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Case Report

Myocerebrohepatopathy spectrum disorder due to *POLG* mutations: A clinicopathological report

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Abstract

We report on the clinical, neuropathological, and genetic findings of a Japanese case with myocerebrohepatopathy spectrum (MCHS) disorder due to polymerase gamma (*POLG*) mutations. A girl manifested poor sucking and failure to thrive since 4 months of age and had frequent vomiting and developmental regression at 5 months of age. She showed significant hypotonia and hepatomegaly. Laboratory tests showed hepatocellular dysfunction and elevated protein and lactate levels in the cerebrospinal fluid. Her liver function and neurologic condition exacerbated, and she died at 8 months of age. At autopsy, fatty degeneration and fibrosis were observed in the liver. Neuropathological examination revealed white matter-predominant spongy changes with Alzheimer type II glia and loss of myelin. Enzyme activities of the respiratory chain complex I, III, and IV relative to citrate synthase in the muscle were normal in the biopsied muscle tissue, but they were reduced in the liver to 0%, 10%, and 14% of normal values, respectively. In the liver, the copy number of mitochondrial DNA compared to nuclear DNA was reduced to 3.3% of normal values as evaluated by quantitative polymerase chain reaction. Genetic analysis revealed compound heterozygous mutations for *POLG* (I1185T/A957V). This case represents the differential involvement of multiple organs and phenotype-specific distribution of brain lesions in mitochondrial DNA depletion disorders.

Keywords: Alpers syndrome; Mitochondrial DNA depletion; Myocerebrohepatopathy spectrum disorder; *POLG*

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1. Introduction

Mitochondrial DNA (mtDNA) depletion syndrome (MDDS), first described in 1991, is defined as a reduction in the mtDNA copy number in different tissues, leading to insufficient synthesis of respiratory chain complexes (RCC) [1]. Clinical manifestations of MDDS involve many organ systems including the central and peripheral nervous system, liver, muscle, and gastrointestinal tract [2]. Human polymerase gamma (*POLG*) is the common causative gene involved in MDDS, whose mutations result in a diverse group of phenotypes, such as Alpers syndrome and myocerebrohepatopathy spectrum (MCHS) disorders, which typically show disease onset during early childhood. Further, several *POLG*-related phenotypes manifesting during adolescence and adulthood are recognized, including progressive external ophthalmoplegia, ataxia-neuropathy spectrum disorders, myoclonus epilepsy myopathy sensory ataxia, and sensory ataxic neuropathy with dysarthria/dysphagia and ophthalmoplegia. Some overlaps in the symptoms between these adult phenotypes exist, and can be additionally accompanied by tremor, parkinsonism, hearing loss, stroke-like episodes, and gastrointestinal symptoms, which are reminiscent of symptoms of mitochondrial diseases with pathomechanisms other than MDDS [3,4].

MCHS, the most severe phenotype of POLG disorders, was recently identified and is defined by the clinical triad of (1) myopathy or hypotonia, (2) developmental delay or dementia, and (3) liver dysfunction [3,5]. Severe, intractable epilepsy is included in the diagnostic hallmarks of Alpers syndrome, but is not characteristic of MCHS. As the number of patients with MCHS disorders is small and detailed clinicopathological findings are unavailable, we herein report the case of a girl with MCHS disorders due to *POLG* mutations. As far as we know, this is the first Japanese case of MCHS disorders with *POLG* mutation.

2. Case report

A girl was born at 40 weeks of gestation to healthy non-consanguineous parents without any abnormalities. The birth weight, height, and head circumference were normative. Early development and growth were unremarkable. At 4 months of age, she developed poor weight gain, emesis, hypotonia, developmental delay, and lethargy. She was admitted to our hospital because of recurrent vomiting at 6 months of age.

On admission, body length was 60.9 cm [−2.2 standard deviation (SD)], body weight was 5600 g (−2.3 SD), and head circumference was 42 cm (+0.2 SD). Hepatomegaly of a hard consistency was observed approximately 3 cm under the costal margin with no associated splenomegaly. She was alert and could establish good eye contact and smile. She showed severe hypotonia and proximal dominant muscular weakness. She could hold neither her head nor limbs up. All deep tendon reflexes were weak.

Although complete blood count and urinalysis were unremarkable, hepatocellular dysfunction was obvious at the time of hospitalization, with the following laboratory test values: aspartate aminotransferase, 390 U/L; alanine aminotransferase, 218 U/L; total bilirubin, 1.6 mg/dL; total bile acids, 172 μmol/L; γ-glutamyl transpeptidase, 179 IU/L; leucine aminopeptidase, 268 IU/L; and cholinesterase, 73 IU/L. Levels of serum creatine kinase and blood glucose were normal. Cerebrospinal fluid (CSF) examination showed elevated protein levels of 304 mg/dL and normal cell count and glucose levels. Lactic acid was elevated in both plasma and CSF, at 15.9 mg/dL and 30.3 mg/dL, respectively. Pyruvic acid was normal in both plasma and CSF. Metabolic screening tests, including urine organic acids, plasma, and urine amino acids, were unremarkable. Initial brain computed tomography (CT) and magnetic resonance imaging performed at 6 months of age were unremarkable. The electroencephalogram showed generalized slow wave activity. Only wave I was identifiable on auditory evoked potentials. Motor nerve and sensory conduction were mildly delayed.

Muscle biopsy findings at 6 months of age showed a variation in fiber type; ragged-red fiber was not observed. Lipid and glycogen storage were not observed. Cytochrome c oxidase staining showed normal findings. Analysis of the RCC enzyme activity revealed no abnormality. No mtDNA mutations were identified.

Soon after admission, difficulty in feeding and vomiting aggravated, and tube feeding along with parenteral nutrition was required. She experienced bouts of diarrhea. Consciousness level decreased progressively, and myoclonic jerks of the right and left arms were infrequently observed. Follow-up CT revealed mild cerebral atrophy at 7 months of age. Hepatocellular dysfunction exacerbated progressively, and she died of multiple organ failure caused by hepatic failure at 8 months of age, despite supplementation of multiple vitamins and coenzyme Q 10, and was autopsied. Two years later, another girl was born to the parents. She had the same clinical course and laboratory findings observed in the present patient and died at 7 months of age. Valproic acid was not used in either patient.

