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Original Article

Next-generation sequencing for mitochondrial diseases: A wide diagnostic spectrum

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Abstract ***Background***: The current diagnostic approach for mitochondrial disorders requires invasive procedures such as muscle biopsy and multiple biochemical testing but the results are often inconclusive. Clinical sequencing tests are available only for a limited number of genes. Recently, massively parallel sequencing has become a powerful tool for testing genetically heterogeneous conditions such as mitochondrial disorders.

Methods: Targeted next-generation sequencing was performed on 26 patients with known or suspected mitochondrial disorders using in-solution capture for the exons of 908 known and candidate nuclear genes and an Illumina genome analyzer.

Results: None of the 18 patients with various abnormal respiratory chain complex (RCC) activities had molecular defects in either subunits or assembly factors of mitochondrial RCC enzymes except a reference control sample with known mutations in SURF1. Instead, several variants in known pathogenic genes including *CPT2*, *POLG, PDSS1*, *UBE3A*, *SDHD*, and a few potentially pathogenic variants in candidate genes such as *MTO1 or SCL7A13* were identified.

Conclusions: Sequencing only nuclear genes for RCC subunits and assembly factors may not provide the diagnostic answers for suspected patients with mitochondrial disorders. The present findings indicate that the diagnostic spectrum of mitochondrial disorders is much broader than previously thought, which could potentially lead to misdiagnosis and/or inappropriate treatment. Overall analytic sensitivity and precision appear acceptable for clinical testing. Despite the limitations in finding mutations in all patients, the present findings underscore the considerable clinical benefits of targeted next-generation sequencing and serve as a prototype for extending the clinical evaluation in this clinically heterogeneous patient group.

Key words mitochondrial disorders, mitochondrial respiratory chain complex enzyme deficiency, next-generation sequencing.

Mitochondrial diseases are likely the most common metabolic diseases of childhood and probably in adults, with an estimated frequency of 1 in 5000 births.[1] Variability in clinical presentation and lack of reliable diagnostic screening makes the diagnosis of mitochondrial diseases challenging. Currently, the diagnosis of mitochondrial disorders relies largely on the enzymatic analysis of the respiratory chain complexes (RCC) in tissues and extensive biochemical analysis, but considerable differences exist between clinical laboratories in their RCC assay protocols and the subsequent interpretation of their results.[2,3] Because current guidelines used in the diagnosis of mitochondrial disorders are heavily weighted upon demonstration of an RCC enzyme deficiency,[4] this variability can have significant effects upon accurate diagnosis and then ultimately affect the quality of patient care.[5]

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Mutations can occur in mitochondrial DNA (mtDNA) or in nuclear genes encoding mitochondrial proteins. So far, more than 170 nuclear genes have been identified as causative for mitochondrial disorders presenting as neuropathy, myopathy, or liver disease.[6] Given that approximately 1500 proteins are likely involved in mitochondrial structure and function,[7] many disease causing gene mutations remain unidentified.

Mitochondrial diseases have been traditionally defined primarily by a disruption of the respiratory chain or of other mitochondrial functions, including organelle dynamics or metabolite transport. Other genetic or metabolic conditions present with similar symptoms such as fatty acid oxidation disorders. This presents a challenge for clinicians when selecting and prioritizing the genes to be sequenced, because sequencing all candidate genes is not feasible with traditional sequencing methods. Thus the diagnostic process is lengthy, often relying upon an invasive biopsy for RCC assay and extensive biochemical testing with significant costs and risks.

In recent years, next-generation sequencing (NGS) has been successfully used for the discovery of the causative genes in

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several Mendelian disorders.[8,9] A few NGS targeted tests for genetic conditions, including mitochondrial disorders, have been developed as well.[10–16]

Although whole-exome sequencing appears to be an attractive choice for diagnostic testing, the current cost of this approach is still prohibitive for routine clinical utilization. In addition, the tremendous number of variations that can be discovered in genes with unknown relationship to mitochondrial function will make data interpretation extremely difficult. There is also a profound ethical concern for finding mutations for late-onset or untreatable disorders. Furthermore, current clinical laboratory standard practice mandates a clear distinction between research and clinical testing.

We previously explored the feasibility of this approach for mitochondrial disorders by sequencing the exons of 362 known and candidate genes.[14] Here, we further expanded the panel to 908 nuclear genes and validated this methodology by analyzing 26 patients with known or highly suspected mitochondrial disease.

Methods

Subjects

The study cohort consisted of 26 unrelated affected patients, with additional testing of siblings and parents when appropriate, following written consent per institutional review board (IRB) approval (Table 1; Fig. 1). We deliberately chose a range of patients whom the clinician is likely to encounter: those with definitive mitochondrial disease; probable disease but without RCC defects; and those who possibly had disease based on limited findings. Two samples were used as reference controls with multiple variants in several genes previously identified by Sanger. Various RCC deficiencies were found in the muscle tissue from 18 patients but no underlying molecular background was defined except for the two controls used in the study. Six patients were included despite normal RCC activity due to their clinical presentations being highly suggestive of mitochondrial disorders, and two patients did not have RCC assayed. Mitochondrial DNA sequencing result was available in 10 patients with no pathogenic alterations (Table 1).

DNA capture and sequencing

A DNA library was prepared for each sample using an Illumina Genome DNA Sample preparation kit (San Diego, CA, USA). The exons of the genes of interest (Table S1) were captured by in-solution hybridization to probes using a custom-made SureSelect kit by Agilent (Santa Clara, CA, USA).

The known/candidate genes include all of the structural components of respiratory chain complexes (89 subunits), as well as other mitochondrial proteins of the following functional groups: respiratory complexes assembly factors, transcription and translation factors, enzymes, and carrier proteins.[6,7] Some of the genes causing secondary inhibition of mitochondrial respiratory chain or similar phenotypes are also included in this panel. Sequencing was performed using the Illumina GAIIX instrument with single reads on one sample per flow-cell lane. For accuracy, one sample was sequenced in duplicate and one in triplicate within one run for intra-assay precision. Two samples were sequenced in two separate independent runs for inter-assay comparison.

Data analysis

Reads were aligned using Burrows-Wheeler Aligner (BWA). Data were analyzed with GATK's Unified Genotyper (version 1.0.4013) and Variant Filtration Walker to filter the variants that meet quality control requirements.[17] Insertion and deletions were analyzed with Dindel (GATK version 1.0.5336).[18] Variants were annotated with genomic coordinates, reference nucleotide, variant nucleotide, number of reads, and percentage of reads containing the variant nucleotides. Analysis of variants, including non-synonymous variants, splice sites variants and small indels was performed by cross-referencing with the dbSNP database and the Human Gene Mutation Database[19] as well as by literature review. Variants of interest were confirmed in probands and subsequently in their parents or siblings by Sanger sequencing. We further analyzed the non-synonymous single nucleotide substitutions with PolyPhen 2 (Polymorphism Phenotyping)[20] to predict the possible impact of amino acid substitutions on the structure and function of a protein.

