min, followed by 30 cycles of 94°C for 20 sec, 60°C for 20 sec, and 72°C for 5 min, with a final extension at 72°C for 11 min. Second-round PCR was performed in a reaction mixture as above except with a 10,000-fold dilution of LA1 amplicon and a 100-fold dilution of LA2 amplicon (total volume of the PCR reaction, 10 μL). Initial denaturation was performed at 96°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 45 sec, and 72°C for 45 sec, with a final extension at 72°C for 10 min.

First- and second-round PCR products were separated by 1% and 2% agarose gels, respectively, then 10 μL of second-round PCR products were incubated with 1 μL of ExoSAP-IT reagent (GE Healthcare UK Ltd., Bucks, U.K.) at 37°C for 30 min to degrade remaining primers and nucleotides. The ExoSAP-IT reagent was then inactivated by incubating at 75°C for 15 min. PCR products were sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI3130xl Genetic Analyzer (Applied Biosystems). Sequence data were compared with the revised Cambridge sequence (GenBank Accession No. NC_012920.1) and sequences present in MITOMAP (http://mitomap.org/MITOMAP) and mtSNP (http://mtsnp.tmig.or.jp/mtsnp/index_e.shtml) using SeqScape software (Applied Biosystems). Whole mtDNA sequencing of seven samples was obtained using an Ion PGM™ sequencer (Life Technologies Corporation, Carlsbad, CA).

Characterization of mtDNA deletions

We searched for mtDNA deletions by focusing on the size of first-round PCR products in agarose electrophoresis. If PCR products were smaller than controls, we suspected mtDNA deletion and performed further analysis. The smaller PCR products were recovered from the gel and amplified by second-round PCR, as described above, and

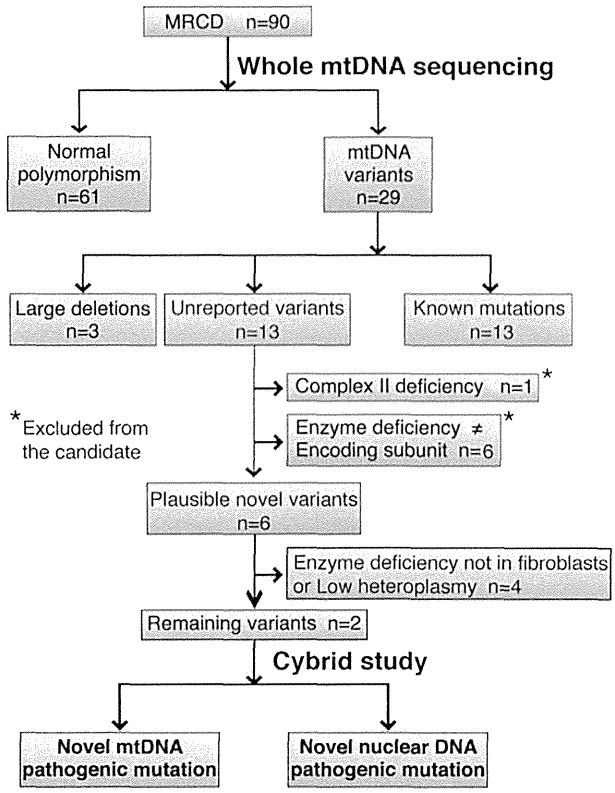

Figure 1. Flow diagram of study analysis. Ninety MRCD patients were analyzed in this study. Sixty-one patients had normal polymorphisms and 29 had mtDNA variants. Of these variants, 13 patients had MRCD causative mutations that had been previously described. We identified three novel large deletions and 13 unreported variants. Of the unreported variants, one patient with complex II deficiency was excluded because complex II is not encoded by mtDNA. Six patients were excluded because their enzyme deficiency pattern did not coincide with the variants found in mtDNA. Four patients were excluded because of the lack of fibroblast enzyme deficiency or low heteroplasmy. The remaining two cases were analyzed by cybrid study.

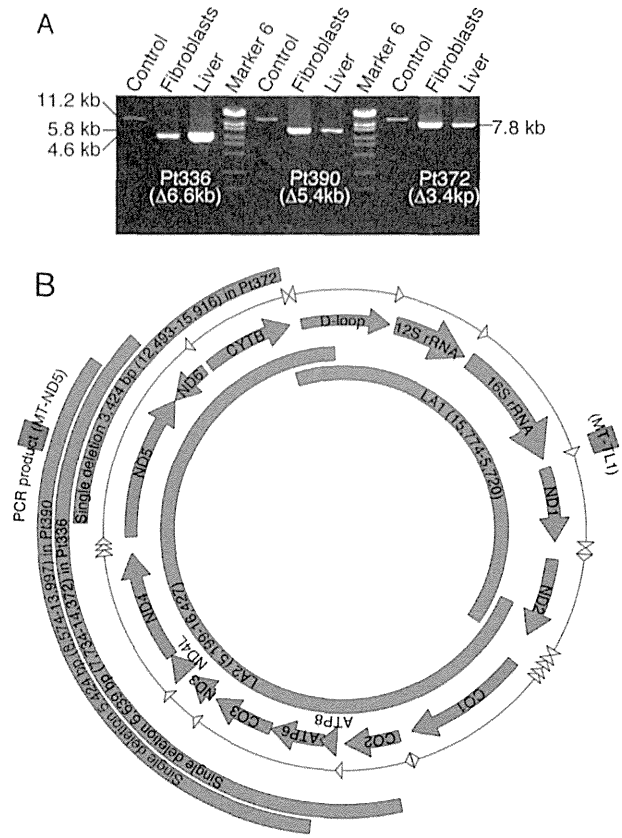

Figure 2. Identification of three large deletions. (A) Characterization of the three novel mtDNA deletions using agarose electrophoresis. First-round PCR products amplified from patient fibroblast and liver DNA clearly showed the presence of mtDNA deletions in Pt336, 390, and 372. Normal mtDNA from an MRCD patient was used as a positive control. (B) Positions of the novel mtDNA deletions are shown in blue. LA1 and LA2 amplification is shown in green. Two red squares represent real-time PCR amplicons MT-ND5 and MT-TL1.

analyzed for an mtDNA deletion. Second-round PCR was performed using fewer (25–26) PCR cycles to avoid untargeted DNA amplification. To identify the location of the deletion, we first compared the density of bands and screened the faint bands with agarose electrophoresis. The precise deletion boundaries were confirmed by sequencing analysis with primers used for second-round PCR that were close to the probable deletion region.

