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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 previously 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.

Correspondence: Akira Ohtake, MD PhD, Department of Pediatrics, Faculty of Medicine, Saitama Medical University, 38 Morohongo, Moroyama, Iruma-gun, Saitama 350-0495, Japan. Email: akira_oh@saitama-med.ac.jp

*Present address: Monash IVF PTY Ltd, Melbourne, Victoria, Australia.

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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.

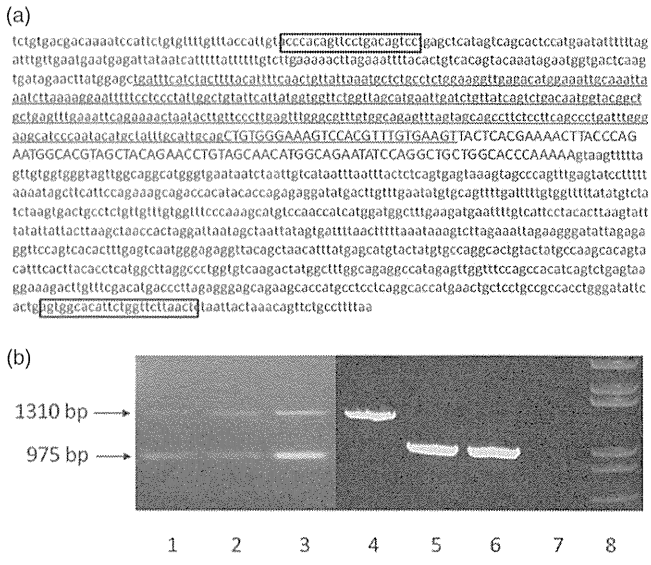

Fig. 1 Genomic sequence determination of 335 bp deoxyguanosine kinase (*DGUOK*) deletion in the family of patients 1 and 2. (a) Capitalization, sequence of exon 2; two rectangles, long-range polymerase chain reaction (PCR) primer sets; underline, 335 bp deletion. The large 335 bp deletion encompassing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

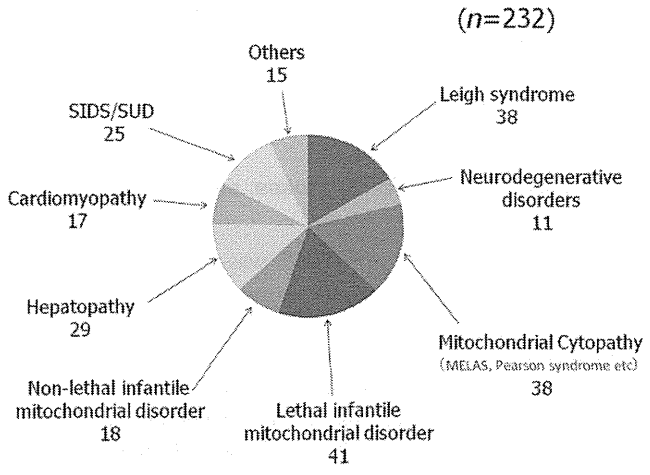

Fig. 2 Clinical diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan. Neurodegenerative disorders, neurodegenerative disorders unclassified to specific diseases. Patients with non-lethal infantile mitochondrial disorder started with symptoms such as lethal infantile mitochondrial disorder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

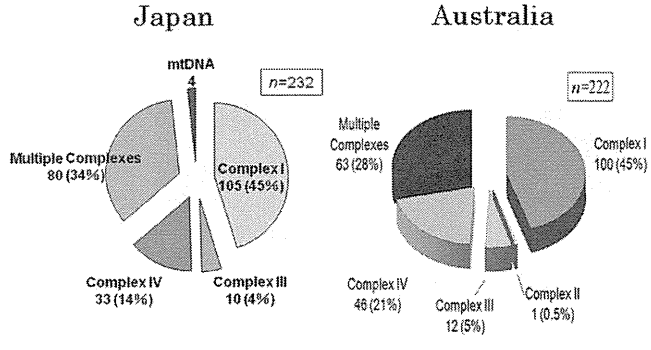

Fig. 3 Percentage distribution of enzymatic diagnoses of mitochondrial respiratory chain disorder (MRCD) in Japan and those reported previously in Australia. The enzymatic diagnosis of MRCD showed similar trends in prevalence between the Japanese and Australian patients,[17] with respiratory chain complex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

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

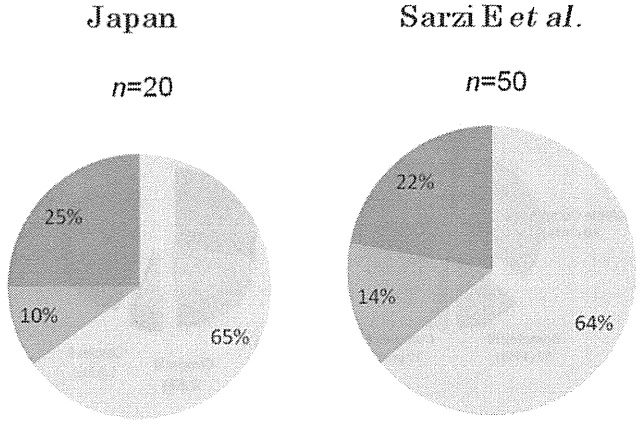

Fig. 4 Percentage distribution of disease types of mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* "Alpers-like" refers simply to delayed-onset hepatocerebral MTDPS, because no true case of Alpers syndrome has yet been identified in Japan. The distribution of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

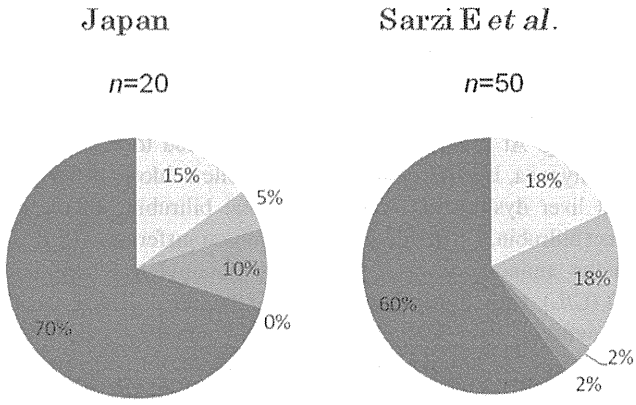

Fig. 5 Percentage distribution of responsible genes for mitochondrial DNA depletion syndrome (MTDPS) in Japan and those reported by Sarzi *et al.* The causative gene was not identified in the majority of patients in each population. Four genes, *DGUOK*, *POLG*, *MPV17*, and *TK2*, contained 40% of the 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.

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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    493   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]

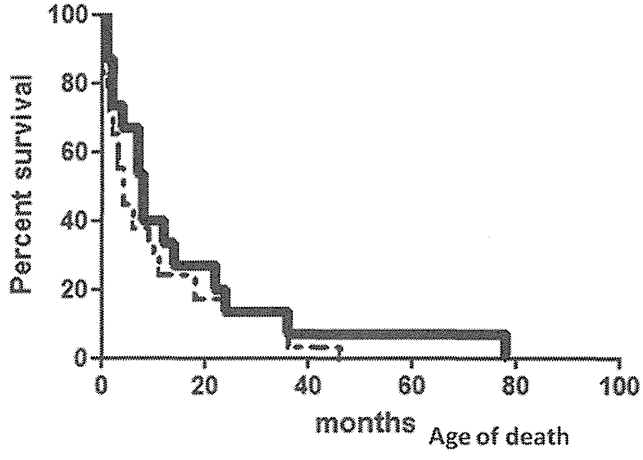

Fig. 7 Comparison of the ages of death (in months) in the two studies. A commonality between the Japanese patients and the Sarzi *et al.* patients[4] was observed. No significant difference in disease severity was identified (log-rank test, $P = 0.3637$; Gehan-Breslow-Wilcoxon test, $P = 0.2667$). (▬)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

Fever of Unknown Origin as the Initial Manifestation of Valproate-Induced Fanconi Syndrome

Fumihito Nozaki MD [a,*], Tomohiro Kumada MD, PhD [a], Takashi Kusunoki MD, PhD [a], Tatsuya Fujii MD, PhD [a], Kei Murayama MD, PhD [b], Akira Ohtake MD, PhD [c]

[a] *Department of Pediatrics, Shiga Medical Center for Children, Moriyama-shi, Shiga, Japan*
[b] *Department of Metabolism, Chiba Children's Hospital, Midori, Chiba, Japan*
[c] *Faculty of Medicine, Department of Pediatrics, Saitama Medical University, Saitama, Japan*