Results

Statistical data

Each lane produced 4500 Mbases on average with approximately >90% of reads mapped to the human reference genome and 60% on targeted areas. Less than 8% of targeted bases had <20 reads of quality score (Q) ≥ 30 (Fig. 2). (Quality score: http://www.illumina.com/truseq/quality_101/quality_scores.ilmn) Of 12 variants previously identified by Sanger sequencing (two pathogenic mutations and 10 polymorphisms in four genes), 11 were detected on NGS (91.6% concordance). One complex insertion/deletion mutation in the *SURF1* gene (c.312_321del10ins2) was identified only as a deletion of nine nucleotides. For analytical sensitivity, 90 variants detected on NGS were further sequenced using the Sanger method and 89 of them were found to be concordant (98.9%). We noticed several false positives when the GATK filtering options[17] were not applied.

In the intra- and inter-assay reproducibility studies, we observed an average coefficient of variation of 1.31% and 1.83%, respectively, in the identification of total coding variants. On average, approximately 300 non-synonymous variants were detected per sample, 92% of them being known single-nucleotide polymorphisms (SNP) or previously identified in the 1000 genomes project.

Confirmed disease-causing or possible disease-causing variants

Selected novel variants or known pathogenic SNP identified in patients are summarized in Table 2.

Patient 1

This female patient was hypotonic at birth and had developmental delays. She had atonic and atypical absence seizures, gait ataxia, muscle weakness in the proximal muscles and dilated

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Table 1 Patient information

Patient no.	Age onset/sex	Chief complaints	Significant laboratory findings	Muscle pathology	Muscle RCC enzymes	Radiology	Others	Modified Walker criteria
1	At birth F	Hypotonia, developmental delay, seizures, ataxia	Normal AA, AC, UOA, lactate; normal COX15, 10, 6B1, SCO1, 2, SURF1, FASTKD2; normal mtDNA copy number in muscle	Normal	Complex IV deficiency: I/III = 77%, I = 76%, II/III = 153%, II = 72%, IV = 0%, CS = 92%	Mildly increased T2 signal in thalamus		Definite: 2 majors 1 minor
2	<6 months F	Died at 16 months of age due to nephrotic syndrome and renal failure, failure to thrive, developmental delay	CoQ10 = 30 pmol/mg protein (66–183); Lactate 11.4 mmol/L (<2.2)	Not available	Not available	Leukoencephalopathy, Lactate peak on MRS	Kidney biopsy: acute tubular epithelial damage	Definite: 2 majors 1 minor
3	<4 months, M	Hypotonia, muscle weakness, gastric reflux	Plasma AC with elevations of several acylcarnitine from various chain lengths; Elevated excretions of EMA, 3-MGA and GA in UOA	Increased lipid, focal absent SDH and COX staining activity, increased mitochondria.	Multiple enzyme deficiency: I/III = 3.7%, I = 88%, II/III = 6%, II = 32%, III = 136%, IV = 52%, CS = 240%	Normal brain MRI; No lactate peak in MRS	CPTII in SF = 0.15 nmol/min per mg (control 0.43); CPT II in muscle =29.2%/ 42.3% of normal	Definite: 2 majors 1 minor
4	<8 months M	Seizure, hypotonia, loss of language at age 3 years, ataxia	Elevated lactate; elevated alanine; Normal CSF, biotinidase, AC, AA, UOA	Normal	Complex III deficiency: I/III = 31%, I = 59%, II/III = 47%, II = 89%, III = 16%, IV = 47%, CS = 107%	Normal brain MRI	*SCN1A* mutation, C>T3733; Used for reference control	Definite: 1 major 2 minors
5	<2 weeks M	Seizure, hypotonia, delayed motor development	Normal CSF, AA, UOA, AC, CDT, PUPY; normal array CGH	Scattered atrophic muscle fibers	Possible multiple deficiency: several low from one center but normal from other diagnostic center	Delayed myelination; diffuse decrease in white matter, thinning of corpus callosum; lactate peaks in MRS		Definite: 1 major 2 minors
6	<3 months M	Infantile spasm, hypotonia, gastrointestinal dysmotility, dystonic movement, obstructive apnea	Normal liver enzymes, normal AA, lactate and pyruvate, normal CSF	Increased lipid droplets, enlarged mitochondria, no COX deficient fibers	Complex IV deficiency: original report was not available in the clinical record	Normal brain MRI		Definite: 2 majors
7	At birth M	Died three days after birth with lactic acidosis, & hypertrophic cardiomyopathy	Lactate 23.1, Pyruvate 0.27; Normal pyruvate dehydrogenase complex activity in skin fibroblasts; normal mtDNA sequence	Not available	Partial complex IV deficiency: skeletal: I = 74%, II = 62%, I/III = 141%, II/III = 98%, IV = 31%, CS = 46% cardiac: I = 31%, II = 64%, I/III = 173%, II/III = 50%, IV = 52%, CS = 57%	Not available	Sister died of similar clinical phenotype (skin fibroblast RCC reportedly normal)	Definite: 1 major 2 minors

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Table 1 *Continued*

Patient no.	Age onset/sex	Chief complaints	Significant laboratory findings	Muscle pathology	Muscle RCC enzymes	Radiology	Others	Modified Walker criteria
8	<1 year F	Developmental delay, hypotonia, dilated cardiomyopathy, seizure, peripheral neuropathy	Mildly elevated GA, 3-MGA and MMA in UOA; normal AA, AC, CoQ10, acid alpha glucosidase; normal mtDNA sequence	Normal	Partial complex IV deficiency: I/III = 33%, I = 56%, II/III = 39%, II = 88%, III = 51%, IV = 30%, CS = 77%	Normal brain MRI	22q13 deletion, possible Phelan-McDermid syndrome	Definite: 1 major 2 minors
9	At birth F	Congenital nystagmus, optic atrophy, hypotonia, developmental delay	CoQ10 0.7 mg/L (0.8–1.5); elevated liver enzymes; normal UOA, AA: Normal mtDNA sequence	Increased numbers of mitochondria on EM	Normal RCC	Mild cerebellar atrophy, hypoplasia of optic nerve and chiasm; small lactate peak in MRS	One older brother with similar clinical phenotype, younger brother normal	Probable: 1 major 1 minor
10	<4 months M	Infantile spasm, gastric reflux, hypotonia, poor visual tracking with normal optic nerve	Normal karyotype; normal AC, CoQ10, pipecolic acid; mtDNA 150% copy number in muscle; normal mtDNA sequence	Increased lipid	Multiple enzyme deficiency: I/III = 0%, I = 43%, II/III = 1%, II = 16%, III = 20%, IV = 30%, CS = 41%	Ventriculomegaly, thin corpus callosum with hypoplastic splenium and rostrum; normal myelination		Definite: 2 majors
11	<2 months F	Horizontal nystagmus, ptosis, axial hypotonia	Elevated lactate; abnormal UOA with elevated 3-MGA; normal array CGH; normal mtDNA sequence;	Normal	Complex IV deficiency: I/III = 85%, I = 143%, II/III = 129%, III = 209%, IV = 21%, CS = 173%	Hyperintensities in basal ganglia	Used for reference control	Definite: 3 majors
12	<1 month M	Seizure, hypotonia, developmental delay, gastric reflux; history of lactic acidosis and hypoglycemia	Normal AA, AC, UOA; normal array CGH	Normal	Possible complex IV deficiency: IV = 6.5%, CS = 182% but second testing center on same specimen showed normal IV activity (57%, CS = 226%)	Normal brain MRI		Possible: 2 minors
13	<6 months F	Gross motor delay, failure to thrive, macrocephaly, ataxia	UOA with mildly elevated Kreb cycle intermediates; normal AC; normal mtDNA sequence	Increased lipid; normal mitochondria structure	Complex IV deficiency: I/III = 60%, I = 78%, II/III = 41%, II = 27%, III = 96%, IV = 22%, CS = 117%	Hyperintensities in basal ganglia		Definite: 2 majors 2 minors
14	<3 months F	Intractable seizure, hypotonia, severe developmental delay	Mildly elevated lactate, mildly increased excretion of 3-MGA and TCA intermediates in UOA; normal karyotype	Normal	Normal RCC	Delayed myelination	Sister died of progressive encephalopathy during infancy.	Possible: 2 minors