2.1. Postmortem examinations

Body weight was 6.0 kg (mean ± SD, 8.0 ± 0.88 kg). The weight of the atrophic liver was 200 g, and the surface was yellowish, irregular, and hard. The lungs were congested and adrenal glands were atrophic. The other visceral organs were unremarkable on macroscopic

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examination. The brain weighed 760 g and showed massive edema and caudal necrosis. Microscopically, hepatocytes and adrenal cortical cells were swollen, and renal tubular cells contained phospholipids and diffuse foam cells. Similar foam cells were also seen in the lungs and cardiac muscle fibers. In the liver, hepatic fibrosis, microvesicular steatosis, and fatty degeneration were observed (Fig. 1). In the central nervous system, a spongy change was noted predominantly in the cerebral white matter, and neuronal loss in the cerebral and cerebellar cortex was mild. Alzheimer type II glia was observed in massive numbers in the cerebral and cerebellar white matter, with a smaller amount in the cerebral cortex and deep gray matter. Neuronal loss, capillary proliferation, and sponginess were prominent in the substantia nigra (Fig. 2). Recent linear necrosis was present in the bilateral caudate nucleus.

2.2. Assay of respiratory chain complex enzyme activity in the liver

The liver samples were immediately frozen at autopsy and stored at −70 °C. Activities of RCC I, II, III and IV were assayed as described previously [6,7]. The percentages of RCC I, II, III and IV activities relative to that of citrate synthase (CS) as a mitochondrial enzyme marker were calculated. Relative enzyme activities of RCC I, III, and IV to CS in the liver were reduced to 0%, 10%, and 14% of normal values, respectively, while that of RCC II was reduced to 29%.

2.3. Analysis of quantitative polymerase chain reaction of mtDNA and DNA sequence of POLG gene

Written informed consent was obtained from the patient's parents in order to perform gene analysis. The quantitative estimation of mtDNA was performed by real-time amplification of fragments of *ND1* in the mtDNA genome, as previously described [7,8]. To determine the overall abundance of mtDNA, we compared the real-time amplification of *ND1* with a single-copy nuclear reference gene (exon 24 of the *CFTR* gene) [7,9]. The ratio of *ND1* to *CFTR* in the liver was reduced to 3.3% (SD, 1.2%) as compared to the control.

Mutation analysis was performed on the genomic DNA using primers designed to amplify the coding exons and the exon-intron boundaries of *POLG* (NM_002693.2). Fragments were analyzed by direct sequencing using ABI 3130XL (Applied Biosystems, Tokyo, Japan). The genetic analysis revealed compound heterozygous mutations in *POLG* (c.2870C>T, p.A957V and c.3554T>C, p.I1185T). The two DNA mutations

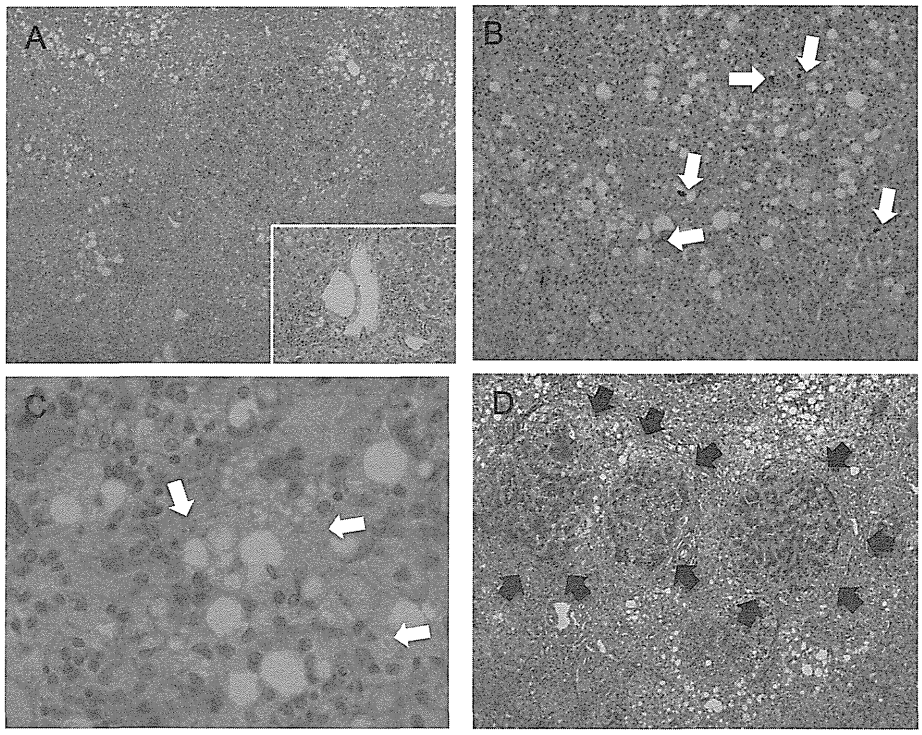

Fig. 1. Pathological findings of the postmortem liver (A–C: hematoxylin & eosin staining, D: Masson trichrome staining). (A) Moderate inflammatory cell infiltration (inset) with destroyed limiting plates and a rather progressive fibrosis with bridging formation in the portal tracts were observed (original magnification, ×40). (B) Swollen hepatocytes containing lipid droplets of various sizes were found. Bile plugs (white arrows in B and C) were noted in the cytoplasm of hepatocytes and dilated canaliculi (×100). (C) Swollen hepatocytes containing lipid droplets of various sizes were found. Bile plugs were noted in the cytoplasm of hepatocytes (×400). (D) A rather progressive fibrosis with bridging formation (arrows) in the portal tracts was found (×40).