Results

Patient characteristics and their mtDNA mutations

A total of 90 patients (49 were men and 41 were women) with MRCD were subjected to whole mtDNA sequencing analysis (Table 1). Eighty-four subjects (93%) were non-consanguineous. Seventy-six subjects (84%) were aged 1 year or younger. We identified 13 previously reported mtDNA mutations, 13 unreported variants, and three novel deletions (Fig. 1). The remaining 61 subjects had normal polymorphisms in their mtDNA (Fig. 1).

Large mtDNA deletions were identified in three patients

Agarose gel electrophoresis of first-round PCR from fibroblast and liver mtDNA clearly showed the presence of mtDNA deletions in Pt336, 390, and 372 (Fig. 2A). The precise deletion sites were confirmed by sequencing analysis. The expected size of the first-round PCR LA2 product in wild-type mtDNA from an MRCD patient was 11.2 kb, which enabled us to estimate the deletion sizes

of Pt336, 390, and 372 as 6639, 5424, and 3424 bp, respectively (Fig. 2A and B). In Pt336, the 6639-bp deletion was located between nucleotides 7734 and 14,372 and was flanked by 5-bp perfect direct repeats. This deletion results in the loss of 15 genes (*CO2, ATP8, ATP6, CO3, ND3, ND4L, ND4, ND5, ND6*, and six *tRNA* genes). The heteroplasmy ratio of this deletion was 9.2% in the fibroblasts (Fb) and 92.6% in the liver (Hep) (Table 2 and Data S1). In Pt390, the 5424-bp deletion was located between nucleotide positions 8574 and 13,997 and was flanked by 11-bp imperfect direct repeats. This deletion results in the loss of 11 genes (*ATP6, CO3, ND3, ND4L, ND4, ND5*, and five *tRNA* genes). The heteroplasmy ratio of this deletion was 44.9% (Fb) and 86.4% (Hep) (Table 2). In Pt372, the 3424-bp deletion was located between nucleotides 12,493 and 15,916 and was flanked by 6-bp imperfect direct repeats. This deletion results in the loss of five genes (*ND5, ND6, CYB*, and two *tRNA* genes). The heteroplasmy ratio of this deletion was 65.7% (Fb), and 89.9% (Hep) (Table 2).

Unreported variants of mtDNA detected in 13 patients

We identified 13 unreported mtDNA variants. Of these, seven were excluded by manual curation (Fig. 1). One of these was excluded because the enzyme deficiency was specific to complex II, which is not encoded by mtDNA. The other six were excluded because their enzyme deficiency pattern did not coincide with the variants found in mtDNA. From the remaining six plausible mtDNA variants, we determined whether they were causative using the following inclusion criteria for further analysis: (1) cells were viable for further assay, (2) mtDNA variants corresponded to the enzyme assay data in the RC subunit, (3) enzyme deficiency was observed in the fibroblasts, and (4) variants had high heteroplasmy ratios (Fig. 1 and Table 2). On the basis of these criteria, we selected two patients whose mtDNA variants (m.14439G>A in *MT-ND6* and m.1356A>G in *12S rRNA*) were suitable for further analysis as shown in Figure 1. The other four patients were excluded because they did not show enzyme deficiency in their fibroblasts or because of low heteroplasmy ratios (Table 2).

m.14439G>A (*MT-ND6*), but not m.1356A>G (12S rRNA), is a causative mutation

The m.14439G>A (*MT-ND6*) variant was observed in fibroblasts from Pt377 (Fig. 3A). PCR- restriction fragment length polymorphism (RFLP) analysis with the *Hpy*188I restriction enzyme found Pt377 fibroblasts to be homoplasmic, and the m.14439G>A variant was not detected in the blood of the patient's parents (Fig. 3A and B). This mutation changes the proline to a serine at amino acid position 79, which is highly conserved among vertebrates (Fig. 3C). ND6 is one of the mtDNA-encoded complex I subunits and alignment of the ND6 protein in different species revealed conservation of amino acids. The activity level of the RC complex I was coincidentally reduced in the patient's fibroblasts (Fig. 4A). To further confirm whether this mutation was causative of mitochondrial dysfunction, we performed cybrid analysis (Data S1). The cybrids showed a reduction in the complex I activity level consistent with the respiratory enzyme assay in the patient's fibroblasts (Fig. 4B). These data strongly support the idea that the m.14439G>A *(ND6)* mutation detected in Pt377 is responsible for the complex I deficiency.

The m.1356A>G (*12S rRNA*) variant was observed in fibroblasts from Pt312, which showed reduced activity levels of RC complex I (Fig. 4A). By mismatch PCR-RFLP-analysis using the *Sty*I restriction enzyme, this variant was determined at a heteroplasmy ratio of 66% in the patient's fibroblasts (Table 2). The cybrids harboring this variant showed a recovery of complex I enzyme activity compared with the original patient's fibroblasts (Fig. 4B). These data suggest that reduced complex I enzyme activity was rescued by nuclear DNA and that this mtDNA variation is not causative. This further indicates that the nuclear gene mutation is the cause of MRCD in this patient.

Identification of the c.55C>T (*NDUFA1*) mutation in Pt312 by whole exome sequencing

To search for the causative nuclear gene mutation in Pt312, we performed whole exome sequencing (Data S1). This identified a single hemizygous mutation (c.55C>T) in exon 1 of the *NDUFA1* gene, which altered the amino acid residue at position 19 from proline to serine (p. P19S). The mutation was confirmed by Sanger sequencing (Fig. 5A). This conserved proline residue lies within the hydrophobic N-terminal side constituting a functional domain that is involved in mitochondrial targeting, import, and orientation of *NDUFA1*.[10,11] SIFT and PolyPhen, which predict the function of non-synonymous variants (http://genetics.bwh.harvard.edu/pph/), also revealed that the p.P19S mutation "probably" damages the function of the NDUFA1 protein (damaging score, 0.956). Alignment of the NDUFA1 protein between different species revealed the conservation of three amino acids, including the proline at position 19, which is highly conserved among vertebrates (Fig. 5B). To further confirm if the complex I deficiency in Pt312 occurred because of the mutation in *NDUFA1*, we overexpressed *NDUFA1* cDNA to determine if the enzyme deficiency