ABSTRACT

BACKGROUND: Valproate-induced Fanconi syndrome is a rare adverse effect of valproate. Severely disabled patients who require tube feeding are reported to be susceptible to valproate-induced Fanconi syndrome. Although most patients with valproate-induced Fanconi syndrome are asymptomatic and detected incidentally with findings such as hypophosphatemia, hypouricemia, increased urinary β2-microglobulin, and generalized hyperaminoaciduria, clinical symptoms such as bone fracture, fever, tachypnea, and edema have been reported. **PATIENT DESCRIPTION:** This 15-year-old, severely disabled, tube-fed, male patient with cytochrome oxidase deficiency had taken valproate for 3 years when he developed fever for 3 weeks. Hypophosphatemia, hypouricemia, hypokalemia, increased urinary β2-microglobulin, and generalized hyperaminoaciduria, as well as hypocarnitinemia, were found, indicating that he had Fanconi syndrome. Valproate was the most likely cause of Fanconi syndrome in this patient. After discontinuation of valproate, the fever resolved immediately, and the laboratory findings normalized. **CONCLUSION:** Valproate-induced Fanconi syndrome should be considered when individuals taking valproate develop fever of unknown origin.

Keywords: Fanconi syndrome, valproate, fever of unknown origin, side effects, valproate-induced Fanconi syndrome

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Introduction

Fanconi syndrome is a generalized dysfunction of the proximal renal tubules that causes urinary excretion of amino acids, glucose, phosphate, bicarbonate, uric acid, and other substances. Valproate (VPA)-induced Fanconi syndrome is a rare adverse effect of VPA.[1] Several case reports have shown that severely disabled, tube-fed patients are vulnerable to VPA-induced Fanconi syndrome.[2,3] Most patients with VPA-induced Fanconi syndrome are diagnosed during routine or incidental laboratory examinations without any obvious symptoms.[2] The case of a severely disabled, tube-fed patient with cytochrome oxidase deficiency who presented with fever of unknown origin that was most likely caused by VPA-induced Fanconi syndrome is presented.

Patient Description

This 15-year-old boy was born at 41 weeks' gestation with a birth weight of 3270 g. His Apgar score was 3 at 1 minute and 6 at 5 minutes. He developed spastic tetraplegia and needed tube feeding. At age 4 months, he presented with infantile spasms. He was diagnosed with probable Leigh syndrome because of high lactate levels in the blood (39.6 mg/dL) and cerebrospinal fluid (34.5 mg/dL), as well as high-intensity signals in bilateral basal ganglia and thalami on T2-weighted magnetic resonance imaging at age 2 years. Pyruvate dehydrogenase complex activities in the lymphocytes and respiratory chain complex activities in the muscle, as well as histopathology of the skeletal muscle, were normal. Screening for known mitochondrial DNA mutations was negative. Seizure control was poor, and he had been on VPA and carbamazepine without l-carnitine supplementation from age 12 years. Two

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* Communications should be addressed to: Nozaki; Department of Pediatrics; Shiga Medical Center for Children; 5-7-30 Moriyama; Moriyama, Shiga 524-0022, Japan.
E-mail address: nozaki-kgw@umin.ac.jp

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months before his admission at age 15 years, the VPA dose was increased (34 mg/kg/day) because of seizure deterioration, and l-carnitine (6 mg/kg/day) was added as a supplement to prevent secondary carnitine deficiency.

He was admitted to our hospital because of high-grade fever (39.1°C) that had lasted for 3 days. Bronchial pneumonia was suspected, and cefotaxime was administered. However, the fever persisted for 3 weeks. His body weight decreased from 16.7 kg at admission to 14.8 kg during the 3 weeks despite sufficient water (1800 mL/day) and caloric intake (1500 kcal/day). The following laboratory examinations were normal: white blood cell count, C-reactive protein, serological tests for viral or mycoplasma infection, antinuclear antibodies, rheumatoid factor, thyroid hormones, lymphocyte stimulation test for VPA, and bacterial and mycotic cultures. However, the erythrocyte sedimentation rate was high (90 mm/hr). Whole-body [F^{18}]-fluorodeoxyglucose positron emission tomography scan did not reveal any findings indicating focal inflammation. On the other hand, hypophosphatemia (1.3 mg/dL; normal 2.6-6.3 mg/dL), hypokalemia (3.3 mEq/L; normal 3.6-5.2 mEq/L), and hypouricemia (1.6 mg/dL; normal 2.0-7.0 mg/dL) were found, indicating the possibility of proximal renal tubular dysfunction. Urinalysis showed proteinuria, glycosuria, elevated β2-microglobulin (4460 μg/L; normal <230 μg/L), and generalized hyperaminoaciduria. These results confirmed Fanconi syndrome. In addition, hypocarnitinemia (19.0 μmol/L) was found despite carnitine supplementation, indicating secondary carnitine deficiency due either to VPA or Fanconi syndrome, or both. VPA was discontinued at 18 days from the start of fever. Four days later, the high fever resolved, and he gained 1 kg of weight. After the fever resolved, the l-carnitine supplement was increased (40 mg/kg/day) to treat hypocarnitinemia. Two months later, laboratory findings associated with Fanconi syndrome were normal. After recovery, a skin biopsy revealed cytochrome oxidase deficiency. The patient was not rechallenged with VPA, and no recurrence of Fanconi syndrome has been evident for more than 2 years.

Discussion

Fanconi syndrome is characterized by a general dysfunction of proximal renal tubules that causes urinary excretion of amino acids, glucose, phosphate, bicarbonate, uric acid, and other substances.[4] Recently, VPA-induced Fanconi syndrome has been recognized mostly in severely disabled patients.[2,3] The fever of unknown origin and weight loss in the present patient were likely the manifestations of VPA-induced Fanconi syndrome due to (1) the patient's vulnerability to this condition due to his diagnosis of cytochrome oxidase deficiency and severe disability requiring tube feeding, and (2) the fact that fever of unknown origin, weight loss, and laboratory findings indicative of Fanconi syndrome normalized after discontinuation of VPA.

A search of the PubMed database and the Japan Medical Abstract Society website for articles using the keywords "Fanconi syndrome" and "valproate" or "valproic acid" identified 20 reports of 49 patients (Table).[1-20] In these, sex and age were described in 37 patients (19 males and 18 females), with ages ranging from 2 to 32 years (median age, 8 years). As previously reported,[3] a high percentage of Japanese patients (36 of 49 patients) was evident. Among the 49 patients identified, 47 were described as being severely disabled (n = 42) or not (n = 5); in addition, feeding was described in 41 of the 49 patients, with 36 reported as being tube-fed. The duration of VPA treatment ranged from 3 months to 21 years (median, 4 years), and the VPA blood levels ranged from 21 to 141 μg/mL (median, 77.6 μg/mL). When VPA was discontinued, 45 of 47 patients recovered completely from VPA-induced Fanconi syndrome.

The duration needed for recovery ranged from 1 week to 18 months (median, 4 months). Two patients developed renal failure or continuing proteinuria despite the discontinuation of VPA.[6,13] Thus, the clinical course of VPA-induced Fanconi syndrome in the present patient was similar to the previous reports. Among the 13 patients in whom serum carnitine levels or carnitine supplementation was described, 3 patients had hypocarnitinemia,[11,16,19] 1 patient had a normal carnitine level,[15] and 9 patients with no description of serum carnitine levels had carnitine supplementation.[12,13,17,18] None of the patients whose serum carnitine levels were described had fever of unknown origin. Thus, there was no apparent association between hypocarnitinemia and prolonged fever, as appeared in the present patient. Furthermore, the weight loss and elevated erythrocyte sedimentation rate found in the present patient were not described in the other reported patients with VPA-induced Fanconi syndrome.

Overall, in 19 of the 49 reported patients, VPA-induced Fanconi syndrome was found on routine or incidental laboratory examinations without any obvious symptoms. In the remaining 30 patients, the initial clinical manifestations led to the diagnosis: 11 had fracture, 9 had fever, 3 had tachypnea, 2 had edema, 2 had weakness, 1 had anorexia, abdominal pain, and myopathy-like symptoms, 1 had hypertension, and 1 had fatigue and confusion. Among the 9 patients with fever,[8,9,14,15,20] 2 had prolonged fever described as fever of unknown origin.[8] In the remaining 7 patients, VPA-induced Fanconi syndrome was diagnosed and treated before the fever became prolonged.