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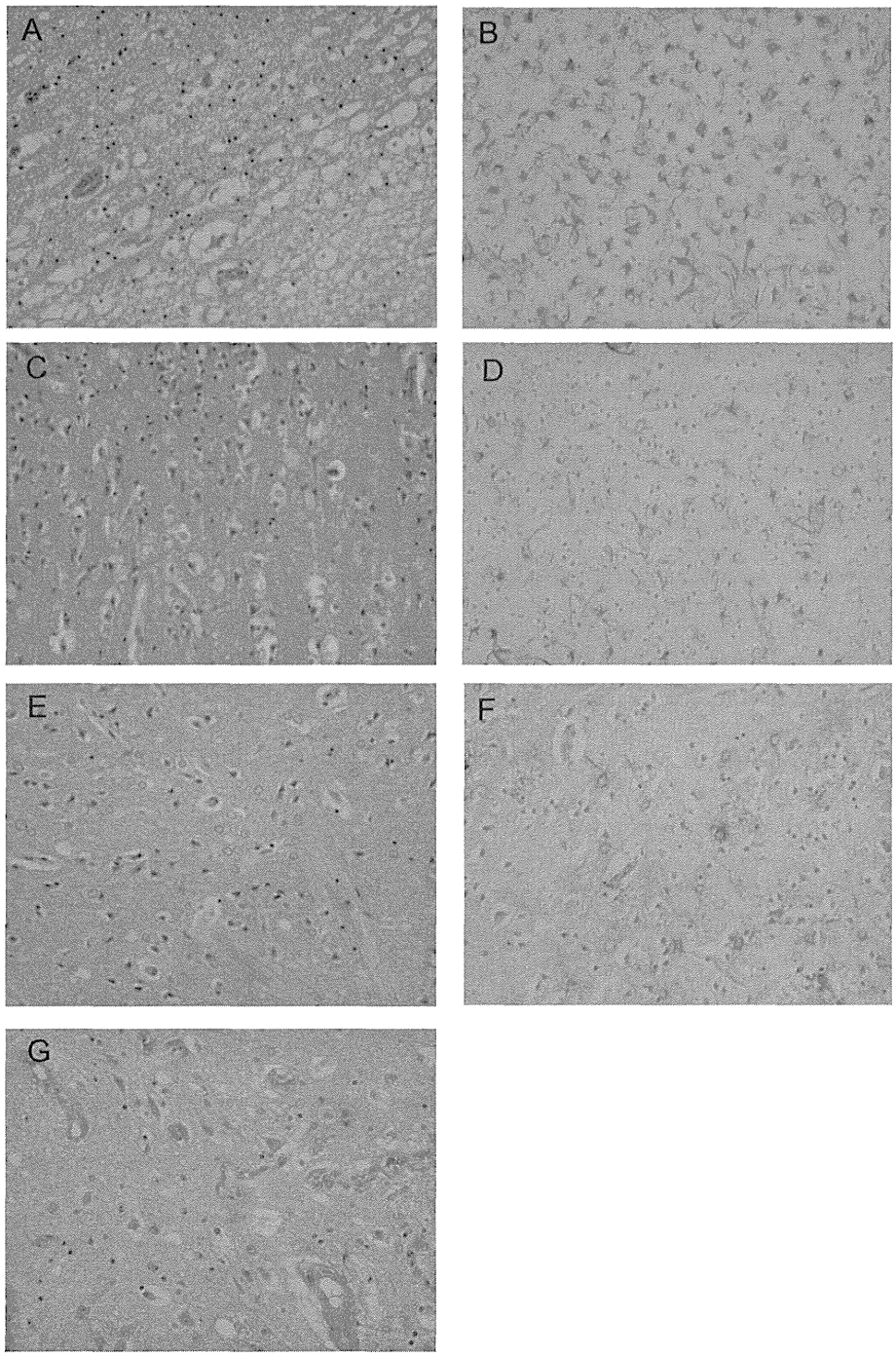

Fig. 2. Pathological findings of the postmortem brain (A, C, E, and G: hematoxylin & eosin staining; B, D, and F: immunohistochemical staining against glial fibrillary acidic protein; original magnification, ×400). Marked spongy changes (A) with Alzheimer type II astrocytosis (B) was observed in the cerebral white matter, and less prominently in the cerebral cortex (C and D) and striatum (E and F). Neuronal loss, sponginess, and capillary proliferation, which were reminiscent of the findings of Leigh syndrome, were noted in the substantia nigra (G).

were not registered in neither of the 1000 Genomes Project Database (http://www.1000genomes.org/), ESP6500 database (http://evs.gs.washington.edu/EVS/) or HGVD (http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html). The amino acid sequences of these two sites (p.A957V and p.I1185T) are well conserved across species, suggesting their importance (Fig. 3). *In silico* analyses were performed using the prediction algorithms SIFT (http://sift.jcvi.org) and PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/). These mutations are predicted to be deleterious by SIFT (0 and 0, respectively) and PolyPhen2 (0.985 and 0.991, respectively) programs. The results of mutation analysis have been reported previously (patient 6 in Ref. [9]).

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Fig. 3. Conservation analysis of mutation sites in *POLG*. The sites of compound heterozygous amino acid mutations (p.957A and p.1185I) are well conserved across species.

3. Discussion

The hetero compound mutations in *POLG* were not found in either of the 1000 Genomes Project Database, ESP6500 database nor HGVD, suggesting that these are pathogenic mutations. The amino acid sequences of these two sites (p.A957V and p.I1185T) are well conserved across species including *Saccharomyces cerevisiae*, indicating their importance (Fig. 3). *In silico* analyses also predicted that these two amino acid mutations are deleterious. Furthermore A957V has been reported by Tang et al. [10]. They reported A957V allele was shared in three unrelated patients and concluded this mutation is pathogenic. The pathogenic mutations in the flanking region of p.1185I; p.1184D [11,12] and p.1186D [13] have been reported, suggesting this region is also important. Thus, we conclude the compound heterozygous mutations of this patient cause the disease.

Alpers syndrome is defined as the clinical triad of (1) refractory, mixed-type seizures that often include a focal component, (2) psychomotor regression, often triggered by intercurrent infection, and (3) hepatopathy with or without acute liver failure. There is an overlap between the phenotypes of MCHS and Alpers syndrome; however, the former usually shows an earlier onset age and more rapid disease progression, while the latter is characterized by intractable epilepsy. Using the "myo-" prefix in MCHS may be confusing since the pathological findings of muscles in this disorder often shows no evidence of mitochondrial myopathy; instead, the hypotonia observed in the triad can be regarded as a symptom of brain dysfunction. Thus, the clinical features of the patient discussed herein were typical of MCHS.