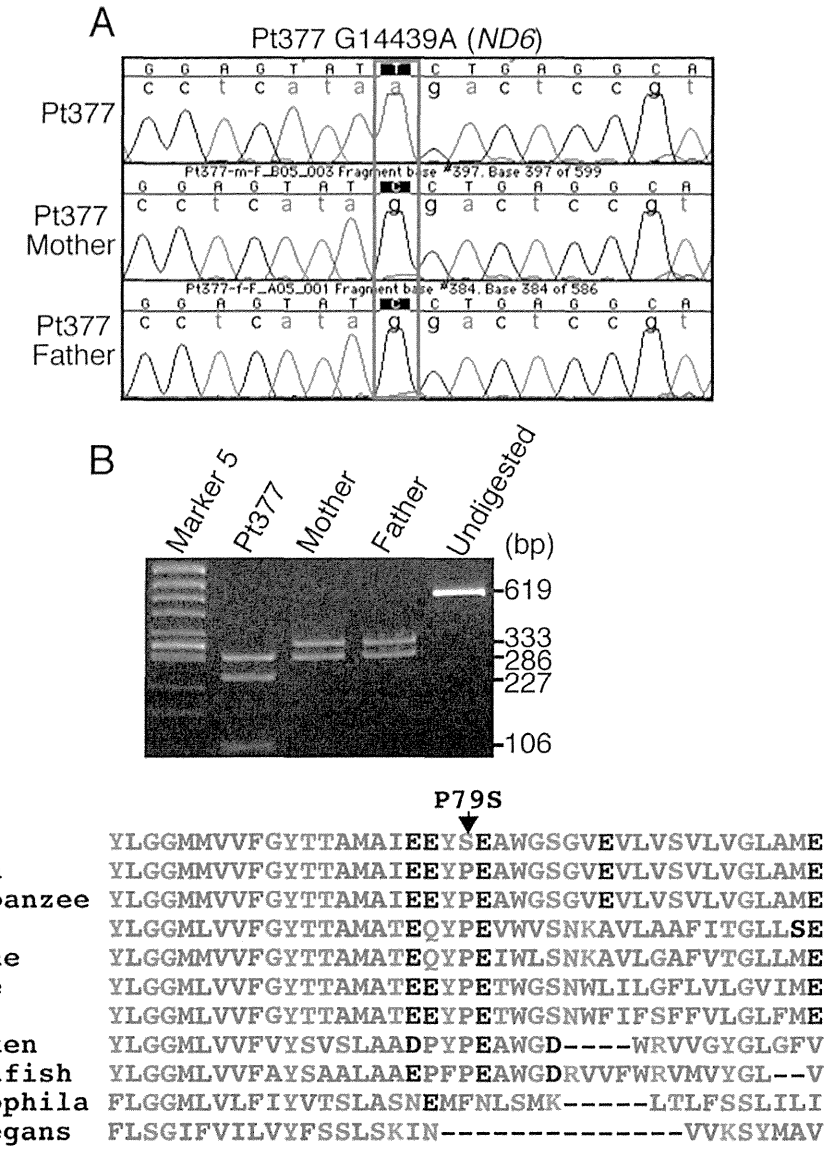

Figure 3. Novel mutation m.14439G>A in Pt377 mtDNA. (A) Trio-sequencing analysis of m.14439G>A (*MT-ND6* p.P79S) change in Pt377 family. Sequence chromatograms show that the m.14439G>A is detectable only in Pt377. (B) PCR-RFLPanalysis using fibroblast mtDNA from Pt377 and blood from both parents. A 619-bp PCR fragment was digested with *Hpy*188I. Wild-type mtDNA was cleaved into two fragments of 333 and 286 bp as shown in "Mother" and "Father", whereas the PCR product containing the m.14439G>A mutation was cleaved into three fragments: 286, 227, and 106 bp ("Pt377"). Undigested = undigested PCR product. (C) Alignment of MT-ND6 protein between different species shows the conservation of amino acid Proline 79. Amino acid sequences of *MT-ND6* gene products were aligned by ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and NCBI/homologene (http://www.ncbi.nlm.nih.gov/homologene).

could be recovered (Data S1). Lentiviral transfection of NDUFA1 resulted in a significant increase in complex I assembly level as determined by blue native polyacrylamide gel electrophoresis. By contrast, lentiviral transfection of control mtTurboRFP did not rescue the phenotype (Fig. 5C). These data indicate that the c.55C>T mutation in *NDUFA1* is responsible for the complex I deficiency in Pt312.

Discussion

MRCD is particularly difficult to diagnose in pediatric cases as the clinical features are highly variable. We, therefore, propose a systematic approach for diagnosing MRCD that starts with a biochemical enzyme assay and is followed by whole mtDNA sequencing. In this study, we performed whole mtDNA sequencing for 90 children with

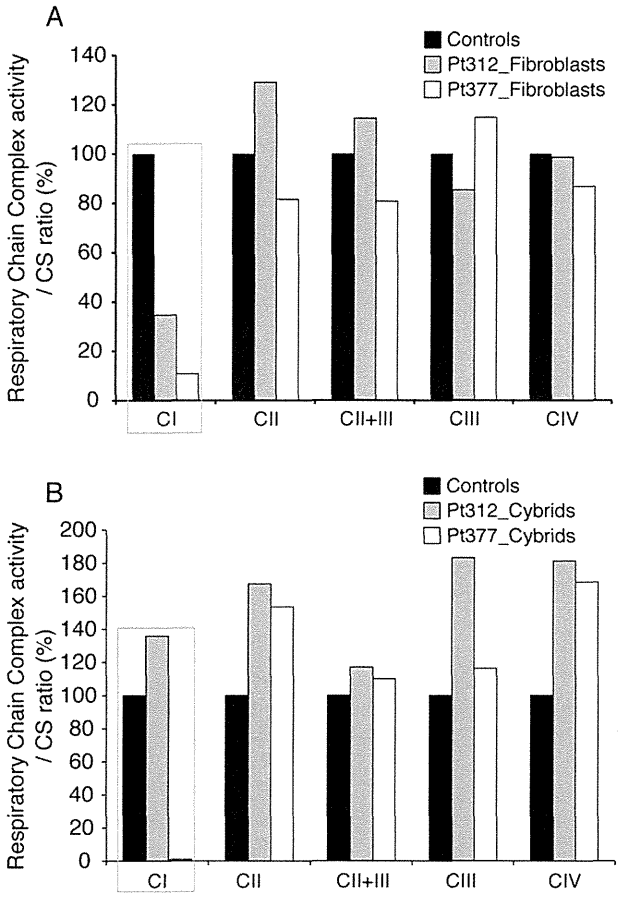

Figure 4. Biochemical assay for respiratory chain enzyme activity in fibroblasts and cybrid cells from Pt377 and Pt312. (A) Respiratory chain complex enzyme activity for CI, CII, CII + III, and CIV in skin fibroblast mitochondria from Pt312 and Pt377 compared with normal controls. The activity of each complex was calculated as a ratio relative to citrate synthase (CS). CI showed a reduction in enzyme activity in Pt312 and 377 fibroblasts. (B) Respiratory chain complex enzyme activity of cybrids established from Pt312 and Pt377 fibroblasts. Cybrids were established from rho0-HeLa cell and Pt312 or Pt377 fibroblasts. The activity of each complex in these cybrids was calculated as a ratio relative to that of citrate synthase (CS).