In the present case, it took time to suspect VPA-induced Fanconi syndrome because fever is a common symptom in severely disabled patients. After infection was ruled out, more time was taken to rule out various other causes. The present case report, therefore, suggests the importance of considering VPA-induced Fanconi syndrome in severely disabled patients on VPA who develop prolonged fever and of measuring serum phosphate, uric acid, and electrolyte levels, as well as urinalysis including β2-microglobulin. The present case also suggested that supplementation with less than 10 mg/kg of carnitine is insufficient in these patients.

Although the precise pathogenic mechanism of VPA-induced Fanconi syndrome remains unknown, a mitochondrial abnormality in the proximal renal tubules is a typical finding of drug-induced Fanconi syndrome. There are at least three hypotheses for the pathogenesis of the renal tubular dysfunction. The first is an inhibition of β-oxidation in the mitochondria of the proximal renal tubules either directly by VPA or indirectly by the secondary carnitine deficiency caused by VPA.[1,5] The second is tubulo-interstitial nephritis (TIN) caused by hypersensitivity to VPA or a direct toxic effect of VPA.[5,8] The third is increased oxidative stress due to the VPA-induced decrease in plasma glutathione peroxidase activity, which causes mitochondrial dysfunction in the tubules.[1] The mechanisms of fever in VPA-induced Fanconi syndrome are unknown. Only two reported patients had prolonged fever of unknown origin, and TIN was found in one patient.[8] Because TIN can cause fever and weight loss,[8] fever of unknown origin and weight loss in the present patient might have been caused by TIN, but it could not be confirmed without renal biopsy. Another possibility is

TABLE.
Previously Published Cases of VPA-induced Fanconi Syndrome

Patient number	Age (y)/ Sex	Clinical remarks	Severe disability	Tube feeding	VPA duration	VPA blood level, μg/mL	Other AEDs	Time to recovery	Opportunity that disclosed FS	Reference
1	27/F	Epilepsy	No	No	5 y	136	None	1 wk	Fatigue, confusion	1
2	6/M	Cerebral palsy	Yes	Yes	5 1/2 y	81.4	PHT, CLB	6 mo	Edema	2
3	15/M	Cerebral palsy	Yes	Yes	14 1/2 y	75	CBZ, CLB	3 mo	Laboratory study	2
4	10/F	Cerebral palsy, lissencephaly	Yes	-	16 mo	69.8	PB, GBP	5 mo	Laboratory study	2
5	9/M	Anoxic encephalopathy	Yes	Yes	Several years	49.3	PB, DZP	4 mo	Laboratory study	2
6	4/F	Perinatal anoxic encephalopathy	Yes	Yes	3 1/2 y	60.2	CBZ	4 mo	Laboratory study	2
7	8/M	West syndrome	Yes	-	7 y	64	CZP, TPM	2 mo	Laboratory study	3
8	6/F	Cerebral retardation	Yes	-	2 1/2 y	-	CLB	14 mo	Weakness	3
9	12/M	Petit mal epilepsy	No	No	19 mo	-	PB	6 mo	abdominal pain, myopathy-like	4
10	10/M	Severe and global neurolgic impairment	Yes	-	10 mo	-	PHT, lorazepam	3 mo	Hypertension	5
11	10/M	Epilepsy	No	No	18 mo	-	None	Renal failure	Laboratory study	6
12	9.5/M	Birth asphyxia	-	-	8 y	-	CZP, CBZ	4 mo	Fracture	7
13	19/M	Cerebral palsy	-	-	-	-	PB	-	Fracture	7
14	15/M	Near-drowning	Yes	Yes	13 y	89.2	None	3 mo	Laboratory study	8
15	6/F	Neonatal asphyxia	Yes	Yes	6 y	73.9	CLB	2 mo	Laboratory study	8
16	6/F	Neonatal asphyxia	Yes	Yes	6 y	119.8	None	2 mo	Laboratory study	8
17	2/F	Early infantile epileptic encephalopathy	Yes	Yes	12 mo	94.7	ZNS, CLB	3 mo	Fever of unknown origin	8
18	4/M	Pachygyria	Yes	Yes	4 y	62	None	18 mo	Laboratory study	8
19	8/F	Neonatal asphyxia	Yes	Yes	8 y	95	None	2 mo	Laboratory study	8
20	13/F	Neonatal asphyxia	Yes	Yes	7 y	141	None	12 mo	Fever of unknown origin	8
21	8/F	Chromosome abnormality	Yes	Yes	6 y	98.96	-	2 mo	fever	9
22	4/M	West syndrome	Yes	Yes	3 y	40	PB, ZNS	9 mo	Laboratory study	10
23	10/M	Partial epilepsy	No	No	12 mo	21	None	18 mo	Laboratory study	10
24	7/M	Lissencephaly	Yes	Yes	7 y	129.7	PB, CZP	2 mo	Edema	10
25	7/F	Near-drowning	Yes	Yes	2 y	57.2	CBZ	15 mo	Laboratory study	10
26	9/F	Hypoxic ischemic encephalopathy	Yes	Yes	8 y	81.6	-	4 mo	Laboratory study	11
27	22/M	West syndrome	Yes	Yes	21 y	-	-	1 mo	Laboratory study	12
28	-	Encephalopathy sequelae	Yes	Yes	2 y	-	ZNS	3 mo	Fracture	13
29	-	Encephalopathy sequelae	Yes	Yes	4 y	-	ZNS	3 mo	Fracture	13
30	-	Chromosome abnormality	Yes	Yes	4 y	-	CBZ, PHT	Proteinuria	Laboratory study	13
31	-	Epilepsy	Yes	Yes	3 y	-	KBr	1 mo	Laboratory study	13
32	-	Brain malformation	Yes	Yes	9 y	-	PB	3 mo	Fracture	13
33	-	Cerebral palsy	Yes	Yes	4 y	-	ZNS	6 mo	Tachypnea	13
34	-	Cerebral palsy	Yes	Yes	8 y	-	none	9 mo	Fracture	13
35	-	Cerebral palsy	Yes	Yes	3 y	-	CLB, ZNS, PB, KBr	9 mo	Fracture	13
36	-	Neurodegenerative disease	Yes	Yes	3 y	-	CZP, PB, DZP	6 mo	Fracture	13
37	-	Cerebral palsy	Yes	Yes	3 y	-	ZNS, PB	2 mo	Fracture	13
38	-	Encephalopathy sequelae	Yes	Yes	3 mo	-	ZNS, PB	6 mo	Tachypnea	13
39	-	Neurocutaneous syndrome	Yes	Yes	12 mo	-	ZNS	6 mo	Tachypnea	13
40	4/M	Congenital myopathy	Yes	Yes	-	-	None	3 mo	Lower respiratory tract infection	14
41	8/F	West syndrome	Yes	Yes	6 y	74.2	PHT, PB, CZP, ZNS	5 mo	Pneumonia	15
42	2/F	Chromosome abnormality	Yes	Yes	2 y	122.7	CBZ, CLB	2 mo	Gastroenteritis	15
43	3/M	Epilepsy	Yes	Yes	3 y	65.6	ZNS, GBP	1 mo	Upper respiratory infection	15
44	8/F	Myoclonic epilepsy	Yes	Yes	7 y	-	CLB	12 mo	Fracture	16
45	14/M	Epilepsy	No	No	2 y	-	-	6 mo	Weakness	17
46	10/F	Partial deletion of chromosome 4p	Yes	Yes	9 y	-	TPM	12 mo	Laboratory study	18
47	2/F	Type 2 Gaucher disease	Yes	Yes	2 y	80.2	CLB, TPM	2 mo	Fracture	19
48	11/M	Cerebral palsy, epilepsy	Yes	-	11 y	-	PB, ZNS CLB, KBr	A couple of weeks	Respiratory illness	20
49	32/F	Neonatal asphyxia	Yes	-	-	-	-	-	Pneumonia	20

Abbreviations:
AEDs = Antiepileptic drugs
CBZ = Carbamazepine
CLB = Clobazam
CZP = Clonazepam
DZP = Diazepam
FS = Fanconi syndrome
GBP = Gabapentin
KBr = Potassium bromide
PB = Phenobarbital
PHT = Phenytoin
TPM = Topiramate
VPA = Valproate
ZNS = Zonisamide
Severe disability means bedridden or wheelchair-bound.

uncoupling or "loose coupling" of oxidative phosphorylation in the mitochondria, which may cause fever and weight loss because of hypermetabolism, as found in Luft's disease.[21] However, valproate is not known to have an uncoupling effect. Our patient had a cytochrome oxidase deficiency, which itself could cause Fanconi syndrome. However, the fact that Fanconi syndrome resolved with VPA withdrawal indicated that the mitochondrial disease was not a direct cause of Fanconi syndrome in the present patient.