Although Wong et al. [3] "*. . .excluded classical Alpers hepatopathy by liver biopsy*" in MHCS, exact pathological findings were not provided by the authors. Differences in the hepatopathy observed in these two phenotypes have not been established; pathological characteristics of the liver in Alpers syndrome include fibrosis, regenerative nodules, hepatocyte dropout, bile duct proliferation, fatty changes, and bile stasis [14]. The findings of the present patient were compatible with those of Alpers syndrome, similar to the case of POLG-related MDDS previously observed [15]. As for the neuropathological findings, Alpers syndrome usually shows a preferential involvement of gray matter, characterized by gliosis, nerve cell loss, spongy degeneration, and accumulation of neural lipids in the cerebral cortex [16]. Alzheimer type II glia, representing hepatic encephalopathy, was also distributed predominantly in the gray matter [17].

A patient exhibiting a clinical evolution from MCHS to Alpers phenotype showed gray matter involvement and microscopic findings similar to those in Leigh syndrome [5], and brain biopsy in another Alpers patient with prominent white matter signal change revealed pathological characteristics typical of Alpers disease with intractable seizures [18]. On the other hand, marked gliosis and sponginess of the white matter without pathological changes in the cerebral cortex was observed in a patient with probable MCHS [17]. Apart from these, we could not find any MCHS cases with a neuropathological description in the literature. The white matter-predominant spongy degeneration with Alzheimer type II astrocytosis in the present patient may therefore be characteristic of MCHS.

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POLG disorders often show elevated levels of lactate both in the serum and CSF as well as elevated levels of hepatic enzymes. However, these findings are not specific for POLG disorders; rather, they are hallmarks of mitochondrial disorders. Analysis of the RCC enzyme activity is the most valuable test for diagnosis of MDDS. However, RCC enzyme activity varies among muscle, liver, kidney, and brain tissues in the same patient [1,19], presumably due to the differential degree of DNA depletion among individual organs. The constituents of complex II are coded by genes in the nuclear, not mitochondrial DNA. In the present patient, the decreased complex II enzyme activity in the biopsied liver may either result from augmented activity of control CS enzyme due to an increase of mitochondria in number, or may be secondary to the damage of hepatocytes with necrotic and fibrotic changes [19]. It is very important to keep in mind that morphological findings and RCC enzyme activities in the muscle are sometimes unremarkable in MCHS patients, even though they show hypotonia or muscular weakness, as in the present case [5,15,20]. Therefore, analysis of RCC enzyme activities in the liver should be considered when Alpers syndrome or MCHS disorders are suspected, even when the morphological findings of muscle or enzyme assay results are unremarkable.

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References

[1] Moraes C, Shanske S, Tritschler HJ, Aprille JR, Andreetta F, Bonilla E, et al. mtDNA depletion with variable tissue expression: a novel genetic abnormality in mitochondrial diseases. Am J Hum Genet 1991;48:492–501.

[2] Sarzi E, Bourdon A, Chrétien D, Zarhrate M, Corcos J, Slama A, et al. Mitochondrial DNA depletion is a prevalent cause of multiple respiratory chain deficiency in childhood. J Pediatr 2007;150(531–4):e6.

[3] Wong L-J, Naviaux RK, Brunetti-Pierri N, Zhang Q, Schmitt ES, Truong C, et al. Molecular and clinical genetics of mitochondrial diseases due to *POLG* mutations. Hum Mutat 2008;29:150–72.

[4] Cohen BH, Naviaux RK. The clinical diagnosis of POLG disease and other mitochondrial DNA depletion disorders. Methods 2010;51:364–73.

[5] Scalais E, Francois B, Schlesser P, Stevens R, Nuttin C, Martin J-J, et al. Polymerase gamma deficiency (POLG): clinical course in a child with a two stage evolution from infantile myocerebrohepatopathy spectrum to an Alpers syndrome and neuropathological findings of Leigh's encephalopathy. Eur J Paediatr Neurol 2012;16:542–8.

[6] Kirby DM, Crawford M, Cleary MA, Dahl HH, Dennett X, Tourburn DR. Respiratory chain complex I deficiency. An underdiagnosed energy generation disorder. Neurology 1999;52:1255–64.

[7] Kaji S, Murayama K, Nagata I, Nagasaka H, Takayanagi M, Ohtake A, et al. Fluctuating liver functions in siblings with MPV17 mutations and possible improvement associated with dietary and pharmaceutical treatments targeting respiratory chain complex II. Mol Genet Metab 2009;97:292–6.

[8] Pagnamenta AT, Taanman JW, Wilson CJ, Anderson NE, Marotta R, Duncan AJ, et al. Dominant inheritance of premature ovarian failure associated with mutant mitochondrial DNA polymerase gamma. Hum Reprod 2006;21:2467–73.

[9] Yamazaki T, Murayama K, Compton AG, Sugiana C, Harashima H, Amemiya S, et al. Molecular diagnosis of mitochondrial respiratory chain disorders in Japan: focusing on mitochondrial DNA depletion syndrome. Pediatr Int 2014;56:180–7.

[10] Tang S, Wang J, Lee NC, Milone M, Halberg MC, Schmitt ES, et al. Mitochondrial DNA polymerase gamma mutations: an ever expanding molecular and clinical spectrum. J Med Genet 2011;48:669–81.

[11] Martikainen MH, Hinttala R, Majamaa K. Novel POLG1 mutations in a patient with adult-onset progressive external ophthalmoplegia and encephalopathy. BMJ Case Reports 2010; doi:10.1336/bcr.01.2010.2604.

[12] González-Vioque E, Blázquez A, Fernández-Moreira D, Bornstein B, Bautista J, Arpa J, et al. Association of novel POLG mutations and multiple mitochondrial DNA deletions with variable clinical phenotypes in a Spanish population. Arch Neurol 2006;63:107–11.

[13] Cheldi A, Ronchi D, Bordoni A, Bordo B, Lanfranconi S, Bellotti MG, et al. *POLG1* mutations and stroke like episodes: a distinct clinical entity rather than an atypical MELAS syndrome. BMC Neurol 2013;13:8.

[14] Nguyen KV, Sharief FS, Chan SS, Copeland WC, Naviaux RK. Molecular diagnosis of Alpers syndrome. J Hepatol 2006;45:108–16.

[15] Kurt B, Jaeken J, Van Hove J, Lagae L, Löfgren A, Everman DB, et al. A novel POLG gene mutation in 4 children with Alpers-like hepatocerebral syndromes. Arch Neurol 2010;67:239–44.

[16] Boustany R, Zucker A. Degenerative disease primarily gray matter. In: Swaiman KF, Ashwal S, Ferriero D, editors. Pediatric neurology: principles & practice. Philadelphia: Mosby; 2006. p. 1326–8.