MRCD, and identified 29 mtDNA variants. Of these, we identified 13 known causative mutations, three large deletions, and further confirmed that m.14439G>A (*MT-ND6*) and c.55C>T (*NDUFA1*) are new causative mutations for MRCD from the results of a cybrid assay, whole exome sequencing, and a complementation study. The diagnosis of MRCD was then confirmed as definite by molecular analysis in these 18 cases.

Whole mitochondrial DNA sequencing identified 13 cases (14%) harboring known causative mtDNA mutations. mt. 10191T>C (*ND3*) and mt. 8993T>C or G (*ATP6*) mutations were detected in three and two patients, respectively (data not shown). Both are common causative mutations of infantile Leigh syndrome. Previous reports found that most common MRCD causative mutations are primarily responsible for adult-onset disease, whereas few are responsible for childhood-onset MRCD;[12,13] only 14% of our cases were attributed to known mtDNA mutations.

Most patients in this study were 1-year old or younger at the onset of disease, with no family history. We used the RC complex enzyme assay to diagnose pediatric patients who had not been diagnosed with MRCD in a clinical setting. Several MRCD cases in children were previously reported to be difficult to diagnose with nonspecific clinical presentations in contrast to the characteristic clinical syndromes such as MELAS and MERRF caused by common mtDNA mutations.[6,12]

We identified three novel deletions that we concluded were causative because they include several genes that could explain the deficiency of the RC enzymes. Generally, most mtDNA deletions share similar structural characteristics, are located in the major arc between two proposed origins of replication (OH and OL; Mitomap), and are predominantly (~85%) flanked by short direct repeats.[14,15] Single mtDNA deletions are reported to be the common causes of sporadic MRCD such as Kearns-Sayre syndrome (KSS), CPEO, and Pearson's syndrome. In this study, all three deletions were located in the major arc and were flanked by repeat sequences, similar to previous studies. Although Pt390 was diagnosed with Pearson's syndrome, the other two patients (Pt336 and Pt372) did not show a common phenotype caused by a single deletion such as KSS, CPEO, or Pearson's syndrome. Therefore, screening by mtDNA size differences is important even in those patients not clinically suspected to have mtDNA deletions.

Manual curation identified six plausible mtDNA variants that had not previously been reported (Fig. 1). We attempted to carry out a functional assay of the two patients whose fibroblasts are enzyme deficient, although it was difficult to apply this strategy to those fibroblasts with normal enzyme activity. In this sense, it is important to collect patients with similar phenotypes and carrying the same mtDNA variants to accurately diagnose the causal mutation. Thus, this study of patients harboring unreported mtDNA variants will be useful in a clinical situation. Of these, the m.14439G>A (*MT-ND6*) variant was experimentally confirmed to be a novel causative mtDNA mutation, while 1356A>G (*12S rRNA*) was confirmed to be non-pathogenic by a cybrid assay. The remaining four novel variants have yet to be experimentally elucidated, but m.5537A>G (*mt-tRNA trp*) in Pt004 is likely to be causative because m.5537AinsT was reported to be disease causing.[16]

ND6 is an mtDNA-encoded complex I subunit that is essential for the assembly of complex I and the maintenance of its structure.[17–19] *ND6* mutations were previ-

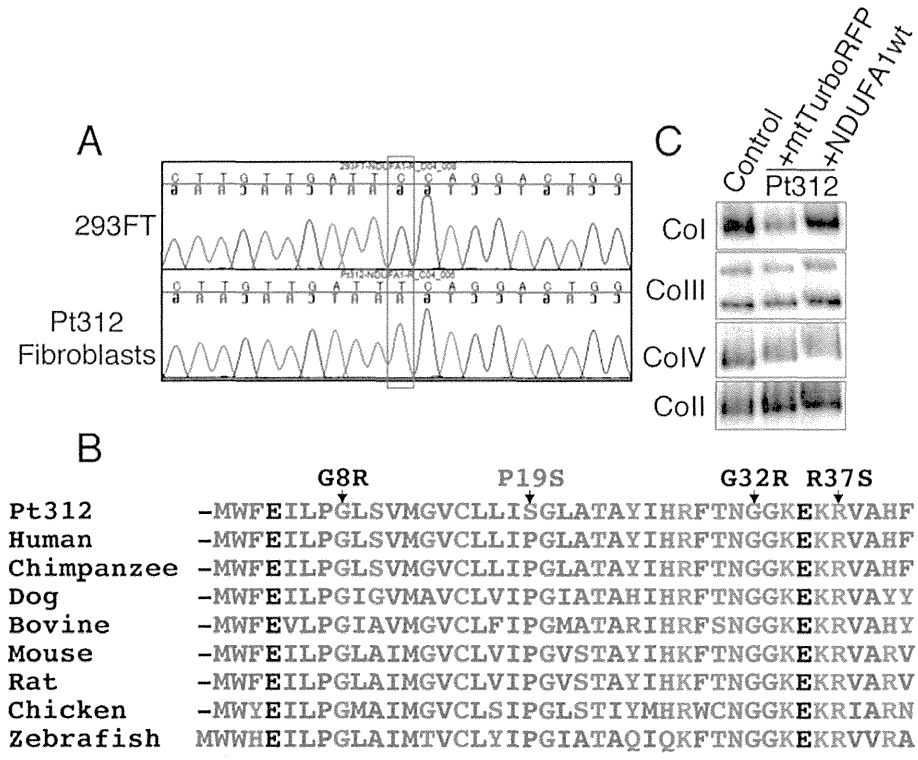

Figure 5. The novel nDNA mutation c.55C>T in *NDUFA1*. (A) Sequence chromatograms showing the c.55C>T (*NDUFA1* p.P19S) mutation in Pt312 and 293FT genomic DNA as a wild-type control. (B) Alignment of amino acid sequences of NDUFA1 subunit between different species shows the high conservation of amino acid Proline 19. G8R, G32R, and R37S show reported pathogenic mutations in *NDUFA1*. (C) Blue native polyacrylamide gel electrophoresis for CI, CII, CIII, and CIV following lentiviral transductions. Transduction of wild-type *NDUFA1* cDNA into Pt312 fibroblasts using recombinant lentivirus rescued complex I assembly levels of the fibroblasts, similar to the transduction of mtTurboRFP into normal fibroblasts (fHDF). As control gene of candidate genes, mtTurboRFP was used which inserted mitochondrial targeting signal sequence to N terminal of TurboRFP protein. By contrast, lentiviral transduction of control mtTurboRFP into Pt312 fibroblasts decreased the assembly level of complex I.