In conclusion, VPA-induced Fanconi syndrome should be considered when patients taking VPA develop fever of unknown origin. Furthermore, individuals taking VPA, especially those who are severely disabled and tube-fed, should be given carnitine supplementation and be periodically screened for Fanconi syndrome.

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COQ4 Mutations Cause a Broad Spectrum of Mitochondrial Disorders Associated with CoQ_{10} Deficiency

Gloria Brea-Calvo,[1,20] Tobias B. Haack,[2,3,20] Daniela Karall,[4,20] Akira Ohtake,[5] Federica Invernizzi,[6] Rosalba Carrozzo,[7] Laura Kremer,[2,3] Sabrina Dusi,[6] Christine Fauth,[8] Sabine Scholl-Bürgi,[4] Elisabeth Graf,[2,3] Uwe Ahting,[2,3] Nicoletta Resta,[9] Nicola Laforgia,[10] Daniela Verrigni,[7] Yasushi Okazaki,[11,12] Masakazu Kohda,[11] Diego Martinelli,[13] Peter Freisinger,[14] Tim M. Strom,[2,3] Thomas Meitinger,[2,3] Costanza Lamperti,[6] Atilano Lacson,[15] Placido Navas,[1] Johannes A. Mayr,[16] Enrico Bertini,[7,21] Kei Murayama,[17,18,21] Massimo Zeviani,[19,21] Holger Prokisch,[2,3,21,*] and Daniele Ghezzi[6,21,*]

Primary coenzyme Q10 (CoQ_{10}) deficiencies are rare, clinically heterogeneous disorders caused by mutations in several genes encoding proteins involved in CoQ_{10} biosynthesis. CoQ_{10} is an essential component of the electron transport chain (ETC), where it shuttles electrons from complex I or II to complex III. By whole-exome sequencing, we identified five individuals carrying biallelic mutations in *COQ4*. The precise function of human COQ4 is not known, but it seems to play a structural role in stabilizing a multiheteromeric complex that contains most of the CoQ_{10} biosynthetic enzymes. The clinical phenotypes of the five subjects varied widely, but four had a prenatal or perinatal onset with early fatal outcome. Two unrelated individuals presented with severe hypotonia, bradycardia, respiratory insufficiency, and heart failure; two sisters showed antenatal cerebellar hypoplasia, neonatal respiratory-distress syndrome, and epileptic encephalopathy. The fifth subject had an early-onset but slowly progressive clinical course dominated by neurological deterioration with hardly any involvement of other organs. All available specimens from affected subjects showed reduced amounts of CoQ_{10} and often displayed a decrease in CoQ_{10}-dependent ETC complex activities. The pathogenic role of all identified mutations was experimentally validated in a recombinant yeast model; oxidative growth, strongly impaired in strains lacking COQ4, was corrected by expression of human wild-type *COQ4* cDNA but failed to be corrected by expression of *COQ4* cDNAs with any of the mutations identified in affected subjects. *COQ4* mutations are responsible for early-onset mitochondrial diseases with heterogeneous clinical presentations and associated with CoQ10 deficiency.

Coenzyme Q (CoQ), or ubiquinone, is a lipophilic component of the electron transport chain (ETC), where it shuttles electrons derived from NADH and $FADH_2$ to ETC complex III (cIII) or ubiquinone-cytochrome c reductase. The main electron donors to CoQ are ETC complexes I (cI) and II (cII) but also include other mitochondrial flavoproteins, for instance, electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial (ETF-dehydrogenase [ETFDH]), which is the terminal component of fatty acid β-oxidation and branched-chain amino acid oxidation pathways. CoQ can also act as an antioxidant and a membrane stabilizer, is a cofactor of additional mitochondrial enzymes (e.g., uncoupling protein UCP1),[1,2] and plays an indispensable role in the de novo pyrimidine biosynthesis as the electron acceptor from dihydroorotate dehydrogenase.[3–5]

CoQ is a 1,4-benzoquinone with a tail of 10 isoprenyl units in humans (CoQ_{10}) but of variable length in other species (e.g., CoQ_6 in yeast). The synthesis of the isoprenoid moieties proceeds via either mevalonate or

[1]Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide – Consejo Superior de Investigaciones Científicas – Junta de Andalucía and Centro de Investigación Biomédica en Red de Enfermedades Raras, 41013 Sevilla, Spain; [2]Institute of Human Genetics, Helmholtz Zentrum München – German Research Center for Environmental Health, 85764 Neuherberg, Germany; [3]Institute of Human Genetics, Technische Universität München, 81675 Munich, Germany; [4]Clinic for Pediatrics, Division of Inherited Metabolic Disorders, Medical University of Innsbruck, 6020 Innsbruck, Austria; [5]Department of Pediatrics, Faculty of Medicine, Saitama Medical University, Saitama 350-0495, Japan; [6]Unit of Molecular Neurogenetics, Foundation of the Carlo Besta Neurological Institute, Istituto di Ricovero e Cura a Carettere Scientifico, 20126 Milan, Italy; [7]Unit for Neuromuscular and Neurodegenerative Disorders, Laboratory of Molecular Medicine, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carettere Scientifico, 00165 Rome, Italy; [8]Division of Human Genetics, Department of Medical Genetics, Molecular and Clinical Pharmacology, Medical University of Innsbruck, 6020 Innsbruck, Austria; [9]Division of Medical Genetics, Department of Biomedical Sciences and Human Oncology, University of Bari Aldo Moro, 70121 Bari, Italy; [10]Neonatology and Neonatal Intensive Care Unit, Department of Biomedical Sciences and Human Oncology, University of Bari Aldo Moro, 70121 Bari, Italy; [11]Division of Translational Research, Research Center for Genomic Medicine, Saitama Medical University, Saitama 350-1241, Japan; [12]Division of Functional Genomics & Systems Medicine, Research Center for Genomic Medicine, Saitama Medical University, Saitama 350-1241, Japan; [13]Unit of Metabolism, Bambino Gesù Children's Hospital, Istituto di Ricovero e Cura a Carettere Scientifico, 00165 Rome, Italy; [14]Department of Pediatrics, Klinikum Reutlingen, 72764 Reutlingen, Germany; [15]Walter Mackenzie Health Sciences Centre, 8440 112 Street NW, Edmonton, AB T6G 2B7, Canada; [16]Department of Pediatrics, Paracelsus Medical University Salzburg, 5020 Salzburg, Austria; [17]Department of Metabolism, Chiba Children's Hospital, Chiba 266-0007, Japan; [18]Chiba Cancer Center Research Institute, Chiba 260-8717, Japan; [19]Mitochondrial Biology Unit, Medical Research Council, Hills Road, Cambridge CB2 0XY, UK
[20]These authors contributed equally to this work
[21]These authors contributed equally to this work
*Correspondence: prokisch@helmholtz-muenchen.de (H.P.), dghezzi@istituto-besta.it (D.G.)
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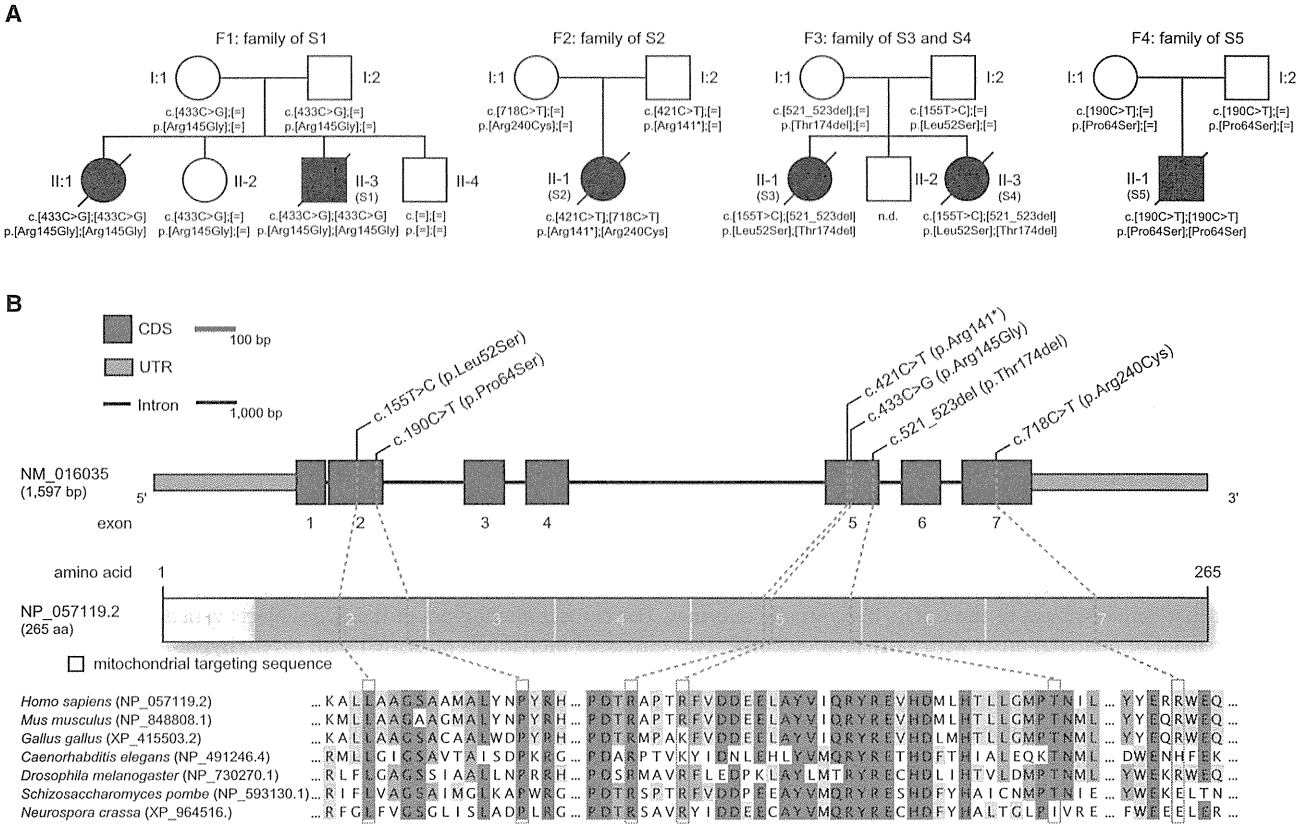