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15	<2 months M	Seizure, hypotonia, choreoathetosis, global developmental delay, failure to thrive	Elevated excretion of 3-MGA and TCA intermediates in UOA, normal array CGH:	Normal	Normal RCC	Increased signals in basal ganglia	Brother with identical phenotype; two maternal male siblings died from unknown etiology	Probable: 1 major 1 minor
16	<1 year M	Developmental delay, hypotonia, athetoid movement	Normal UOA, AA, AC, CDT, PUPY; normal array CGH; normal mtDNA genome sequence	Normal	Normal RCC	Normal brain MRI	Sister with identical phenotype	Possible: 2 minors
17	<1 week M	Developmental delay, failure to thrive, hypotonia	Elevated 3-MGA in UOA; normal mtDNA genome sequence	Normal	Normal RCC	Normal brain MRI	Brother with identical phenotype	Possible: 2 minors
18	At birth M	Seizure; GERD; hypotonia; ataxia	Dicarboxylic aciduria in UOA, elevated liver enzymes, normal AC	Increased glycogen; normal mitochondria	Complex I deficiency: I/III = 8%, I = 132%, II/III = 71%, III = 148%, III = 107%, IV = 76%, CS = 130%	Normal brain MRI		Definite: 2 majors 1 minor
19	<4 weeks M	Central apnea, seizure, developmental delay, hypothermia, poor visual attention	Normal UOA, AA, AC, lactate, copper, creatine/ guanidinoacetate, VLCFA: normal array CGH; normal Prader Willi methylation test	Normal	Normal RCC	Increased signals in internal capsules		Possible: 2 minors
20	At birth M	Autism spectrum disorder, developmental delay, seizure, ataxia, hemihypertrophy, microcephaly	Elevated 3-MGA in UOA, normal AC, CoQ10; normal array CGH	Increased number of mitochondria with abnormal cristae	Complex I deficiency; I/III = 17%, I = 51%, II/III = 23%, II = 41%, III = 35%, IV = 40%, CS = 55%	Normal brain MRI, lactate peak on MRS		Definite: 2 major 1 minor
21	<6 weeks F	Developmental delay, microcephaly, failure to thrive, GERD, dystonia, athetoid movement	Normal urine UOA, CoQ10, PUPY, lactate; normal array CGH; normal mtDNA genome sequence	Variable size of mitochondria	Multiple enzyme deficiency; I/III = 0%, I = 39%, II/III = 20%, II = 90%, III = 111%, IV = 31%, CS = 84%	Delayed myelination	Parents were first cousins, brother with mild cognitive delay	Definite: 2 majors 1 minor
22	<9 months F	Seizures, developmental delay, autistic features	Normal UOA, PA, AC, CoQ10	Normal	Complex I deficiency: I/III = 14%, I = 97%, II/III = 36%, II = 90%, III = 101%, IV = 39%, CS = 111%			Probable: 1 major 1 minor

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Table 1 *Continued*

Patient no.	Age onset/sex	Chief complaints	Significant laboratory findings	Muscle pathology	Muscle RCC enzymes	Radiology	Others	Modified Walker criteria
23	<3 years M	Asperger, hypotonia, muscle cramping, lipodystrophy	Episodic CK elevation; normal alpha glucosidase (Pompe); negative mtDNA depletion panel; normal mtDNA sequence; mtDNA copy number <20% of normal in muscle	Increased mitochondrial number; increased SDH staining; abnormal cristae	Multiple enzyme deficiency: I/III = 16.2%, II/III = 22.3%, II = 278%, III = 36%, IV = 43%, CS = 120%	Normal brain MRI	Sister with similar clinical phenotype	Definite: 2 majors 2 minors
24	<2 years M	Intractable seizures; s/p temporal lobectomy	Normal AA, AC, UOA, CDT, CoQ10, VLCFA; normal mtDNA sequence	Abnormal cristae with irregular mitochondria	Complex III deficiency: Activity ratio to CS I = 0.009 (0.01–0.055), II = 0.07 (0.003–0.035), III = 0 (0.009–0.06), IV0.07 (0.06–0.260, CS = 0.095 (0.08–0.26)	Left hippocampal atrophy	Sister died of seizure and liver dysfunction	Definite: 2 major 1 mino
25	<8 years M	Sudden regression with muscle coordination and strength, ataxia	Elevated alanine in plasma AA; normal AC, UOA; normal spinal cerebellar atrophy panel; normal α-galactosidase (Fabry)	Not available	Not available	Normal brain MRI		Possible: 2 minors
26	<5 months F	Episodic left-sided hemiparesis; myoclonus; dysphagia; ataxia; delayed development	Normal Lactate, AA, UOA, AC, CDT, CoQ10; normal array CGH	Normal	Complex IV deficiency: I/III = 35%, I = 50%, II/III = 50%, II = 30%, III = 65%, IV = 18% CS = 112%	Normal brain MRI; scattered lactate peaks in MRS	Adopted	Definite: 2 majors 1 minor

Muscle RCC enzyme reported as % to normal control. One patient (24) reported as ratio to CS. 3-MGA, 3-methylglutaconic; AA, amino acid; AC, acylcarnitine; CDT, carbohydrate deficient transferrin; CGH, comparative genomic hybridization; CoQ10, coenzyme Q10; CSF, cerebrospinal fluid; EM, electron microscopy; EMA, ethylmalonic; GA, glutaric; GERD, gastroesophageal reflux disease; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; PUPY, purine pyrimidine; RCC, respiratory chain complex; SF, skin fibroblast;UOA, urine organic acid; VLCFA, very long chain fatty acid.