[17] Kollberg G, Moslemi AR, Darin N, Nennesmo I, Bjarnadottir I, Uvebrant P, et al. POLG1 mutations associated with progressive encephalopathy in childhood. J Neuropathol Exp Neurol 2006;65:758–68.

[18] Bao X, Wu Y, Wong LJ, Zhang Y, Xiong H, Chou PC, et al. Alpers syndrome with prominent white matter changes. Brain Dev 2008;30:295–300.

[19] de Vries M, Rodenburg RJ, Morava E, van Kaauwen E, ter Laak H, Mullaart R, et al. Multiple oxidative phosphorylation deficiencies in severe childhood multi-system disorders due to polymerase gamma (*POLG1*) mutations. Eur J Pediatr 2007;166:229–34.

[20] Ferrari G, Lamantea E, Donati A, Filosto M, Briem E, Carrara F, et al. Infantile hepatocerebral syndromes associated with mutations in the mitochondrial DNA polymerase-gamma A. Brain 2005;128:723–31.

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

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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

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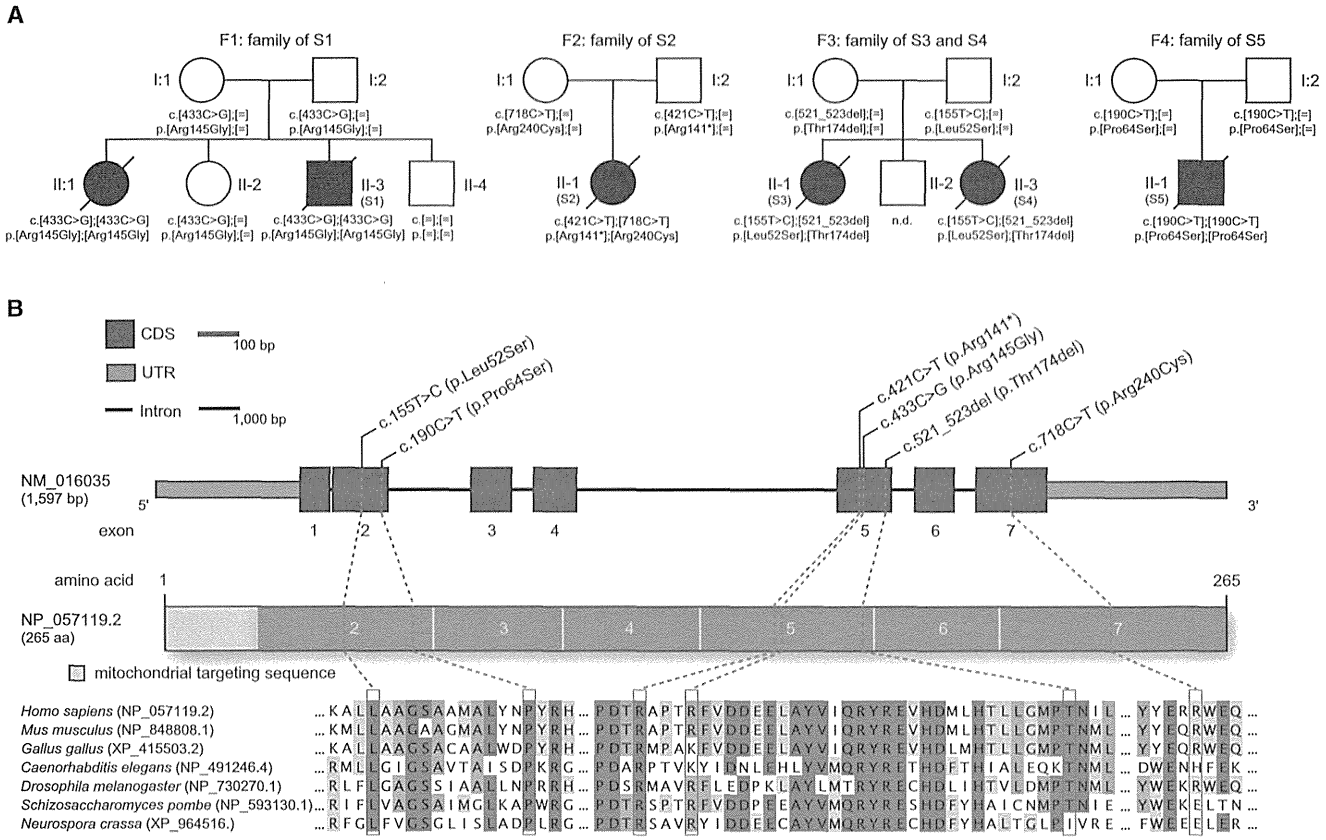

Figure 1. Pedigrees of Investigated Families and *COQ4* Structure and Conservation of Identified Mutations
(A) Pedigrees of four families affected by mutations in *COQ4*. The mutation status of affected and unaffected family members is indicated by closed and open symbols, respectively.
(B) *COQ4* structure 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

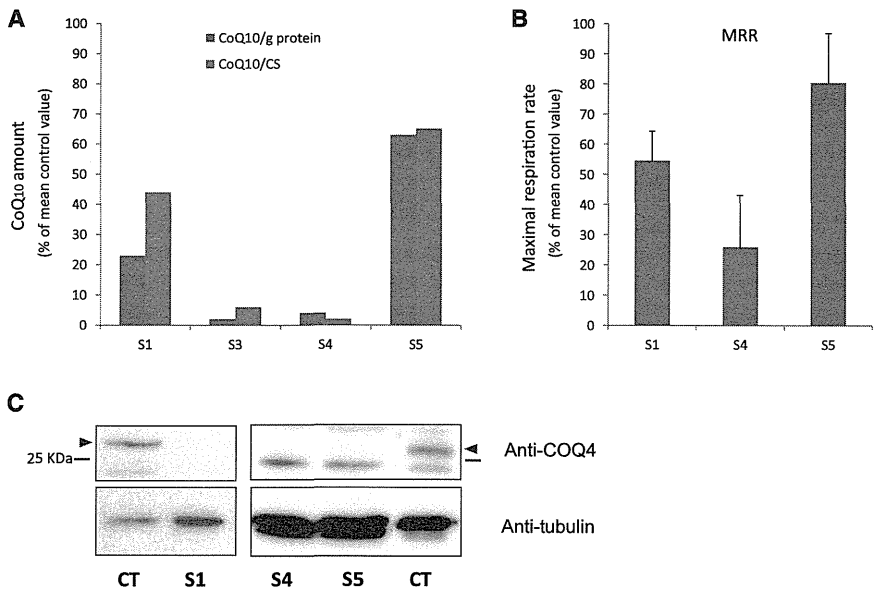

Figure 2. Biochemical Studies in *COQ4* Mutant Muscle and Fibroblasts

(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:y*COQ4* 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]