Figure 1. Pedigrees of Investigated Families and *COQ4* Structure and Conservation of Identified Mutations
(A) Pedigrees of four families affected by mutations in *COQ4*. The mutation status of affected and unaffected family members is indicated by closed and open symbols, respectively.
(B) *COQ4* structure showing the identified mutations. The structure of the gene product, COQ4, is also shown with known domains and localization and conservation of amino acid residues affected by the mutations. Intronic regions are not drawn to scale.

2-C-methyl-D-erythritol 4-phosphate pathways, whereas the aromatic precursor of the CoQ benzoquinone ring is p-hydroxybenzoate, derived from tyrosine.[6] After the isoprenoid "tail" is bound to the aromatic "head," the ring undergoes sequential modification. At least ten enzymes participate in CoQ biosynthesis; in yeast, and possibly mammals as well, these enzymes are all localized in mitochondria.

Primary CoQ_{10} deficiency is the biochemical signature of a group of rare, clinically heterogeneous autosomal-recessive disorders caused by mutations in several genes encoding proteins involved in CoQ_{10} biosynthesis.[7] Mutations in *COQ2* (MIM 609825), *COQ6* (MIM 614647), *ADCK3* (*COQ8* [MIM 606980]), *ADCK4* (MIM 615573), *COQ9* (MIM 612837), *PDSS1* (MIM 607429), and *PDSS2* (MIM 610564) have been reported in subjects with severe infantile mitochondrial syndromes associated with severe tissue CoQ_{10} deficiency, whereas the genetic bases underpinning adult-onset CoQ_{10} deficiency remain mostly undefined.[8,9] *COQ4* (MIM 612898) codes for a ubiquitously expressed 265-amino-acid protein that is peripherally associated with the mitochondrial inner membrane on the matrix side;[10] the precise function of human COQ4 is not known, but the yeast ortholog seems to play a structural role crucial in the stabilization of a multiheteromeric complex including several, if not all, of the CoQ biosynthetic enzymes.[11]

We report here the identification of pathogenic biallelic *COQ4* mutations in a total of five individuals from four families; these subjects were part of a cohort of severe mitochondrial cases where the CoQ_{10} defect was not anticipated. The family pedigrees are shown in Figure 1A.

Subject 1 (S1; II-3, family 1), a boy, was the third of four siblings and was born to healthy, non-consanguineous Italian parents after an uncomplicated pregnancy and elective cesarean delivery. His oldest sister (II-1), who presented with bradycardia and hypotonia, died at birth, and his 16-year-old second sister and his 5-year-old brother are alive and well. At birth, S1 had a weight of 3,410 g, a length of 49.5 cm, and a head circumference of 34.5 cm. Apgar scores were 7 and 10 at 1 and 5 min after birth, respectively. At birth, his condition appeared critical, given that he showed severe hypotonia, areflexia, acrocyanosis, bradycardia, and respiratory insufficiency. Ultrasound examination revealed markedly decreased motility of the left ventricle with an ejection fraction of 20%–25%. No evidence of hepatic or renal impairment was observed. Dobutamine infusion via an umbilical venous catheter was

Table 1. Mitochondrial ETC Activities in Muscle

	Subject	cI/CS[a]	cI+III/CS[a]	cII/CS[a]	cII+III/CS[a]	cIII/CS[a]	cIV/CS[a]	CS[b]
Muscle biopsy	S1[c]	36	24	N	34	N	N	64
	S2[c]	6	ND	42	43	10	30	57
	S3	N	N	N	55	N	50	54
	S4	145	N	N	N	222	189	109
	S5	<5	ND	N	30	50	N	65

Abbreviations are as follows: N, value in the control range; ND, not done; cI, complex I; cII, complex II; cIII, complex III; cIV, complex IV; cI+III, coupled activity of complexes I and III; and cII+III, coupled activity of complexes II and III. The analyses were performed in different laboratories, and the reference values are diverse (they usually range between 60% and 140% of the mean control value). The values of ETC complex activities out of the control range (specific to each enzymatic activity and to each laboratory) are reported.
[a]Mean control value (%) of CS-normalized ETC complex activities.
[b]Percentage of mean control value.
[c]Sample from autopsy.

ineffective, and the baby died 4 hr after birth. His blood glucose level was normal, as were renal and hepatic parameters; plasma creatine kinase was moderately elevated (861 U/l; normal value [n.v.] < 400), and blood lactate was extremely high (20.1 mM; n.v. < 2). Analysis of urinary organic acids showed elevated levels of 2-OH glutaric acid, whereas plasma and urinary amino acids were within normal ranges. The autopsy examination revealed left ventricular hypoplasia with septum hypertrophy and a patent ductus arteriosus. No brain examination was performed.

The activities of the ETC complexes in autoptic skeletal-muscle homogenate showed severe defects of both coupled cI+cIII and cII+cIII reactions, normalized to citrate synthase (CS), and a decrease in CS-normalized cI (Table 1). In both liver and cultured fibroblasts, the CS-normalized activities of each of the individual ETC complexes were in the control range. Although the coupled cI+cIII activity cannot be reliably assayed in cultured cells,[12] the coupled cII+cIII activity was clearly decreased in S1 fibroblasts (65% of the control mean).

S2 (II-1, family 2) was born at the 34th week of gestation and was the female first child of non-consanguineous Japanese parents. Her birth weight was 1,120 g (−2.2 SDs). Apgar scores were 7 and 8 at 1 and 5 minutes after birth, respectively. There was no family history of neurological or cardiac disease. The pregnancy was complicated by severe intrauterine growth delay and ultrasound-documented hypertrophic cardiomyopathy. On S2's first day of life, she became apnoeic and was intubated as a result of respiratory failure. She initially displayed moderate lactic acidosis, but soon after her admission to Neonatal Medical Center, her lactic acidosis rapidly worsened (blood lactate = 11.2–18.8 mM; n.v. < 2); her hypertrophic cardiomyopathy evolved into severe heart failure, leading to death at the age of 1 day.

The metabolic profile (urinary and plasmatic amino acids, organic acids, and acylcarnitines) showed no significant findings. A liver autoptic specimen showed a severe deficiency of cI (cI/CS ratio = 2.9%); autoptic skeletal-muscle homogenate also showed a cI deficiency together with less pronounced reductions of other ETC complexes (Table 1).