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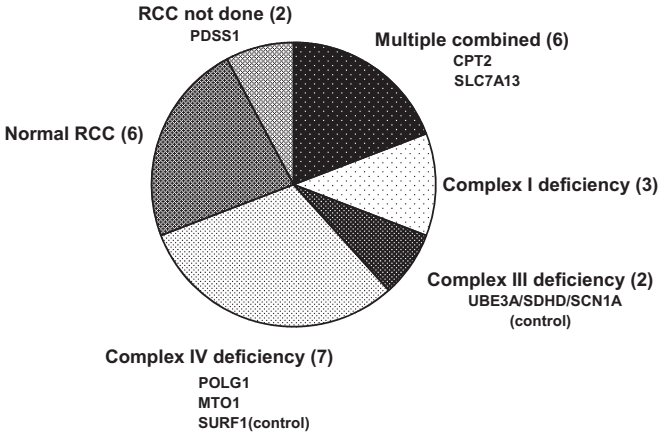

Fig. 1 Subject categories and the genes found mutated. Modified Walker criteria: definite, $n = 17$; probable, $n = 3$; possible, $n = 6$. RCC, respiratory chain complex.

cardiomyopathy. Magnetic resonance imaging (MRI) performed at ages 2 and 4 years was normal. On muscle biopsy, pathology was unrevealing but RCC testing demonstrated complete absent complex IV activity.

On clinical sequencing, no alterations in any of the catalytic or known assembly factors of complex IV were found, but two previously reported mutations in polymerase gamma 1 (*POLG1*) were identified (p.Trp748Ser, p.Arg852Cys; Table 2). They were inherited separately from the parents. These two mutations have been associated with Alpers-Huttenlocher syndrome.[21] In addition, two previously known *POLG1* polymorphisms (p.Gly11Asp; p.Glu1143Gly) were also detected.[56] p.Glu1143Gly was in *cis* with p.Trp748Ser mutation.[57] This ecogenetic single-nucleotide variant can partially rescue *in vitro POLG* activity.[58] The p.Arg852Cys polymorphism was in *cis* with p.Gly11Asp. *POLG1* mutations can be associated with deficiencies of various respiratory chain enzyme complexes in muscle.[59,60]

Patient 2

This young girl presented with developmental delay, nephrotic syndrome, and failure to thrive, and subsequently died at 16 months of age due to renal failure. Brain MRI showed multifocal, near-symmetric patchy regions of white matter-restricted diffusion, and high fluid attenuation inversion recovery (FLAIR) signal within the posterior temporal and occipital periventricular white matter and brainstem. The level of coenzyme Q10 (CoQ10) was significantly reduced at 30 pmol/mg protein in white blood cell (control range, 66–183). Sanger sequencing of *COQ2*, the gene encoding an enzyme that functions in the final steps in the biosynthesis of CoQ10, showed only multiple polymorphisms. We thus used this sample as a reference control and at the same time we searched for mutations responsible for the phenotype. The patient was found to be a compound heterozygote for two novel variants (p.Arg221Term and p.Ser370Arg) in prenyl (decaprenyl) diphosphate synthase, subunit 1 (*PDSS1*; Table 2). The protein encoded by this gene is an enzyme that elongates the prenyl side-chain of CoQ10 and defects in this gene have previously been described to cause CoQ10 deficiency.[61]

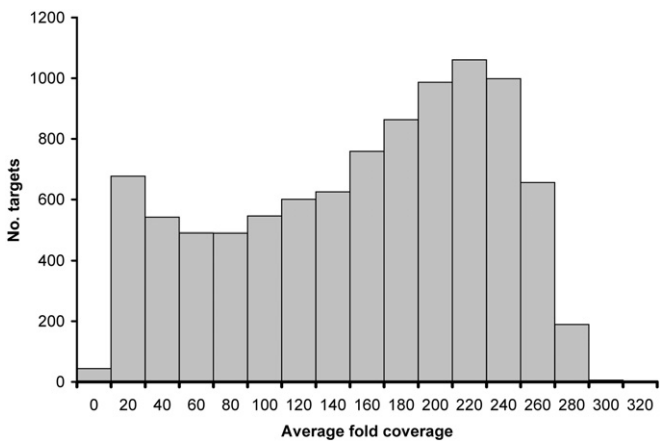

Fig. 2 Targeted exons coverage. Aligned sequences had a quality score ≥30.

Patient 3

This boy presented with poor oral intake and gastroesophageal reflux, hypotonia, and muscle weakness at 4 month of age. Muscle biopsy showed significant reduction of several RCC enzymes with increased citrate synthase activity. Histochemistry showed scattered cytochrome oxidase-negative fibers, lipid droplets in some fibers, and succinate dehydrogenase negative staining. Serum lactate levels had continually been elevated. Urine organic acid analysis in repeated analysis showed mildly elevated excretions of ethylmalonic acid, glutaric acid and dicarbolyxlic acids suggestive of possible mitochondrial dysfunction. The concentrations of C8–C18 acylcarnitine species were moderately elevated, in particular, C18:1 acylcarnitine was prominent. The profile was difficult to interpret but it was thought to reflect mitochondrial dysfunction given multiple enzyme deficiencies in muscle tissue. We found two novel alterations (p.Pro21His and p.Glu33His) in the *CPT2* gene encoding carnitine palmitoyltransferase type 2 (CPTII, Table 2). The CPTII enzyme is part of the mechanism by which long-chain fatty acids are transferred from the cytosol to the mitochondrial matrix to undergo beta-oxidation. In order to determine the functional significance of the novel variants, CPTII enzyme activity was measured in skin fibroblasts and skeletal muscle and found to be reduced in both, confirming CPTII deficiency. Reduced RCC activity and increase in citrate synthase activity have been reported in some patients with CPTII deficiency.[62,63]

Patient 4

This patient was one of two controls used in this study given that several genes were previously sequenced by Sanger as part of diagnostic evaluations. Due to the presence of unexplained clinical presentations, we further analyzed entire genes. This young boy presented with febrile and afebrile seizures at 6 months of age with hypotonia, language delay, delayed milestones and ataxia. Seizures became intractable to multiple medications, but finally responded to ketogenic diet with dramatic improvement in motor skills and language development. Brain MRI was normal and biochemical testing showed mildly elevated lactate and

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Table 2 Selected variants identified in known and candidate genes for mitochondrial disorders