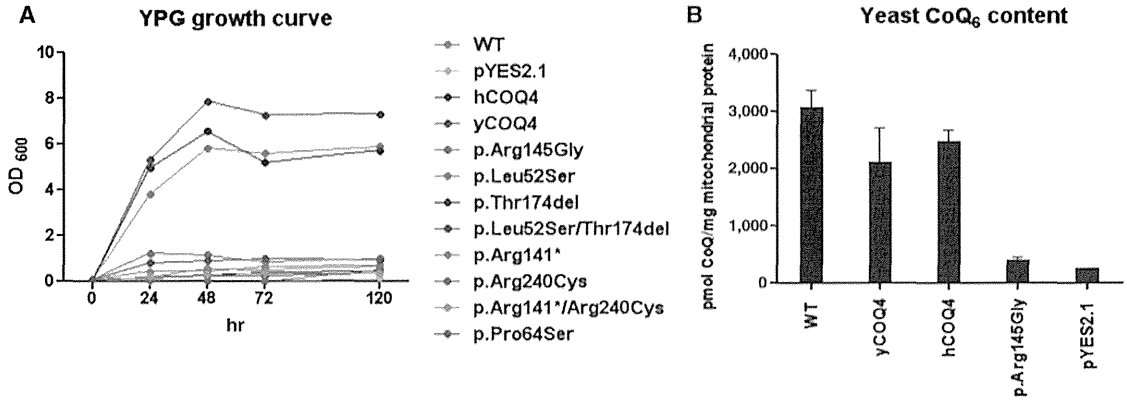

Figure 3. Yeast Studies

(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.

Acknowledgments

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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

References

1. Turunen, M., Olsson, J., and Dallner, G. (2004). Metabolism and function of coenzyme Q. Biochim. Biophys. Acta *1660*, 171–199.
2. Echtay, K.S., Winkler, E., and Klingenberg, M. (2000). Coenzyme Q is an obligatory cofactor for uncoupling protein function. Nature *408*, 609–613.
3. Miller, R.W., and Curry, J.R. (1969). Mammalian dihydroorotate—ubiquinone reducatse complex. II. Correlation with cytochrome oxidase, mode of linkage with the cytochrome chain, and general properties. Can. J. Biochem. *47*, 725–734.
4. Schmelzer, C., Lorenz, G., Rimbach, G., and Döring, F. (2007). Influence of Coenzyme Q_10 on release of pro-inflammatory chemokines in the human monocytic cell line THP-1. Biofactors *31*, 211–217.
5. Bentinger, M., Tekle, M., and Dallner, G. (2010). Coenzyme Q—biosynthesis and functions. Biochem. Biophys. Res. Commun. *396*, 74–79.
6. Kawamukai, M. (2009). Biosynthesis and bioproduction of coenzyme Q10 by yeasts and other organisms. Biotechnol. Appl. Biochem. *53*, 217–226.
7. Quinzii, C.M., Emmanuele, V., and Hirano, M. (2014). Clinical presentations of coenzyme q10 deficiency syndrome. Mol Syndromol *5*, 141–146.
8. Zeviani, M., and Carelli, V. (2007). Mitochondrial disorders. Curr. Opin. Neurol. *20*, 564–571.
9. Duncan, A.J., Bitner-Glindzicz, M., Meunier, B., Costello, H., Hargreaves, I.P., López, L.C., Hirano, M., Quinzii, C.M., Sadowski, M.I., Hardy, J., et al. (2009). A nonsense mutation in COQ9 causes autosomal-recessive neonatal-onset primary coenzyme Q10 deficiency: a potentially treatable form of mitochondrial disease. Am. J. Hum. Genet. *84*, 558–566.
10. Casarin, A., Jimenez-Ortega, J.C., Trevisson, E., Pertegato, V., Doimo, M., Ferrero-Gomez, M.L., Abbadi, S., Artuch, R., Quinzii, C., Hirano, M., et al. (2008). Functional characterization of human COQ4, a gene required for Coenzyme Q10 biosynthesis. Biochem. Biophys. Res. Commun. *372*, 35–39.
11. Marbois, B., Gin, P., Gulmezian, M., and Clarke, C.F. (2009). The yeast Coq4 polypeptide organizes a mitochondrial protein complex essential for coenzyme Q biosynthesis. Biochim. Biophys. Acta *1791*, 69–75.
12. Spinazzi, M., Casarin, A., Pertegato, V., Salviati, L., and Angelini, C. (2012). Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. Nat. Protoc. *7*, 1235–1246.
13. Haack, T.B., Haberberger, B., Frisch, E.M., Wieland, T., Iuso, A., Gorza, M., Strecker, V., Graf, E., Mayr, J.A., Herberg, U., et al. (2012). Molecular diagnosis in mitochondrial complex I deficiency using exome sequencing. J. Med. Genet. *49*, 277–283.
14. Elstner, M., Andreoli, C., Klopstock, T., Meitinger, T., and Prokisch, H. (2009). The mitochondrial proteome database: MitoP2. Methods Enzymol. *457*, 3–20.
15. Ohtake, A., Murayama, K., Mori, M., Harashima, H., Yamazaki, T., Tamaru, S., Yamashita, Y., Kishita, Y., Nakachi, Y., Kohda, M., et al. (2014). Diagnosis and molecular basis of mitochondrial respiratory chain disorders: exome sequencing for disease gene identification. Biochim. Biophys. Acta *1840*, 1355–1359.
16. Invernizzi, F., D'Amato, I., Jensen, P.B., Ravaglia, S., Zeviani, M., and Tiranti, V. (2012). Microscale oxygraphy reveals OXPHOS impairment in MRC mutant cells. Mitochondrion *12*, 328–335.
17. Ogasahara, S., Engel, A.G., Frens, D., and Mack, D. (1989). Muscle coenzyme Q deficiency in familial mitochondrial encephalomyopathy. Proc. Natl. Acad. Sci. USA *86*, 2379–2382.
18. Lamperti, C., Naini, A., Hirano, M., De Vivo, D.C., Bertini, E., Servidei, S., Valeriani, M., Lynch, D., Banwell, B., Berg, M., et al. (2003). Cerebellar ataxia and coenzyme Q10 deficiency. Neurology *60*, 1206–1208.
19. Mignot, C., Apartis, E., Durr, A., Marques Lourenço, C., Charles, P., Devos, D., Moreau, C., de Lonlay, P., Drouot, N., Burglen, L., et al. (2013). Phenotypic variability in ARCA2 and identification of a core ataxic phenotype with slow progression. Orphanet J. Rare Dis. *8*, 173.
20. Rötig, A., Appelkvist, E.-L., Geromel, V., Chretien, D., Kadhom, N., Edery, P., Lebideau, M., Dallner, G., Munnich, A., Ernster, L., and Rustin, P. (2000). Quinone-responsive multiple respiratory-chain dysfunction due to widespread coenzyme Q10 deficiency. Lancet *356*, 391–395.
21. Heeringa, S.F., Chernin, G., Chaki, M., Zhou, W., Sloan, A.J., Ji, Z., Xie, L.X., Salviati, L., Hurd, T.W., Vega-Warner, V., et al. (2011). COQ6 mutations in human patients produce nephrotic syndrome with sensorineural deafness. J. Clin. Invest. *121*, 2013–2024.
22. Ashraf, S., Gee, H.Y., Woerner, S., Xie, L.X., Vega-Warner, V., Lovric, S., Fang, H., Song, X., Cattran, D.C., Avila-Casado, C., et al. (2013). ADCK4 mutations promote steroid-resistant nephrotic syndrome through CoQ10 biosynthesis disruption. J. Clin. Invest. *123*, 5179–5189.
23. Van Maldergem, L., Trijbels, F., DiMauro, S., Sindelar, P.J., Musumeci, O., Janssen, A., Delberghe, X., Martin, J.J., and Gillerot, Y. (2002). Coenzyme Q-responsive Leigh's encephalopathy in two sisters. Ann. Neurol. *52*, 750–754.
24. Lalani, S.R., Vladutiu, G.D., Plunkett, K., Lotze, T.E., Adesina, A.M., and Scaglia, F. (2005). Isolated mitochondrial myopathy associated with muscle coenzyme Q10 deficiency. Arch. Neurol. *62*, 317–320.
25. Doimo, M., Desbats, M.A., Cerqua, C., Cassina, M., Trevisson, E., and Salviati, L. (2014). Genetics of coenzyme q10 deficiency. Mol Syndromol *5*, 156–162.
26. Aeby, A., Sznajer, Y., Cavé, H., Rebuffat, E., Van Coster, R., Rigal, O., and Van Bogaert, P. (2007). Cardiofaciocutaneous (CFC) syndrome associated with muscular coenzyme Q10 deficiency. J. Inherit. Metab. Dis. *30*, 827.
27. Yubero, D., O Callaghan, M., Montero, R., Ormazabal, A., Armstrong, J., Espinos, C., Rodríguez, M.A., Jou, C., Castejon, E., Aracil, M.A., et al. (2014). Association between coenzyme Q 10 and glucose transporter (GLUT1) deficiency. BMC Pediatr. *14*, 284.
28. Musumeci, O., Naini, A., Slonim, A.E., Skavin, N., Hadjigeorgiou, G.L., Krawiecki, N., Weissman, B.M., Tsao, C.Y., Mendell,