Sisters S3 (II-1, family 3) and S4 (II-3, family 3) are the first and third, respectively, of three siblings and were born to healthy, non-consanguineous Austrian parents. Their brother (II-2) is a healthy, unaffected boy. S3 and S4 were born prematurely at gestational ages of 32 weeks (birth weight = 1,550 g) and 34 weeks (birth weight = 2,170 g), respectively.

Performed at the 20th week of gestation, prenatal organ screening of S3 revealed a suspected malformation of the cerebellum. A postnatal cranial ultrasound showed cerebellar hypoplasia. After birth, she showed distal arthrogryposis, but no other dysmorphic features. At birth, she suffered from respiratory-distress syndrome, and a few hours later, a severe myoclonic epileptic encephalopathy ensued; blood lactic acid at 36 hr of age was 6.4 mM and rose to 14 mM prior to her death by multiorgan failure on the third day of life. Echocardiography showed a normal heart. Metabolic investigations (amino acids in plasma, acylcarnitine profile, and standard newborn screening) were essentially normal. Analysis of organic acids in urine showed excretion of glycerol and 2-OH-glutarate. In frozen postmortem muscle (obtained within 30 min after death), ETC enzyme activities were slightly decreased (Table 1). An autopsy of the brain revealed severe olivopontocerebellar and thalamic hypoplasia and scattered cavitations in the white matter; the visceral organs appeared normal for the gestational age.

Six years later, prenatal organ screening of the sister, S4, showed cerebellar hypoplasia, suggesting the same disease as in S3. Similar to her sister, S4 suffered from neonatal respiratory distress. No dysmorphic features were present. Echocardiography was normal. A cranial ultrasound confirmed cerebellar hypoplasia. Six hours after birth, epileptic encephalopathy ensued; blood lactic acid was 3.5 mM at 2 hr of age and rose to 9 mM at death on the second day of life. Metabolic investigations showed normal newborn-screening results and a normal acylcarnitine profile. Amino acids in plasma were grossly elevated but showed no specific pattern. Analysis of urinary organic acids showed excretion of a "mitochondrial dysfunctional pattern" with malate, fumarate, and 2-OH-glutarate, as

well as vitamin B6 metabolites and N-acetyl-tyrosine. Analysis of frozen postmortem muscle showed elevated levels of ETC activities (Table 1). In both girls, blood glucose concentration and renal and hepatic parameters were in the normal range.

S5 (II-1, family 4) is an 18-year-old young man and is the only offspring of healthy Italian parents who deny consanguinity and originate from a medium-size town in southern Italy. Pregnancy was normal, and delivery was via cesarean section because of a podalic presentation. He was born at term, and his weight at birth was 4,100 g. Weight and motor development were reportedly normal in his first year of life, but he started to show slowly progressive motor deterioration after the age of 10 months, when he manifested unsteadiness in maintaining acquired sitting position. He achieved the ability to walk with a spastic ataxic gait at 3 years of age but lost ambulation by 6 years of age and has been wheelchair bound since then. At 12 years of age, he started manifesting epileptic seizures in the form of prolonged right-side hemiclonic seizures. MRI showed bilateral increased signal intensity in fluid-attenuated-inversion-recovery and T2-weighted sequences in both occipital-cortical and juxtacortical areas (Figures S1A–S1D). Around the same period, he started to have swallowing difficulties. He was admitted for extensive investigation. Thorough blood tests excluded liver and kidney involvement and did not show lactic acidosis. A specific pattern of organic aciduria was excluded. Electrophysiological examination showed a sensory motor polyneuropathy with slowed conduction velocities. During a 5-year follow-up, he showed a slowly progressive downhill course with recurrent treatment-resistant seizures, worsened swallowing impairment, progressive scoliosis, and cognitive deterioration. A muscle biopsy was performed when he was 12 years old. Spectrophotometric assays of the ETC complexes in muscle homogenate showed virtually undetectable cI/CS ratios and reduced cII+cIII/CS and cIII/CS ratios. The other ETC complex activities were within control limits (Table 1). Since the age of 15 years, he has used a percutaneous-endoscopic-gastronomy tube and has developed severe scoliosis with a Cobb angle of 75°. Control MRI performed when he was 17 years old showed cerebellar atrophy, widening of ventricular brain spaces, and scars from cortical necrotic lesions in both occipital areas (Figures S1E–S1H).

In agreement with the Declaration of Helsinki, informed consent for genetic and biochemical studies was signed by the parents of all subjects, and the ethics committee of the Technische Universität München approved the study.

We performed whole-exome sequencing (WES) to investigate the molecular bases of the mitochondrial disease presentations of S1, S4, and S5, as described previously.[13] Coding DNA sequences were enriched with a SureSelect Human All Exon 50 Mb V4 or V5 Kit (Agilent) and subsequently sequenced on a HiSeq2500 system (Illumina). Read alignment to the human reference assembly (UCSC Genome Browser hg19) was done with the Burrows-Wheeler Aligner (version 0.7.5), and single-nucleotide variants and small insertions and deletions were identified with SAMtools (version 0.1.19). On the basis of the rare disease phenotype and a pattern concordant with autosomal-recessive inheritance, we sought genes carrying rare (minor allele frequency [MAF] < 0.1% in 4,500 control exomes) variants predicted to be compound heterozygous or homozygous. We then prioritized variants in genes coding for proteins with known or predicted mitochondrial localization.[14] This filtering strategy led to the identification of recessive variants in *COQ4*, coding for a mitochondrial protein involved in CoQ_{10} biosynthesis,[10] in all three subjects. In S2, we used the SeqCap EZ Library (version 1.0; Roche NimbleGen). Details on the bioinformatics pipeline and variant filtering have been reported recently.[15] Sequencing statistics are provided in Table S1.

We identified *COQ4* mutations (RefSeq accession number NM_016035.3) in four individuals (Figure 1). In S1, we identified a homozygous missense variant, c.433C>G (p.Arg145Gly). Both parents and a healthy sister are heterozygous carriers, and a healthy brother has two reference alleles. No material was available from the deceased sister. S2 was found to be compound heterozygous for a nonsense variant on the paternal allele and a missense variant on the maternal allele: c.[421C>T];[718C>T], p.[Arg141*];[Arg240Cys]. S4 was found to be compound heterozygous for a missense mutation and an exon 5 in-frame deletion: c.[155T>C];[521_523delCCA], p.[Leu52Ser];[Thr174del]. Both variants were also confirmed in the DNA of S3, whereas the parents are heterozygous for only one variant each (the father carries the missense mutation, and the mother carries the deletion). In S5, we identified a homozygous mutation, c.190C>T (p.Pro64Ser). Both parents are heterozygous for this mutation.

None of the identified variants are present in our exome database, which contains 4,500 samples, or in public SNP databases, including dbSNP, the NHLBI Exome Sequencing Project Exome Variant Server, and the Exome Aggregation Consortium (ExAC) Browser. The only exception is the c.718C>T variant (rs143441644), which is reported to have an extremely low frequency (MAF = 0.00023; 28/12,0330 alleles) in the ExAC Browser. Moreover, all missense changes are predicted to be deleterious by several bioinformatics tools (Table S2).

Because of the identified genetic defects, we tested CoQ_{10} levels in available specimens from the subjects. In a muscle biopsy from S1, we detected a clear reduction of CoQ_{10} (32.9 nmol CoQ_{10}/g protein; n.v. = 101–183; 1.16 nmol CoQ_{10}/CS; n.v. = 1.75–3.46). In fibroblasts from S1, the levels of CoQ_{10} were also lower than CoQ_{10} levels in neonatal control fibroblasts (54% of control mean). In frozen muscle from S3, CoQ_{10} was reduced (13.5 nmol CoQ_{10}/g protein; n.v. = 160–1,200; 0.3 nmol CoQ_{10}/CS; n.v. = 2.7–7); in muscle from S4, CoQ_{10} was profoundly reduced (25.7 nmol CoQ_{10}/g protein; n.v. = 160–1,200; 0.1 nmol CoQ_{10}/CS; n.v. = 2.7–7), whereas in S5 muscle, the amount of CoQ_{10} was slightly decreased