P	Type of variant	Gene	Variant	OMIM/inheritance	Disorder	Prediction†/comments‡
1	Disease-causing variant	*POLG* [NM_002693]	c.2243G>GC p.Trp748Ser dbSNP:113994097 c.2554C>CT p.Arg852Cys dbSNP:144500145	157640/203700/258450/ dominant/recessive	Progressive external ophthalmoplegia; Alpers disease; Ataxia; Seizure	Known mutation[21] MAF unknown Known mutation[21] MAF < 0.0%
2	Disease-causing variant	*PDSS1* [NM_014317]	c.661C>CT p.Arg221Term c.1108A>AC p.Ser370Arg	607426/recessive	CoQ10 synthesis defect	Damaging Probably damaging
	Heterozygote variant	*GLDC* [NM_000170]	c.2380G>GA p.Ala794Thr dbSNP:141933811	605899/recessive	Glycine-encephalopathy	Benign; MAF 0.6%
3	Disease-causing variant	*CPT2* [NM_000098]	c.62C>CA p.Pro21His	608836/recessive	Fatty acid oxidation defect	Benign
		CPT2 [NM_000098]	c.99G>GC p.Glu33His	608836/recessive	Fatty acid oxidation defect	Possibly damaging
	Heterozygote variant	*CAV3* [NM_033337]	c.233C>CT p.Thr78Met dbSNP:72546668	611818 Dominant/recessive 192600 606072/dominant	Dilated cardiomyopathy and limb girdle muscular dystrophy (LGMD)-1C Long QT syndrome 9/hypertrophic cardiomyopathy/ rippling muscle disease	Questionable known mutation[22,23] MAF 0.1%
		ALDH6A1 [NM_005589]	c.755C>CT p.Pro252Leu dbSNP:139579994	603178/recessive	Methylmalonate-semialdehyde-dehydrogenase deficiency	Probably damaging; MAF 0.4%
4	Disease-causing variant	*SCN1A* [NM_001165963]	c.3733C>CT p.Arg1245Term	604233/607208/dominant	Generalized epilepsy with febrile seizures plus, type 2/Dravet syndrome	Reference control sample Known mutation[24]
	Possible disease-causing variants	*UBE3A* [NM_130838]	c.351A>AG p.Arg118Gly	105830/imprinted	Angelman syndrome	Benign
		SDHD [NM_003002.1]	c.149A>AG p.His50Arg dbSNP:11214077	168000/612359/dominant	Paraganglioma Cowden-like syndrome	Known mutation[25,26] MAF 0.9%
	Heterozygote variant	*SLC22A5* [NM_003060]	c.287G>GC p.Gly96Ala	212140/recessive	Carnitine deficiency, systemic primary	Probably damaging

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5	Possible disease-causing variants	*SLC7A13* [NM_138817]	c.520A>AT p.Ile174Phe dbSNP:140320705 c.154G>GA p.Val52Ile dbSNP:139761067		Solute carrier family 7 (anionic amino acid transporter), member 13; candidate gene	Probably damaging; Present in mother; MAF 0.1% Benign; Present in father; MAF < 0.0%
	Likely non-disease causing variant	*KIF1B* [NM_183416]	c.3017A>AG p.Glu1006Gly dbSNP:148481786	118210/dominant	Charcot-Marie-Tooth 2A1	Benign: Present in asymptomatic mother; MAF 0.5%
	Heterozygote variant	*VARS2* [NM_020442]	c.104A>AG p.His35Arg c.1510C>CT p.Pro504Ser		Candidate gene: Mitochondrial valyl-tRNA synthetase	Possibly damaging; Present in mother Probably damaging; Present in mother
		HMGCS2 [NM_005518]	c.595G>GA p.Val199Ile dbSNP:150140061	605911/recessive	Mitochondrial HMG-CoA synthase deficiency	Possibly damaging; MAF < 0.0%
6	Possible disease-causing variant	*MTO1* [NM_133645]	c.176G>GC p.Gly59Ala c.922A>AG p.Thr308Ala dbSNP:145043138		Mitochondrial translation optimization 1, candidate gene	Probably damaging; Present in mother Possibly damaging; Present in father; MAF 0.3%
	Variants of unknown significance	*PDX1* [NM_000209]	c.694G>GA p.Gly232Ser c.697G>GT p.Glu233Term	260370/ 222100/ 125850/recessive	Agenesis of the pancreas/diabetes mellitus	Possibly damaging; Present in father Possibly damaging; Present in father
	Heterozygote variant	*TK2* [NM_004614]	c.220C>CT p.Arg74Trp	609560/recessive	Mitochondrial DNA depletion	Possibly damaging
		GCDH [NM_000159]	c.1204C>CT p.Arg402Trp dbSNP:121434369	231670/recessive	Glutaric acidemia I	Known mutation[27] MAF unknown
		TYMP [NM_001953]	c.1393G>GA p.Ala465Thr dbSNP:112723255	603041/recessive	Mitochondrial DNA depletion syndrome 1	Questionable known mutation[28] MAF 3.0%
7	Likely non-disease causing variant	*FASTKD2* [NM_014929]	c.29G>GC p.Ser10Thr dbSNP:147727753 c.149A>AG p.Lys50Arg dbSNP:141447598	220110/recessive	Complex IV deficiency	Possibly damaging; Present in mother and a healthy sibling MAF 0.4% Benign; Present in father and a healthy sibling MAF 0.2%
	Heterozygote variant	*AFG3L2* [NM_006796]	c.2314C>CT p.Leu772Phe dbSNP:117182113	610246/dominant	Spinocerebellar ataxia-28	Present in mother; MAF 0.3%
		KIF1B [NM_183416]	c.3092T>TC p.Ile1031Thr dbSNP:149267056	118210/dominant	Charcot-Marie-Tooth-disease-type-2A1	Benign; Present in father; MAF 0.4%

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Table 2 *Continued*

P	Type of variant	Gene	Variant	OMIM/inheritance	Disorder	Prediction[†]/comments[‡]
8	Heterozygote variant	*MFN2* [NM_014874.2]	c.2113G>GA p.Val705Ile dbSNP: 142271930	118210/608507 dominant	Charcot-Marie-Tooth 2A2	Questionable known mutation;[29–31] Present in asymptomatic mother; MAF 0.4%
		NDUFS8 [NM_002496]	c.484G>GA p.Val162Met	256000/recessive	Leigh syndrome	Probably damaging
		CLN8 [NM_018941]	c.200C>CT p.Ala67Val	600143/61003/recessive	Neuronal ceroid lipofuscinosis type 8	Probably damaging
9	Heterozygote variant	*MFN2* [NM_014874.2]	c.2113G>GA p.Val705Ile dbSNP: 142271930	118210/608507 dominant	Charcot-Marie-Tooth 2A1	Questionable known mutation:[29–31] Present in asymptomatic mother; MAF 0.4%
		ACAD8 [NM_014384]	c.512C>CG p.Ser171Cys dbSNP:113488591	611283/recessive	Isobutyryl-CoA dehydrogenase deficiency	Known mutation[32] MAF 0.8%
		ACADM [NM_000016]	c.127G>GA p.Glu43Lys dbSNP:147559466	201450 /recessive	Medium-chain acyl-CoA dehydrogenase deficiency	Known mutation[33] MAF 0.2%
10	Heterozygote variant	*MAPT* [NM_016835]	c.671T>TG p.Val224Gly dbSNP:141120474	601104/260540/600274/ dominant	Supranuclear palsy progressive type 1 Parkinson-dementia syndrome Frontotemporal dementia	Benign. Likely non-disease causing variant. MAF 0.3%
		CLN8 [NM_018941]	c.685C>CG p.Pro229Ala dbSNP:150047904	600143/61003/recessive	Neuronal ceroid lipofuscinosis type 8	Possibly damaging; MAF 0.1%
		NPC2 [NM_006432]	c.88G>GA p.Val30Met dbSNP:151220873	607625/recessive	Niemann-Pick disease type C2	Known mutation[34] MAF 0.1%
		TYMP [NM_001953]	c.1393G>GA p.Ala465Thr dbSNP:112723255	603041/recessive	Mitochondrial DNA depletion syndrome 1	Questionable known mutation[28] MAF 3.0%
		CAPN3 [NM_000070]	c.706G>GA p.Ala236Thr dbSNP: 1801449	253600/recessive	Limb-girdle muscular dystrophy-2A	Questionable known mutation[35] MAF 21.8%
		SLC22A5 [NM_003060]	c.1463G>GA p.Arg488His dbSNP:28383481	212140/recessive	Carnitine deficiency	Known mutation[36] MAF 0.4%