ously found to be associated with Leigh syndrome[20] and MELAS,[21] and this gene region is also reported to be a hot spot for LHON mutations.[22] Mitochondrial *12S rRNA* is a hot spot for mutations associated with aminoglycoside ototoxicity and non syndromic hearing loss, although mutations in this gene have not been reported to cause syndromic mitochondrial disorders.[23] We found that the m.14439G>A mutation altered an evolutionarily conserved proline to a serine in the hydrophilic inner membrane space of the ND6 protein[22] (Fig. 3C). As this mutation was homoplasmic in the patient's fibroblasts and absent from the blood of unaffected parents (Fig. 3A and B), this suggests that it developed de novo.

Exome sequencing in this study identified a single hemizygous change (c.55C>T, p.P19S) in exon 1 of the X-linked *NDUFA1* gene. To date, three missense mutations (G8R,[10] G32R,[24] and R37S[10]) have been reported in *NDUFA1* that are associated with neurological symptoms. *NDUFA1* was shown to interact with the subunits encoded by mtDNA during the complex I assembly process.[11]

Cybrid study is a powerful tool for detecting pathogenicity of either mtDNA or nDNA origin, although patients' cells showing RC enzyme deficiency are inevitable. Nevertheless, a major limitation of this technique is the length of time to establish transmitochondrial cybrids. We would, therefore, propose a systematic approach for diagnosing MRCD that starts with a biochemical enzyme assay and is followed by whole mtDNA sequencing. For patients with no apparent putative mtDNA mutations, whole exome sequencing is a powerful tool to diagnose nuclear gene mutations especially in cases when molecular diagnosis leads to appropriate genetic counseling.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplementary methods.

REPORT

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

Robert Kopajtich,[1,31] Thomas J. Nicholls,[2,31] Joanna Rorbach,[2,31] Metodi D. Metodiev,[3,31] Peter Freisinger,[4] Hanna Mandel,[5] Arnaud Vanlander,[6] Daniele Ghezzi,[7] Rosalba Carrozzo,[8] Robert W. Taylor,[9] Klaus Marquard,[10] Kei Murayama,[11] Thomas Wieland,[1,12] Thomas Schwarzmayr,[1,12] Johannes A. Mayr,[13] Sarah F. Pearce,[2] Christopher A. Powell,[2] Ann Saada,[14] Akira Ohtake,[15] Federica Invernizzi,[7] Eleonora Lamantea,[7] Ewen W. Sommerville,[9] Angela Pyle,[16] Patrick F. Chinnery,[16] Ellen Crushell,[17] Yasushi Okazaki,[18,19] Masakazu Kohda,[18] Yoshihito Kishita,[19] Yoshimi Tokuzawa,[19] Zahra Assouline,[20] Marlène Rio,[20] François Feillet,[21] Bénédict Mousson de Camaret,[22] Dominique Chretien,[3] Arnold Munnich,[3,20] Björn Menten,[23] Tom Sante,[23] Joël Smet,[6] Luc Régal,[24] Abraham Lorber,[25] Asaad Khoury,[25] Massimo Zeviani,[2,7] Tim M. Strom,[1,12] Thomas Meitinger,[1,12,26,27,28] Enrico S. Bertini,[8] Rudy Van Coster,[6] Thomas Klopstock,[28,29,30] Agnès Rötig,[3] Tobias B. Haack,[1,12] Michal Minczuk,[2,*] and Holger Prokisch[1,12,*]

Respiratory chain deficiencies exhibit a wide variety of clinical phenotypes resulting from defective mitochondrial energy production through oxidative phosphorylation. These defects can be caused by either mutations in the mtDNA or mutations in nuclear genes coding for mitochondrial proteins. The underlying pathomechanisms can affect numerous pathways involved in mitochondrial physiology. By whole-exome and candidate gene sequencing, we identified 11 individuals from 9 families carrying compound heterozygous or homozygous mutations in *GTPBP3*, encoding the mitochondrial GTP-binding protein 3. Affected individuals from eight out of nine families presented with combined respiratory chain complex deficiencies in skeletal muscle. Mutations in *GTPBP3* are associated with a severe mitochondrial translation defect, consistent with the predicted function of the protein in catalyzing the formation of 5-taurinomethyluridine (τm^5U) in the anticodon wobble position of five mitochondrial tRNAs. All case subjects presented with lactic acidosis and nine developed hypertrophic cardiomyopathy. In contrast to individuals with mutations in *MTO1*, the protein product of which is predicted to participate in the generation of the same modification, most individuals with *GTPBP3* mutations developed neurological symptoms and MRI involvement of thalamus, putamen, and brainstem resembling Leigh syndrome. Our study of a mitochondrial translation disorder points toward the importance of posttranscriptional modification of mitochondrial tRNAs for proper mitochondrial function.

Defects of the mitochondrial respiratory chain underlie a diverse group of human disorders characterized by impaired oxidative phosphorylation (OXPHOS). The generation of a functional respiratory chain requires the coordinated expression of both the nuclear genome and mitochondrial DNA (mtDNA). Defective translation of mtDNA-encoded proteins, caused by mutations in either the mitochondrial or nuclear genomes, represents a rapidly expanding group of human disorders, which often manifest as severe infantile combined OXPHOS deficiencies.[1]