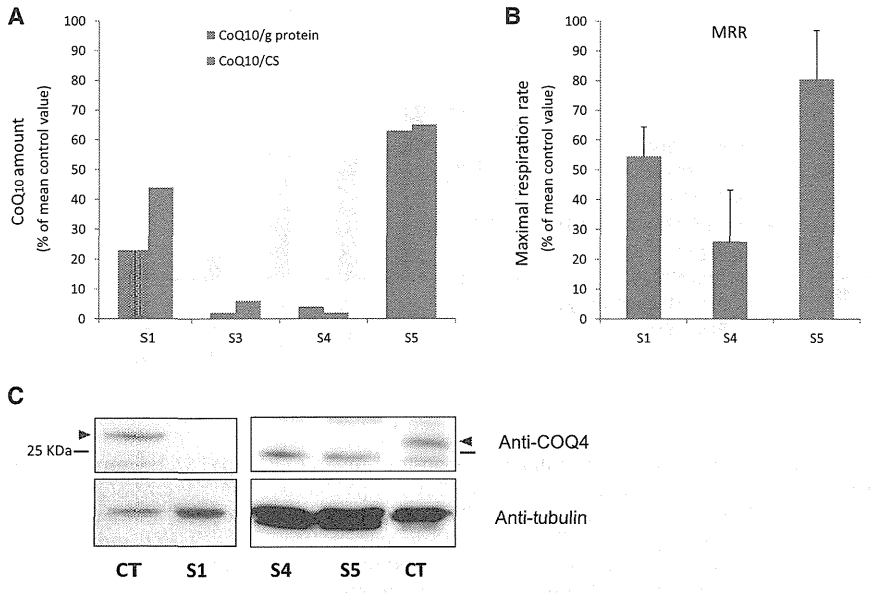

Figure 2. Biochemical Studies in *COQ4* Mutant Muscle and Fibroblasts

(A) CoQ_{10} in muscle from affected subjects S1 and S3–S5 is reported as a percentage of the mean of control values (the analyses were performed in different laboratories, and the reference values are diverse; see text). Data are reported after normalization to protein content or CS activity.

(B) Maximal respiration rate (MRR) measured in fibroblasts from subjects S1, S4, and S5; MRR values are expressed as percentages of MRR values obtained in control fibroblasts. The graphs represent the mean values from two independent experiments, each with six to eight replicates. Error bars represent the SD.

(C) Immunoblot analysis of COQ4 in fibroblasts from subjects S1, S4, and S5 and control individuals (Ct). Arrowheads indicate the band corresponding to COQ4. An antibody against tubulin was used as a loading control.

(88.9 μg CoQ_{10}/g protein; n.v. = 101–183; 1.70 μg nmol CoQ_{10}/CS; n.v. = 1.75–3.46) (Figure 2A). No residual sample from the muscle biopsy of S2 was available. Together, these findings are consistent with a deleterious role of the mutations identified in *COQ4*.

By Seahorse micro-oxygraphy,[16] we detected that maximal respiratory rates were lower in S1, S4, and S5 fibroblasts than in control cells (Figure 2B). Moreover, a drastic decrease in the amount of COQ4 was detected by immunoblot analysis in S1, S4, and S5 fibroblasts (Figure 2C), confirming that the identified *COQ4* nucleotide variants are deleterious.

The *Saccharomyces cerevisiae* ortholog of human *COQ4* is *yCOQ4*; *yCOQ4*-null strains have been reported to be effectively complemented by human *COQ4*.[10] In order to functionally test the effect of all the mutations found in our cohort, we transformed a *COQ4*-null strain (*Δcoq4*) by inserting the following *hCOQ4* variants into the multicopy pYES2.1 vector: pYES:$hCOQ4^{WT}$ (human wild-type [WT]), pYES2.1 (empty vector), pYES:$yCOQ4^{WT}$ (positive control), pYES:$hcoq4^{p.Arg145Gly}$ (mutation c.433C>G), pYES:$hcoq4^{p.Arg141*}$ (c.421C>T), pYES:$hcoq4^{p.Arg240Cys}$ (c.718C>T), pYES:$hcoq4^{p.Leu52Ser}$ (c.155T>C), pYES:$hcoq4^{p.Thr174del}$ (c.521_523delCCA), and pYES:$hcoq4^{p.Pro64Ser}$ (c.190C>T). In addition, to replicate the compound-heterozygous condition found in probands of families 2 and 3, we transformed the *Δcoq4* strain via a pYES construct harboring both the c.155T>C and the c.521_523delCCA mutations (pYES:$hcoq4^{p.Leu52Ser/p.Thr174del}$) and a pYES construct expressing the c.421C>T and c.718C>T mutations (pYES:$hcoq4^{p.Arg141*/p.Arg240Cys}$). A WT strain transformed with the pYES2.1 empty vector was also included as an additional control. In order to reveal a possible respiratory defect, we compared the growth of our transformant strains cultured in either glucose (a fermentable carbon source) or glycerol (a non-fermentable carbon source) after inducing gene expression with galactose for 4 hr. Notably, whereas the growth of the pYES2.1:$hCOQ4^{WT}$ transformant strain was comparable to that of the pYES2.1:$yCOQ4^{WT}$ transformant strain, the strains transformed with the *hCOQ4* mutant vectors grew as slowly as that transformed with pYES2.1 (Figure 3A). This result clearly indicates that each mutation reported in our probands leads to a virtually complete loss of function of the corresponding protein, COQ4. Next, we found that the CoQ_6 content in one *Δcoq4* mutant strain, $hcoq4^{p.Arg145Gly}$, was markedly decreased, whereas *Δcoq4* strains transformed with either pYES2.1:*yCOQ4* or pYES2.1:*hCOQ4* had CoQ_6 levels similar to those in the WT strain (Figure 3B). This result indicates that mutant $hcoq4^{p.Arg145Gly}$ impairs CoQ biosynthesis.

Primary CoQ_{10} deficiency, caused by genetic defects in CoQ_{10} biosynthesis, is a clinically heterogeneous condition associated with a spectrum of different phenotypes, including encephalomyopathic forms with seizures and/or ataxia,[17–19] multisystem infantile forms with encephalomyopathy and renal failure,[20] nephrotic syndrome with sensorineural deafness,[21,22] adult Leigh syndrome,[23] and isolated myopathic forms.[24] Mutations in seven genes encoding proteins involved in CoQ_{10} biosynthesis have been reported in single families or in a few singleton cases;[25] the genetic defect has not been determined in most of the cases of CoQ_{10} deficiency, and only a few data are available regarding specific genotype-phenotype correlations. Secondary CoQ_{10} deficiency has been reported in association with glutaric aciduria type IIC (MIM 231680), caused by mutations in *ETFDH* (MIM 231675; encoding electron-transfer dehydrogenase); ataxia-oculomotor apraxia syndrome (MIM 208920), caused by mutations in *APTX* (MIM 606350; encoding aprataxin); a cardiofacio-cutaneous syndrome caused by a mutation in *BRAF* (MIM 115150; encoding serine/threonine-protein kinase B-Raf)[26]; and glucose transporter GLUT1 deficiency.[27]

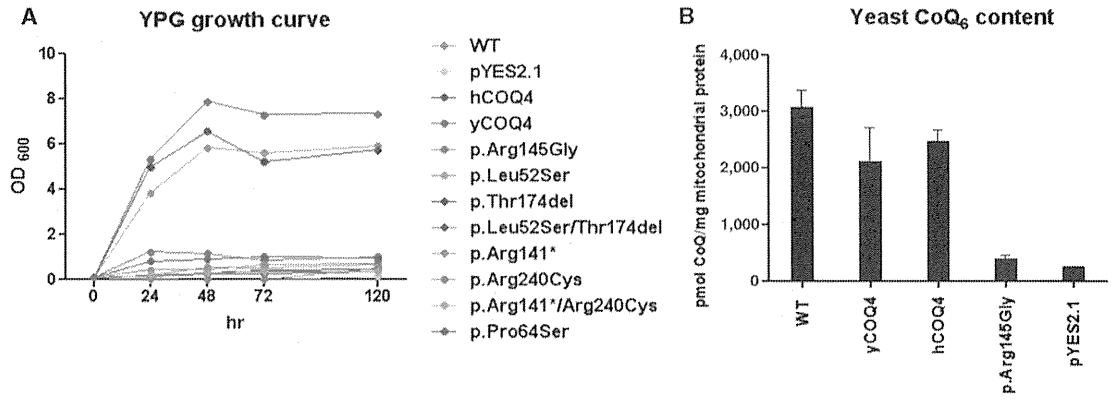

Figure 3. Yeast Studies

(A) Glycerol (YPG) growth of transformed *ΔCOQ4* yeast with the different mutated versions of human *COQ4* (pYES2.1, empty vector; hCOQ4, pYES:*hCOQ4*^{WT}; yCOQ4, pYES:*yCOQ4*^{WT}; c.433C>G, pYES:*hcoq4*$^{p.Arg145Gly}$; c.421C>T, pYES:*hcoq4*$^{p.Arg141*}$; c.718C>T, pYES:*hcoq4*$^{p.Arg240Cys}$; c.155T>C, pYES:*hcoq4*$^{p.Leu52Ser}$; c.521_523delCCA, pYES:*hcoq4*$^{p.Thr174del}$; c.190C>T, pYES:*hcoq4*$^{p.Pro64Ser}$; c.155T>C and c.521_523delCCA, pYES:*hcoq4*$^{p.Leu52Ser/p.Thr174del}$; and c.421C>T and c.718C>T, pYES:*hcoq4*$^{p.Arg141*/p.Arg240Cys}$). WT indicates the wild-type yeast transformed with the YES2.1 empty vector. Cells were grown in selective medium for 16 hr, induced in galactose for 4 hr, and inoculated in YPG at 0.1 U of optical density (OD) at 600 nm. Growth at 30°C was monitored over 5 days by measurement of OD cultures at 600 nm.