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11	Disease-causing variant	*SURF1* [NM_003172]:	c.845_846delCT/het p.Ser282Cys-fs-Term9	256000/recessive	Leigh syndrome	Reference control sample Known mutation[37]
			c.312_321delTCTGC CAGCCinsAT/het p.Leu105Term Known mutation[37]	256000/recessive		Detected as deletion of 9 with NGS
	Heterozygote variant	*SUOX* [NM_000456]	c.1A>AG p.Met1Val	272300/recessive	Sulfocysteinuria	Benign
		XDH [NM_000379]	c.3736C>CT p.Arg1246Cys dbSNP:142329784	252150/recessive	Molybdenum cofactor deficiency	Probably damaging MAF < 0.0%
12	Heterozygote variant	*ACADVL* [NM_000018]	c.194C>CT p.Pro65Leu dbSNP:28934585	201475/recessive	Acyl-CoA dehydrogenase, very long-chain, deficiency	Questionable known mutation[38] MAF 3.1%
		ATP7B [NM_000053]	c.4301C>CT p.Thr1434Met dbSNP:60986317	277900/recessive	Wilson Disease	Questionable known mutation[39] MAF 0.4-2.0%
		GLDC [NM_000170]	c.2203G>GT p.Val735Leu dbSNP:143119940	605899/recessive	Glycine encephalopathy	Possibly damaging; MAF 0.3%
		FH [NM_000143]	c.1431_1433dupAAA	606812/recessive	Fumarase deficiency	Known mutation[40]
13	Likely non-disease causing variant	*SLC12A3* [NM_000339]	c.334G>GT p.Glu112Term c.2782C>CT p.Arg928Cys dbSNP:12708965	263800/recessive	Gitelman syndrome	Novel mutation; Inherited from mother Questionable known mutation[41] Present in father; No clinical signs in the patient; MAF 3.7%
	Heterozygote variant	*ACADVL* [NM_000018]	c.865G>GA p.Gly289Arg	201475/recessive	Acyl-CoA dehydrogenase, very long-chain, deficiency	Known mutation[38]
		TYMP [NM_001953]	c.1393G>GA p.Ala465Thr dbSNP:112723255	603041/recessive	Mitochondrial DNA depletion syndrome 1	Questionable known mutation[28] MAF 3.0%
	X-linked Heterozygote	*AIFM1* [NM_004208]	c.892G>GA p.Arg298Trp	300816/X-linked	Combined oxidative phosphorylation deficiency-6	Possibly damaging; Female carrier; No skewed X-chromosome inactivation observed
14	Heterozygote variant	*GLDC* [NM_000170]	c.319A>AG p.Met107Val dbSNP:138454333	605899/recessive	Glycine-encephalopathy	Benign; MAF 0.1%
		TXNRD2 [NM_006440]	c.1150C>CT p.Gly384Ser	recessive	Dilated cardiomyopathy[42]	Benign
		TYMP [NM_001953]	c.929-6_929-3delCCGC	603041/recessive	Mitochondrial DNA depletion syndrome 1	Known mutation[43]

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Table 2 *Continued*

P	Type of variant	Gene	Variant	OMIM/inheritance	Disorder	Prediction†/comments‡
15	Heterozygote variant	*YARS2* [NM_001040436]	c.535A>AC p.Lys179Gln dbSNP:147630375	613561/recessive	myopathy, lactic acidosis, and sideroblastic anemia-2	Benign; MAF < 0.0%
		ELOVL4 [NM_022726]	c.814G>GC p.Glu272Gln dbSNP:148919174	600110/dominant	Stargardt disease 3	Benign. Not present in affected brother; No clinical signs; MAF 0.9%
		LRPPRC [NM_133259]	c.4078G>GA p.Ala1360Thr dbSNP:147302249	220111/recessive	French-Canadian type of Leigh syndrome	Probably damaging; MAF unknown
		SPAST [NM_014946]	c.863C>CT p.Thr288Ile	182601/dominant	Spastic paraplegia, type 4	Benign; Not present in affected brother
	X-linked, Hemizygote	*PDHA1* [NM_000284]	c.844A>C p.Met282Leu dbSNP:2229137	312170/X-linked	Pyruvate decarboxylase deficiency	Questionable known mutation[44] MAF 4.3%
16	Heterozygote variant	*GFM1* [NM_024996]	c.1343A>AG p.Asp448Gly dbSNP:146951325	609060/recessive	Combined oxidative phosphorylation deficiency-1	Benign; MAF 0.1%
		HADHA [NM_000182]	c.1072C>CA p.Gln358Lys dbSNP:2229420	609015/recessive	Mitochondrial trifunctional protein deficiency	Questionable known mutation;[45] Seen in control samples[13] MAF 2.5%
17	Heterozygote variant/ Likely non-disease causing	*DMPK* [NM_004409]	c.1631C>CT p.Thr544Met dbSNP:146680240	160900/dominant	Myotonic dystrophy 1	Benign; MAF 0.9%
18	Heterozygote variant	*HADHA* [NM_000182]	c.1528C>CG p.Glu510Gln	609015/recessive	Mitochondrial trifunctional protein deficiency	Known mutation[46]
		MOCS1 [NM_005943]	c.853G>GA p.Glu285Lys dbSNP:140243105	252150/recessive	Molybdenum cofactor deficiency	Benign; MAF < 0.0%
19	Heterozygote variant	*HFE* [NM_000410]	c.502G>GC p.Glu168Gln dbSNP:146519482	235200/recessive	Neonatal hemochromatosis	Known mutation[47] MAF < 0.0%
		TUFM [NM_003321]	c.622G>GA p.Glu208Lys dbSNP:143189885	610678/recessive	Combined oxidative phosphorylation deficiency-4	Benign MAF < 0.0%
20	Heterozygote variant	*TYMP* [NM_001953]	c.1393G>GA p.Ala465Thr dbSNP:112723255	603041/recessive	Mitochondrial DNA depletion syndrome 1	Questionable known mutation[28] MAF 3.0%
		AGXT [NM_000030]	c.1020A>AG p.Ile340Met dbSNP: 4426527	259900/recessive	Primary-hyperoxaluria-type-I	Questionable known mutation[48] MAF 13.7%
		CPT1A [NM_001876]	c.823G>GA p.Ala275Thr dbSNP:2229738	255120/recessive	Carnitine palmitoyltransferase deficiency I	Questionable known mutation[49] Seen in control samples[13] MAF 3.5%