[1]Institute of Human Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Neuherberg, Germany; [2]MRC Mitochondrial Biology Unit, Hills Road, Cambridge CB2 0XY, UK; [3]INSERM U1163, Université Paris Descartes-Sorbonne Paris Cité, Institut Imagine, 75015 Paris, France; [4]Department of Pediatrics, Klinikum Reutlingen, 72764 Reutlingen, Germany; [5]Metabolic Unit, Children's Hospital, Ramban Health Care Campus, 31096 Haifa, Israel; [6]Department of Pediatric Neurology and Metabolism, University Hospital Ghent, 9000 Ghent, Belgium; [7]Unit of Molecular Neurogenetics, Fondazione IRCCS (Istituto di Ricovero e Cura a CarattereScientifico) Istituto Neurologico "Carlo Besta," 20126 Milan, Italy; [8]Unità di Malattie Neuromuscolari e Neurodegenerative, Laboratorio di Medicina Molecolare, Dipartimento di Neuroscienze, IRCCS Ospedale Pediatrico Bambino Gesù, 00165 Roma, Italy; [9]Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne NE2 4HH, UK; [10]Department of Neuropediatrics, Klinikum Stuttgart, 70176 Stuttgart, Germany; [11]Department of Metabolism, Chiba Children's Hospital, Chiba 266-0007, Japan; [12]Institute of Human Genetics, Technische Universität München, 81675 Munich, Germany; [13]Department of Pediatrics, Paracelsus Medical University Salzburg, 5020 Salzburg, Austria; [14]Monique and Jacques Roboh Department of Genetic Research and the Department of Genetics and Metabolic Diseases, Hadassah-Hebrew University Medical Center, 91120 Jerusalem, Israel; [15]Department of Pediatrics, Faculty of Medicine, Saitama Medical University, Saitama 350-0495, Japan; [16]Wellcome Trust Centre for Mitochondrial Research, Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne NE1 3BZ, UK; [17]Metabolic Paediatrician, National Centre for Inherited Metabolic Disorders, Temple Street Children's University Hospital, Dublin 1, Ireland; [18]Department of Translational Research, Research Center for Genomic Medicine, Saitama Medical University, Saitama 350-1241, Japan; [19]Department of Functional Genomics & Systems Medicine, Research Center for Genomic Medicine, Saitama Medical University, Saitama 350-1241, Japan; [20]Departments of Pediatrics and Genetics, Hôpital Necker-Enfants Malades, 75015 Paris, France; [21]Service de médecine infantile, Hôpitald'Enfants de Brabois, CHU de Nancy, 54511 Vandoeuvre-les Nancy, France; [22]Service des Maladies Héréditaires du Métabolisme, CHU de Lyon, 69677 Bron, France; [23]Center for Medical Genetics, Ghent University, Ghent University Hospital, 9000 Ghent, Belgium; [24]Department of Pediatrics, Metabolic Center, University Hospital Leuven, 3000 Leuven, Belgium; [25]Department of Pediatric Cardiology, Ramban Medical Center, 31096 Haifa, Israel; [26]DZHK (German Centre for Cardiovascular Research), partner site Munich, 81675 Munich, Germany; [27]Munich Heart Alliance, 80802 Munich, Germany; [28]Munich Cluster for Systems Neurology (SyNergy), 80336 Munich, Germany; [29]German Research Center for Neurodegenerative Diseases (DZNE), 80336 Munich, Germany; [30]Department of Neurology, Friedrich-Baur-Institute, Ludwig-Maximilians-University, 80336 Munich, Germany
[31]These authors contributed equally to this work
*Correspondence: michal.minczuk@mrc-mbu.cam.ac.uk (M.M.), prokisch@helmholtz-muenchen.de (H.P.)
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The mitochondrial genome contains a total of 37 genes, 13 of which encode protein subunits of the respiratory chain complexes and the ATP synthase. Translation of these genes is achieved by the organelle's own protein synthesis machinery, of which only the RNA components (rRNAs and tRNAs) are encoded by mtDNA. All protein factors required for mitochondrial translation are encoded in the nucleus and must be imported after their synthesis in the cytoplasm. Mitochondrial (mt-) tRNAs require extensive posttranscriptional modifications before achieving translation competency. Modifications to tRNAs might contribute to their proper folding, stability, or decoding capacity. In mitochondria a minimal set of 22 different tRNAs is used to translate all codons.[2] Modifications to the wobble position of the anticodon loop of mt-tRNAs play an important role in ensuring correct mRNA-tRNA interactions. In ten mt-tRNA species, all of which correspond to two codon sets, four different types of modified nucleotides have been identified at the wobble position.[3,4] One of these modifications is 5-taurinomethyluridine (τm^5U), found at position 34 (U34) of mt-tRNAsLeu^{UUR}, Trp, Gln, Lys, and Glu, which has been suggested to be synthesized cooperatively by GTPBP3 and MTO1.[5] In addition to τm^5 U, mt-tRNAs Gln, Lys, and Glu also contain a 2-thiouridine modification at U34 (s^2U), introduced by TRMU (also known as MTU1). This results in a 5-taurinomethyl-2-thiouridine ($\tau m^5 s^2 U$) modification in these mt-tRNA molecules. Modifications of U34 have been proposed to modulate either the accuracy or the efficiency of translation.[6,7] Three types of mutations affecting U34 have been associated with human mitochondrial disease: (1) mutations in the mt-tRNAs;[8] (2) mutations in *TRMU* (MIM 610230) affecting U34 2-thiouridylation and leading to acute infantile liver failure resulting from combined OXPHOS deficiency;[9] and (3) more recently, mutations in *MTO1* (MIM 614667) found to underlie cases of hypertrophic cardiomyopathy and lactic acidosis, associated with impaired mitochondrial translation rate and reduced respiratory chain activities.[10,11]

Whole-exome sequencing (WES) of 790 individuals with suspected mitochondriopathy in five centers identified eight index case subjects (plus two affected siblings) with homozygous or two heterozygous rare variants (minor allele frequency < 0.1%) in *GTPBP3* (MIM 608536), with no such case being found in 11,295 control subjects. This presents a genome-wide significant enrichment in *GTPBP3* (RefSeq accession number NM_032620.3) mutation load in samples from individuals with the clinical diagnosis "mitochondrial disease" ($p < 3.2 \times 10^{-10}$, Fisher exact test) in comparison to nonmitochondrial disorder samples. In addition, when filtering for genes coding for mitochondrial proteins,[12] in several individuals *GTPBP3* was the only gene with two mutations. Further evidence for the pathogenic role of *GTPBP3* mutations was derived from follow-up candidate gene sequencing of 18 individuals with similar phenotypes, which identified two more index cases. Collectively, mutations in *GTPBP3* were detected in 12 individuals from 10 families. However, segregation analysis of a single affected individual (#66654) revealed that the two identified heterozygous mutations in *GTPBP3* affected the same allele, leaving genetic evidence about 11 individuals from 9 families (Figure 1).

Written informed consent was obtained from all individuals investigated or their guardians, and the ethics committee of the Technische Universität München approved the study.