J.R., Shanske, S., et al. (2001). Familial cerebellar ataxia with muscle coenzyme Q10 deficiency. Neurology *56*, 849–855.
29. DiMauro, S., Schon, E.A., Carelli, V., and Hirano, M. (2013). The clinical maze of mitochondrial neurology. Nat Rev Neurol *9*, 429–444.
30. Salviati, L., Trevisson, E., Rodriguez Hernandez, M.A., Casarin, A., Pertegato, V., Doimo, M., Cassina, M., Agosto, C., Desbats, M.A., Sartori, G., et al. (2012). Haploinsufficiency of COQ4 causes coenzyme Q10 deficiency. J. Med. Genet. *49*, 187–191.
31. Aberg, F., Appelkvist, E.L., Dallner, G., and Ernster, L. (1992). Distribution and redox state of ubiquinones in rat and human tissues. Arch. Biochem. Biophys. *295*, 230–234.
32. Forsgren, M., Attersand, A., Lake, S., Grünler, J., Swiezewska, E., Dallner, G., and Climent, I. (2004). Isolation and functional expression of human COQ2, a gene encoding a polyprenyl transferase involved in the synthesis of CoQ. Biochem. J. *382*, 519–526.
33. Hanaki, Y., Sugiyama, S., Ozawa, T., and Ohno, M. (1991). Ratio of low-density lipoprotein cholesterol to ubiquinone as a coronary risk factor. N. Engl. J. Med. *325*, 814–815.
34. Langsjoen, P.H., Langsjoen, P.H., and Folkers, K. (1990). Long-term efficacy and safety of coenzyme Q10 therapy for idiopathic dilated cardiomyopathy. Am. J. Cardiol. *65*, 521–523.
35. Manzoli, U., Rossi, E., Littarru, G.P., Frustaci, A., Lippa, S., Oradei, A., and Aureli, V. (1990). Coenzyme Q10 in dilated cardiomyopathy. Int. J. Tissue React. *12*, 173–178.
36. Mortensen, S.A., Vadhanavikit, S., and Folkers, K. (1984). Deficiency of coenzyme Q10 in myocardial failure. Drugs Exp. Clin. Res. *10*, 497–502.
37. Silver, M.A., Langsjoen, P.H., Szabo, S., Patil, H., and Zelinger, A. (2003). Statin cardiomyopathy? A potential role for Co-Enzyme Q10 therapy for statin-induced changes in diastolic LV performance: description of a clinical protocol. Biofactors *18*, 125–127.
38. Ghirlanda, G., Oradei, A., Manto, A., Lippa, S., Uccioli, L., Caputo, S., Greco, A.V., and Littarru, G.P. (1993). Evidence of plasma CoQ10-lowering effect by HMG-CoA reductase inhibitors: a double-blind, placebo-controlled study. J. Clin. Pharmacol. *33*, 226–229.
39. Mortensen, S.A., Rosenfeldt, F., Kumar, A., Dolliner, P., Filipiak, K.J., Pella, D., Alehagen, U., Steurer, G., and Littarru, G.P.; Q-SYMBIO Study Investigators (2014). The effect of coenzyme Q10 on morbidity and mortality in chronic heart failure: results from Q-SYMBIO: a randomized double-blind trial. JACC Heart Fail *2*, 641–649.
40. Scalais, E., Chafai, R., Van Coster, R., Bindl, L., Nuttin, C., Panagiotaraki, C., Seneca, S., Lissens, W., Ribes, A., Geers, C., et al. (2013). Early myoclonic epilepsy, hypertrophic cardiomyopathy and subsequently a nephrotic syndrome in a patient with CoQ_{10} deficiency caused by mutations in para-hydroxybenzoate-polyprenyl transferase (COQ2). Eur. J. Paediatr. Neurol. *17*, 625–630.