(B) Yeast mitochondrial CoQ_6 levels. Purified mitochondria lipid extraction and high-performance-liquid-chromatography quantification of CoQ_6 was performed in the *ΔCOQ4* strain transformed with the empty vector (pYES2.1), WT yeast (yCOQ4), or human (hCOQ4) or *hcoq4*$^{p.Arg145Gly}$ (c.433C>G) *COQ4* genes. A WT strain transformed with the empty vector was included as a positive control. Error bars represent the SD.

Interestingly, although the mechanisms linking these heterogeneous genetic conditions to a decrease in CoQ_{10} remain obscure, most of these individuals benefitted from CoQ_{10} supplementation.[28,29]

We found six *COQ4* mutations in five affected subjects from four unrelated families. All these individuals carried homozygous or compound-heterozygous mutations, clearly indicating that the resulting disease is an autosomal-recessive trait. Two alleles carried nonsense mutations, which are both transmitted by descent in combination with missense *COQ4* mutations to different individuals (S2 and sisters S3 and S4) and are predicted to lead to a truncated and aberrant COQ4. Given that the heterozygous parents carrying the nonsense mutations are alive and well, it is unlikely that COQ4 haploinsufficiency is pathogenic, even though a previous study reported on a boy carrying a de novo heterozygous deletion, including *COQ4*, in chromosomal region 9q34.[30] Because the biosynthetic pathway of CoQ is conserved throughout evolution from human to *Saccharomyces cerevisiae*, we modeled in yeast the mutations found in our subjects. Using this system, we demonstrated that each mutation, or the allelic combinations found in S2 and siblings S3 and S4, was associated with a severe defect of oxidative growth. In parallel, we also showed that COQ4 was strongly reduced in mutant fibroblast cell lines from S1, S4, and S5. In the skeletal muscle of S1 and S3–S5, the CoQ_{10} content was reduced as well. Taken together, these results demonstrate the pathogenic role of the *COQ4* mutations found in our cohort.

In keeping with the essential role of COQ4, four of our five subjects had a prenatal or perinatal onset with a fatal outcome in the first days of life. S1 and S2 presented with severe hypotonia, bradycardia, and respiratory insufficiency at birth; in S2, hypertrophic cardiomyopathy had been evident since fetal development. A markedly different, albeit equally severe, clinical presentation dominated by premature delivery, antenatal cerebellar hypoplasia, neonatal respiratory-distress syndrome, and epileptic encephalopathy characterized sisters S3 and S4. Rapidly progressive, severe lactic acidosis was a common feature in all four affected newborn subjects and is likely to have determined their fatal outcome. Involvement of the heart has been very rarely documented in CoQ_{10}-deficient subjects, often as part of multisystem phenotypes, where cardiomyopathy develops later than brain, muscle, or kidney impairment.[20] For instance, a homozygous nonsense mutation in *COQ9* was described in a baby who presented with neonatal lactic acidosis and later developed hypertrophic cardiomyopathy as part of a multisystem disease including intractable seizures, global developmental delay, and renal tubular dysfunction.[9] In spite of his early onset, the clinical course of S5 was slowly progressive and dominated by neurological deterioration with hardly any involvement of other organs, including the heart and kidneys.

Although the link between specific genetic defects and phenotypes is often unclear in mitochondrial disorders, organs with the highest energy requirements, such as the heart, kidneys, and brain, have the highest CoQ_{10} concentrations[31] and are the most frequently affected by CoQ_{10} deficiency. The level of expression of *COQ* genes in different cells seems to correlate poorly with the primarily affected tissue or organ; for instance, *COQ2*, mutations of which typically cause renal impairment, has expression

levels that are relatively higher in skeletal muscle and the heart than in other organs,[32] whereas *COQ4*, mutated in our subjects with cardiac or brain failure, is ubiquitously expressed and has relatively higher levels in the liver, lungs, and pancreas.

Because cardiomyocytes have a remarkably high energy requirement, and cardiomyopathy is quite common in individuals with various inherited mitochondrial disorders, the cardiac involvement in subjects with mutations in *COQ* genes can be overlooked. Indeed, the crucial role of CoQ_{10} in cardiomyocyte function has been recognized for a very long time; for instance, myocardial biopsies from individuals with congestive heart failure[33] or cardiomyopathy[34,35] show low CoQ_{10} levels, which correlate with the severity of heart damage.[36] Moreover, statins, cholesterol-lowering drugs that inhibit HMG-CoA reductase (the key enzyme common to the biosynthesis of both cholesterol and CoQ_{10}) can cause CoQ_{10} deficiency, ultimately leading to cardiomyopathy;[37] interestingly, this harmful side effect can be overcome by oral CoQ_{10} supplementation.[38] Moreover, long-term CoQ_{10} treatment of individuals with chronic heart failure is safe, improves symptoms, and reduces major adverse cardiovascular events.[39] These observations all converge on a strict association between CoQ_{10} deficiency and cardiomyopathy.

Notably, S3–S5 showed no sign of heart involvement, whereas the clinical phenotype was dominated by encephalopathy with seizures and a more progressive, but mainly neurological, syndrome is the clinical hallmark of S5, indicating the heterogeneity of the clinical presentations associated with *COQ4* defects. The variable specificity of organ failure (e.g., heart versus brain) in the neonatal cases of our cohort could be due to the fulminant course of the disease, which prevented the deployment of multisystem involvement. In support of this view, although cardiomyopathy dominated the clinical picture, the presence of severe hypotonia and hyporeflexia suggests concomitant involvement of the nervous system in S1 and S2 as well. Clinical heterogeneity was accompanied by an equally striking variability of the biochemical findings, which ranged from multiple (S1 and S5) to isolated (S2 and S3) ETC defects in muscle and fibroblasts to hardly any detectable defect at all (S4). This biochemical diversity could be due to differences in individual adaptive responses to reduced CoQ_{10} availability or could reflect the striking tissue specificity observed in the clinical presentations, but at the moment, a mechanistic explanation for these observations is lacking. Poor correlation with the clinical and biochemical phenotypes has also been reported for other genes related to CoQ_{10} biosynthesis. For instance, mutations in *COQ2*, the first mutated gene identified in affected individuals with primary CoQ_{10} deficiency, have been associated with a wide range of clinical presentations, often including nephrotic syndrome but also including fatal neonatal multisystemic disorder, Leigh syndrome, myoclonic epilepsy, hypertrophic cardiomyopathy, deafness, and adult-onset multisystem atrophy.[25,40] In any case, the identification of *COQ4* mutations in subjects with such a wide spectrum of clinical and biochemical abnormalities is a further indication of the advantage of unbiased screening such as WES for the identification of genes newly associated with mitochondrial disorders.

Unfortunately, the fulminant fatal outcome in S1–S4 was so rapid that it prevented both the diagnosis of CoQ_{10} deficiency and the start of CoQ_{10} supplementation. Prompt diagnosis is a main challenge for syndromes of primary CoQ10 deficiency but is very important given that co-factor deficiencies are virtually the only group of mitochondrial disorders for which beneficial pharmacological treatment is currently available. Treatment of the long-surviving subject, S5, has now started and will hopefully provide some useful indication of its efficacy in the near future.

Supplemental Data

Supplemental Data include one figure and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2014.12.023.

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Web Resources

The URLs for data presented herein are as follows:

Exome Aggregation Consortium (ExAC) Browser, http://exac.broadinstitute.org/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, http://evs.gs.washington.edu/EVS/
OMIM, http://www.omim.org
UCSC Genome Browser, http://genome.ucsc.edu

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