Individual #49665 (family F1, Figure 1A) is a boy born to consanguineous parents from the UAE. He presented at the age of 10 years with mild intellectual disability, fatigability, mild hypertrophic cardiomyopathy, and visual impairment. At presentation he measured 134 cm with a body weight of 25 kg. Clinical examination revealed slight dyspnea when climbing stairs and mild intellectual disability. Plasma lactate was consistently elevated (3.0 to 7.2 mmol/l, reference < 2.1 mmol/l). Electroencephalogram, hearing test, and visual-evoked potentials showed no abnormalities. Electrocardiography (ECG) revealed signs of left ventricular hypertrophy confirmed by echocardiography. There was no obstruction of the left ventricular outflow tract. He had a pale optic disc on both sides but visual acuity and visual field could not be examined. Brain MRI was normal, but MR spectroscopy revealed lactate peaks in the parietal and precentral cortex. Respiratory chain (RC) measurement in muscle revealed a significant reduction of complex I and IV activities. He was substituted with CoQ_{10} (200 mg/day), riboflavin (400 mg/day), carnitine (1 g/day), and a fat-rich diet (60% of daily caloric intake). A follow-up examination 1 year after the initial presentation showed no significant changes of his clinical signs/symptoms.

His 17-year-old elder brother, individual #36349 (family F1, Figure 1A), had a very similar clinical picture.

Individual #66143 (family F2, Figure 1A), a boy, is the second child of healthy unrelated parents of Arab-Moslem origin from Israel. He presented at the age of 2 years with sudden respiratory failure. Heart ultrasonography indicated a hypertrophic cardiomyopathy and congestive heart failure. His cardiac symptoms improved on treatment with furosemide, spironolactone, carvedilole, and digoxin. In addition, a high-dose vitamin treatment (100 mg/day riboflavin, 100 mg/day vitamin B1, and 60 mg/day CoQ_{10}) was initiated. RC enzyme measurement in muscle revealed a significant reduction of complex I and IV activities. On follow-up examinations (over 3 years), the child's psychomotor development is normal and his parents reported that he is active like his peers. Digoxin and spironolacton treatment was stopped and his recent echocardiography revealed a stable condition of the heart including normal global function of left ventricle with no further hypertrophy of interventricular septum and no pulmonary hypertension.

Individual #72425 (family F3, Figure 1A) was a girl born to unrelated parents. At 3 months of age, she had feeding difficulties and failure to thrive. At the age of 7 months,

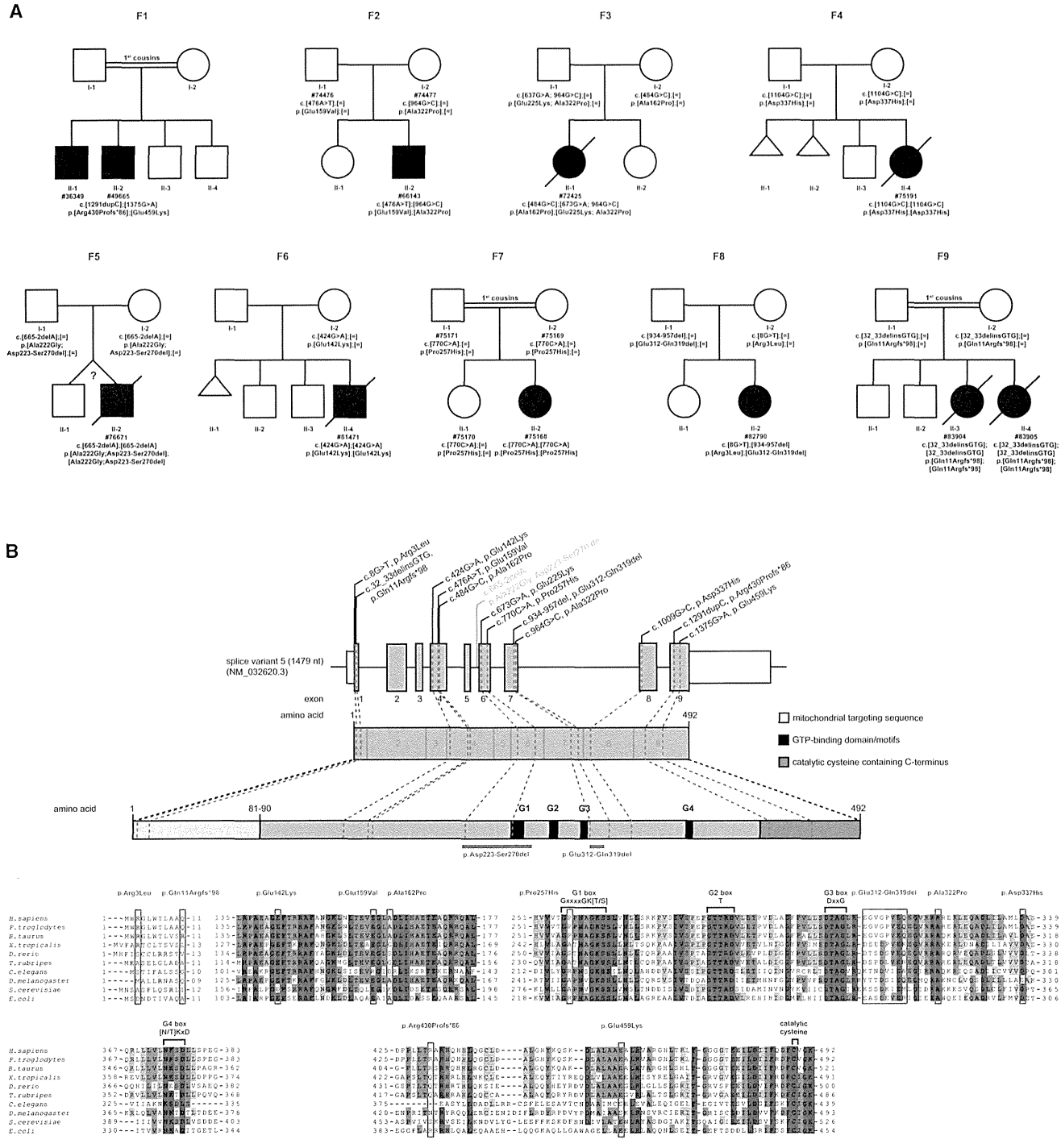

Figure 1. ***GTPBP3* Mutation Status and Gene Structure**
(A) Pedigrees of nine families with mutations in *GTPBP3*.
(B) Gene structure of *GTPBP3* with known protein domains of the gene product and localization and conservation of amino acid residues affected by mutations. Black and orange text indicate exonic and intronic variants. Intronic regions are not drawn to scale. Coloring in the sequence alignment represents the identity of amino acid residues.

she developed recurrent cough and fever and was admitted to the emergency room with severe fatigue, pallor, and progressive malaise. Blood exams showed leukocytosis, and 2 days later her general condition worsened, showing cyanosis and hyporeactivity. Echocardiography showed severe dilated cardiomyopathy with an ejection fraction of 20% that was unresponsive to therapy. She had severe refractory hyperlactatemia (23.3 mmol/l, reference range 0.5–2.3 mmol/l). Histochemical and spectrophotometric analysis of the muscle biopsy showed a severe complex IV deficiency. She died 10 days after admission from cardiac failure.