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21	Heterozygote variant	*PUS1* [NM_025215]	c.401T>TC p.Met134Thr	600462/recessive	Mitochondrial myopathy and sideroblastic anemia (MLASA)	Possibly damaging
		DGUOK [NM_080916]	c.509A>AG p.Gln170Arg dbSNP:74874677	251880/recessive	mtDNA depletion syndrome, hepatocerebral form	Questionable known mutation[50] Seen in control samples[13] MAF 1.23%
		CPT1A [NM_001876]	c.823 G>GA p.Ala275Thr dbSNP:2229738	255120/recessive	Carnitine palmitoyltransferase deficiency I	Questionable known mutation[49] Seen in control samples[13] MAF 3.5%
22	Heterozygote variant	*TFR2* [NM_003227]	c.1403G>GA p.Arg468His dbSNP:80338885	604250/recessive	Hemochromatosis type 3	Known mutation[51] MAF 0.1%
		PINK1 [NM_032409]	c.344A>AT p.Gln115Leu dbSNP:148871409	605909/recessive	Early onset Parkinson disease 6	MAF not available
23	Heterozygote variant	*MCCC1* [NM_020166]	c.1782C>CG p.Asp594Glu	210200/recessive	3-Methylcrotonyl-CoA-carboxylase-1-deficiency	Benign
24	Heterozygote variant	*FOXRED1* [NM_017547]	c.857T>TC p.Ile286Thr dbSNP:148955548	252010/recessive	Complex I deficiency	Benign MAF not available
		HAX1 [NM_006118]	c.626A>AG p.Lys209Arg	610738/recessive	Severe congenital neutropenia 3	
25	Heterozygote variant	*BCKDHA* [NM_000709]	c.452C>CT p.Thr151Met dbSNP:34442879	248600/recessive	Maple syrup urine disease, type Ia	Known mutation[52] MAF 0.5%
26	Heterozygote variant	*PARK2* [NM_004562]	c.1310C>CT p.Pro437Leu dbSNP:149953814	600116/recessive	Juvenile Parkinson disease 2	Known mutation[53,54] MAF 0.2%
		NUBPL [NM_025152]	c.815-27T>TC	252010/recessive	Complex I deficiency	Known mutation[15,55] MAF 0.4%
		ACAD8 [NM_014384]	c.512C>CG p.Ser171Cys dbSNP:113488591	611283/recessive	Isobutyryl-CoA dehydrogenase deficiency	Known mutation[32] MAF 0.8%

[†]Prediction by Polyphen2-HumVar model (no prediction is available for stop mutations). [‡]MAF (minor allele frequency) obtained from dbSNP (1000Genome phase 1 genotype data or other population listed at http://www.ncbi.nlm.nih.gov/snp).

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alanine. Muscle biopsy was suggestive of complex III deficiency but electron microscopy and histochemical findings were normal. Sanger sequencing of *SCN1A* showed a known pathological nonsense mutation.[24] Because the clinical features of severe myoclonic epilepsy resemble those of mitochondrial diseases, we included *SCN1A* in the list of genes to be sequenced and we confirmed the mutation in this sample. We searched for additional variants that may explain the change in RCC activity but we did not find mutations in the subunits of complex III or known assembly factors. In addition to the mutation in the *SCN1A* gene, we detected a known pathogenic variant (p.His50Arg) in succinate dehydrogenase complex, subunit D (*SDHD*) that has been found in patients affected by pheochromocytoma[25] and Cowden-like syndrome.[26] The patient also carried a novel variant (p.Arg118Gly) in the gene encoding ubiquitin-protein ligase E3A (*UBE3A*) – known to cause Angelman syndrome. Reduced activity of respiratory complex III has been recently described in a UBE3A-deficient mouse model.[64] These findings demonstrate the need for future screening and treatment for complications related to the potential development of Angelman syndrome and SDHD-related tumors.

Patient 5

This patient developed intractable seizures at 23 days of life with hypotonia. He had delayed global development and had been diagnosed with failure to thrive, requiring gastrointestinal tube nutrition. Brain MRI was abnormal with diminished white matter with prominence of ventricles. Muscle enzyme analysis produced discrepant results at two national mitochondrial RCC testing centers, with one center showing moderately reduced complex I/III (17.1%) and IV (36%) while the other center reported complete normal results on the same specimen. Muscle pathology was unrevealing. We found that the patient was a compound heterozygote for two novel missense variants (p.Ile174Phe and p.Val52Ile) in the gene for solute carrier family 7 (sodium-independent aspartate/glutamate transporter) member 13 (*SLC7A13*) and confirmed on parental samples. Considering that another glutamate transporter (*SLC25A22*) has been recently reported responsible for myoclonic seizures,[65] we suspect that the alterations in this gene could be responsible for the clinical symptoms. Both variants are in the dbSNP database but are present in the population at a very low frequency (Table 2).

Patient 6

The patient developed normally until the age of 3 months, when he had his first seizure. Seizures rapidly developed into infantile spasms, which did not respond to treatment. His motor and cognitive development was significantly affected. He had cortical visual impairment on examination. His lower extremities were hypertonic (scissoring lower extremities) while his axial structures remained hypotonic. He also had choriform athetoid movements. Muscle biopsy showed that complex IV was deficient with normal CS activity. On muscle pathology, no cytochrome c oxidase-negative fibers were seen. Electron microscopy of muscle tissue showed increased numbers of lipid droplets, and glycogen content was unremarkable. Some of the mitochondria were enlarged. Brain MRI was normal. Genetic sequencing of *SURF1* and *POLG1* gene were negative.

No significant alterations were found in the gene encoding RCC catalytic units or known assembly factors, but the patient was found to be a compound heterozygote for two novel variants in the gene for mitochondrial translation optimization 1 (*MTO1*), confirmed to be *in trans* on parental samples (Table 2). This protein is involved in mitochondrial tRNA modification, and mutations in *MTO1* cause respiratory deficiency and impaired mitochondrial RNA metabolism in *Saccharomyces cerevisiae*.[66,67] This patient's variants occur in highly conserved residues and are predicted to be damaging. A dramatic impairment on mitochondrial protein translation would explain the histopathological and biochemical findings.