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Supplemental Data

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

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SUPPLEMENTAL DATA

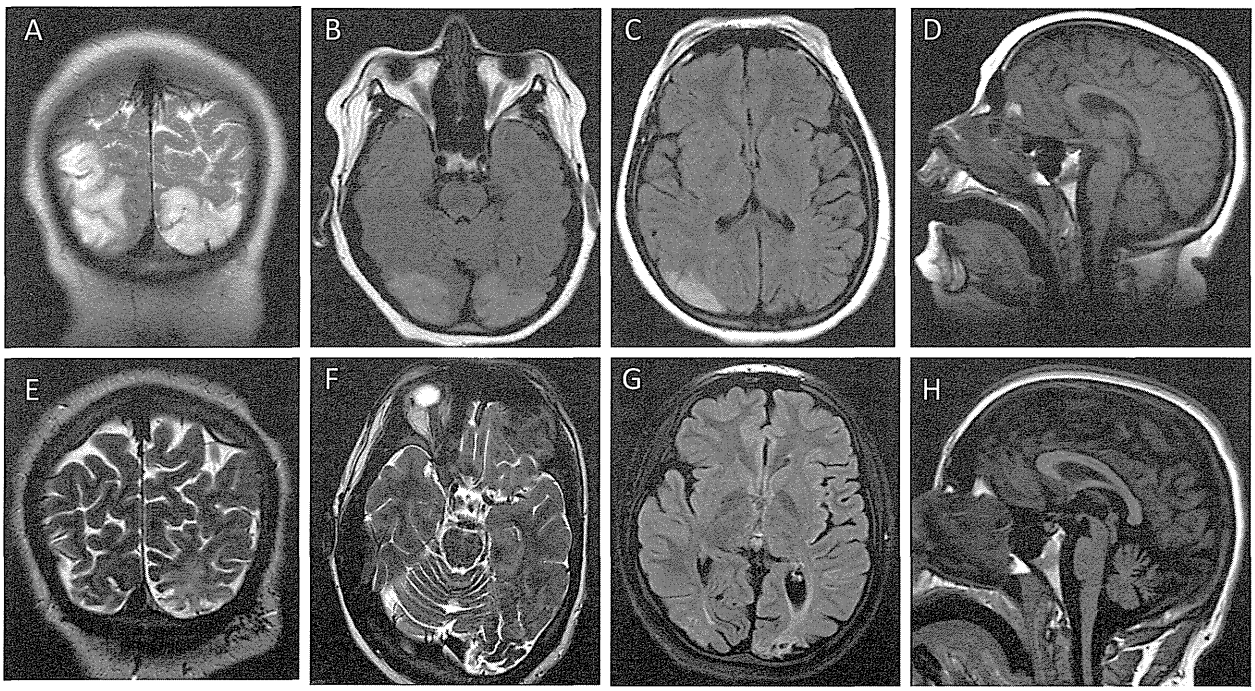

Figure S1. Brain MRI of subject 5.

Two serial brain MRI images of subject S5 obtained at age 12 years (A-D) and at age 17 years (E-H). A and E are coronal T2 weighted sections to show abnormal hyperintense cortical areas corresponding to infarct-like lesions of the occipital lobes at two different stages of progression to cortical atrophy with scars (E-G); The same occipital abnormal hyperintense cortical areas are shown in axial sections B and C (FLAIR weighted), F (T2 weighted) and G (FLAIR weighted). Notice that no abnormality is present in basal ganglia and brainstem. Finally, figures D and H are sagittal T1 sections showing progressive cerebellar atrophy of the vermis.

Id	Type	Reads	Mapped	Percent	Seq (Gb)	on bait	Avg cov	Cov 1x	Cov 4x	Cov 8x	Cov 20x
S1	SureSelect50Mbv5	106627996	105933934	99.35	10.77	78.48	132.44	99.92	99.72	99.35	97.38
S2	SeqCapEZ V1	130889538	125611825	95.97	13.81	62.71	155.83	98.36	97.32	96.41	93.80
S4	SureSelect50Mbv5	90821087	90214603	99.33	9.17	78.99	113.62	99.80	99.52	99.05	96.55
S5	SureSelect50Mbv4	125822167	124979728	99.33	12.71	73.98	146.50	99.95	99.80	99.55	98.22

Table S1: Exome Sequencing Statistics

Avg: average; Cov: coverage

Nucleotide Change	Amino acid Change	Subject	Status	Polyphen2	SIFT	PMUT	Mutation taster
c.[433C>G]	p.[Arg145Gly]	S1	Homozygous	Probably damaging	Deleterious	Pathological	Disease causing
c.[718C>T]	p.[Arg240Cys]	S2	CE with a nonsense mutation p.[Arg141*]	Probably damaging	Deleterious	Pathological	Disease causing
c.[155T>C]	p.[Leu52Ser]	S3, S4	CE with a deletion p.[Thr174del]	Probably damaging	Deleterious	Neutral	Disease causing
c.[190C>T]	p.[Pro64Ser]	S5	Homozygous	Probably damaging	Deleterious	Neutral	Disease causing

Table S2: In silico prediction of pathogenicity for *COQ4* mutations

CE: compound heterozygous.

REPORT

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

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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]

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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,