Individual #75191 (family F4, Figure 1A), a girl, was born to nonconsanguineous parents after an uneventful pregnancy of 40 weeks. The mother had had two miscarriages at 6 and 8 weeks and had a healthy son aged 16 months. In the first hours after birth, individual #75191 developed mild stridor and dyspnea which rapidly worsened. She fed poorly and became less responsive, and a Kussmaul breathing pattern was seen. She was transferred to a specialist center and was found to be severely hypotonic, moving very little, either spontaneously or after stimulation. She had hyperlactatemia (23 mmol/l), hypoglycemia (18 mg/dl), hyperammonemia (135 μmol/l, control value 11–48 μmol/l), and hyperlactaturia. She progressively developed respiratory insufficiency and bradycardia. Cardiac ultrasound showed apical right ventricular hypertrophy and an open duct of Botalli with minor shunting. Fractional shortening was 28% (mildly decreased). Cerebral ultrasound showed a minimal grade I bleeding, and the cerebral matter appeared mildly hyperechogenic. She died of asystolia at day 1. A muscle biopsy performed immediately after death showed decreased activities of RC complexes I and IV.

Individual #76671 (family F5, Figure 1A) was the second boy of nonconsanguineous parents. The infant was born at 41 weeks of gestation from a twin pregnancy. Generalized hypotonia and difficulty in suction was noted since birth and he rapidly developed failure to thrive. He acquired head control at the age of 7 months but parents reported normal cognitive skills. At the age of 9 months he was admitted to the intensive care unit for acute aspiration pneumonia that required intubation. Laboratory test revealed a metabolic acidosis with hyperlactatemia (5.2 mmol/l) and brain MRI showed bilateral thalamic T2-weighted hyperintense abnormalities with low diffusion. Analysis of a muscle biopsy revaled a clear reduction in histochemical cytochrome *c* oxidase activity and decreased complex I and IV enzyme activities. The cardiological examination disclosed hypertrophic cardiomyopathy and a Wolff-Parkinson-White pre-excitation syndrome (MIM 194200). The baby died after 15 days of hospitalization with clinical signs of heart failure.

Individual #81471 (family F6, Figure 1A) was a boy born to nonconsanguineous Romanian parents at 34 weeks gestation (birth weight 2.18 kg). His mother had premature and prolonged (85 hr) rupture of membranes before delivery, and the baby was treated with i.v. antibiotics before being discharged home on day 7. He was readmitted to hospital on day 25 with weight loss (2.23 kg). He was hypothermic and jaundiced and initial blood analysis showed profound metabolic acidosis. He was treated with i.v. antibiotics for presumed sepsis. The acidosis did not resolve, and serum lactate was elevated (11.0 mmol/l). ECG was abnormal and echocardiography showed concentric left ventricular hypertrophy. CSF lactate was 12.4 mmol/l (normal range 0.9–2.4 mmol/l) prompting bicarbonate treatment. Brain MRI showed abnormal diffusion of the subthalamic nuclei extending down to the brain stem. There was abnormal T2 signal in the midbrain and basal ganglia bilaterally. On examination he was thin but not dysmorphic. He was mildly jaundiced and had puffy feet. There was little spontaneous movement but normal muscle bulk and he was distinctly hypotonic. Feeding through a nasogastric tube was established but he did not become responsive despite high caloric intake. He developed recurrent apnea and died aged 5 weeks. Biochemical analysis performed in muscle revealed a significant decrease of RC complexes I and IV.

Individual #75168 (family F7, Figure 1A) is the second girl of first-cousin parents from India. She was first seen at the age of 2 years with development delay. She was able to walk but she couldn't speak. She received occupational and speech therapy. During a febrile illness when she was 3 years old, she had an acute metabolic failure with hyperlactatemia and hyperlactatorachia. She recovered but had epileptic seizures and more severe intellectual disability. Brain MRI showed pronounced bilateral hyperintensities affecting the whole thalamus and extending to the mesencephalon. Hyperlactatemia (>10 mmol/l) and hyperlactatorachia (6 mmol/l) were noticed. RC activity in muscle was normal as well as PDH complex tested by immunoblot. The girl was treated with qa carnitine 3 × 350 mg/day, CoQ_{10} 3 × 50 mg/day, vitamins B1 3 × 50 mg/day and B6 3 × 50 mg/day, and bicarbonate 4 × 1 g. Epilepsy was in good control with levetiracetame 40 mg/kg/day and a high-fat diet. The girl is in a special school for children with developmental delay. Her general condition is good. She is always in a good temper. Development is delayed about 1.5 years. She has continual hyperlactatemia (8–10 mmol/l).

Individual #82790 (family F8, Figure 1A) is a girl born at 40 weeks of gestation with normal birth weight to nonconsanguineous Japanese parents. At the age of 1 year, she developed frequent epileptic seizures, and she was medicated with phenobarbital. Severe developmental delay was noted and at the age of 15 months she was admitted to children's hospital. Her weight gain (9.25 kg, −0.06 SD) is within the normal range, but she developed severe muscle hypotonia. There is no cardiac involvement by ECG and echocardiogram. Hyperlactatemia was noted (5.72–6.49 mmol/l) whereas metabolic profiling of amino acids, urinary organic acids, and acylcarnitine was normal. RC analysis in muscle showed a significant decrease in complexes I and IV activities. Brain MRI showed bilateral hyperintensities in the putamen and weakly also in the anterior thalamus. A lactate peak was detected on $[H^+]$-MR spectroscopy. She is now 2 years of age and still presents with a severe global developmental delay.

Individual #83904 (family F9, Figure 1A) was the second child of consanguineous, healthy parents of Turkish origin. She was born at 39 weeks of gestational age (birth weight 2,740 g, length 49 cm, head circumference 32 cm). Shortly after birth, she presented with Wolff-Parkinson-White syndrome. Cardiac ultrasound was normal. Treatment was started with amiodarone and she