Misannotated mutations

In several samples, we found variants that have previously been annotated as pathogenic mutations, but the current patients did not present with symptoms attributed to those mutations. This study underscores the fact that many variants may have been misannotated in the literature and that NGS is now bringing this issue to light.[13] For instance, a known pathogenic variant, p.Val705Ile, in mitofusin 2 (*MFN2*) gene was found in two patients.[29–31] Mutations in this protein, which participates in mitochondrial fusion, cause two disorders of the peripheral nervous system: Charcot-Marie-Tooth disease type 2A2 (CMT2A2), and hereditary motor and sensory neuropathy VI. The current patients, however, did not present with peripheral neuropathy and this same variant was also detected in multiple asymptomatic family members.

Another previously reported mutation, p.Met282Leu (dbSNP:2229137) in *PDHA1*, was found in two male siblings, but these patients did not show any symptoms of pyruvate dehydrogenase complex enzyme deficiency. Additionally, their mother was of Asian heritage and this variant has been a frequently observed polymorphism in the Asian population

Another known pathogenic variant, p.Thr78Met in caveolin 3 (*CAV3*), the muscle-specific form of the caveolin protein family, was detected in one patient. This mutation has been reported in patients with recessive dilated cardiomyopathy and limb girdle muscular dystrophy (LGMD)-1C[22] and in heterozygote patients affected by long-QT syndrome.[23] The present patient, however, had a normal electrocardiogram and echocardiogram and no evidence of cardiomyopathy.

We detected several other variants that appear to be questionable mutations because they were previously reported as pathogenic in the literature (some listed in Table 2). Based on the high allelic frequency of those variants in the population available in the dbSNP, we concluded that these alterations are most likely benign polymorphisms.[13]

Discussion

The NGS technology has already had considerable impact on basic research and is now quickly being translated into clinical practice. There are now several clinical genetic tests available from commercial labs using NGS technology. The use of targeted

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gene panels allows a more simplified analysis and interpretation as compared to whole exome sequencing. Due to the complexity of data analysis and interpretation, and, most of all, the stringent regulations required for clinical testing and ethics considerations, whole exome sequencing may not be readily applicable to clinical testing in the very near future.

In this study, we investigated 26 patients with either confirmed or suspected mitochondrial disorders. Due to the genetic heterogeneity in mitochondrial disorders, we focused on those patients with mitochondrial RCC deficiencies to determine if they were primarily caused by nuclear defects in mitochondrial RCC catalytic units or assembly factors. Our assumption was that many of these patients with multiple RCC deficiencies likely had assembly factor or modulator defects, given the hypothesized unknown numbers of assembly or modulator factors. In the present patient cohort, 24 of them had muscle biopsy and RCC assay and 18 of them had various RCC deficiencies. None of the patients with multiple RCC deficiencies had structural RCC defects on sequencing. This is somewhat surprising but gives credence to possible other factors contributing to RCC dysfunction other than structural gene mutations. Indeed, we found several alterations in other well-known genes including *POLG1, PDSS1, CPTII, SDHD and UBE3A*. The alterations in candidate genes, *SLC7A13* and *MTO1* are highly suspicious given that other studies have shown pathogenicity in genes with a similar function. Additionally, their clinical symptoms appear consistent with the present findings but at this point we do not have a functional validation of their pathology. The RCC enzyme deficiencies in the present patients may be mostly secondary to molecular defects affecting mitochondrial function, rather than being caused by mutations in RCC subunits or assembly factors. It is also possible that the culprit genes were not included in the present study, given that more assembly factors for complex I are expected to be discovered in the next several years.[68] In contrast, we do not know whether the RCC enzyme deficiencies detected by *in vitro* assays are significant enough to cause clinical problems or if they may be a result of assay artifact of interference.[3]

Overall, the present results are in agreement with a recent study on a group of patients with complex I deficiency in which a molecular diagnosis could be reached in only 22% of the cases by sequencing 103 genes (81 of them nuclear).[15] Together with the present results, this indicates that the clinical spectrum of mitochondrial disease could be much broader than previously thought. It also means that there is a very high chance of misdiagnosis and inappropriate treatment if testing is limited to RCC enzyme assay or focused sequencing of RCC subunits and assembly factors.

We observed multiple protein coding variants in each individual, some of which could potentially impact on disease. These oligogenic alterations appear to be a major challenge in interpretation. These various combinations of mutations may not be uncommon but could individually or collectively lead to an exceedingly complex clinical pattern as highlighted by the patient who was found to have alterations in multiple genes, *SCN1A*, *UBE3A* and *SDHD*. Ecogenetic single nucleotide variants (ENSV) are an interesting concept that has been highlighted by mutations in *POLG*.[69] These ENSV may be responsible for some complex clinical findings and therefore continued study is needed in some of the questionable variants. Nevertheless, pathogenic effects of the detected mutations, especially missense mutations, should be functionally validated in the future and highlight the importance of the need for developing high-throughput model systems.

One interesting finding related to a known *MFN2* dominant mutation identified in two patients: one patient with a normal RCC and the other with low complex IV enzyme activity. Given the absence of relevant symptoms and family studies, this *MFN2* alteration most likely is a non-significant variant possibly misannotated in the literature as pathogenic. This finding underscores the need to carefully re-evaluate human mutation databases.[13] Two novel heterozygote variants in kinesin family member 1B (*KIF1B*), a gene involved in mitochondria transport and associated with dominant CMT2A1, were found in two patients. In both cases, one asymptomatic parent also carried the same variant, making these variants less significant although incomplete penetrance cannot be entirely excluded. A few other reported mutations were found but not thought to be significant as listed in Table 2.

The current standard diagnostic approach for suspected mitochondrial patients often requires an invasive procedure – a muscle biopsy – for histopathology, electron microscopy and RCC enzyme analysis. Based on the clinical judgment, biochemical abnormalities, and muscle biopsy, many of these patients are then treated with mitochondrial vitamin cocktails, which include high dose of antioxidants (vitamin E and C), α-lipoic acid, CoQ10, creatine, and L-carnitine. Clinical trials have been difficult to design and implement due to the inherent genetic variability of patients labeled as having 'mitochondrial disease' and the large patient numbers required for statistical significance. As a result, the benefits of these treatments are often unclear or inconsistent.[70] Considering the present data, treatments may have limited or no benefit in some patients with secondary RCC deficiency. Conversely, knowing the specific molecular defects involved will facilitate the development of appropriate therapeutic interventions and improve efficacy and cost-effectiveness.

The present results were comparable with other NGS studies showing high analytical sensitivity.[13] The precision was acceptable with high accuracy in inter- and intra-assay comparison. The depth of coverage was appropriate for most of the target bases although approximately 8% of targets did not pass the quality indicator of 20 reads and Q ≥30 (where Q is the base quality capped by the read mapping quality assigned by the GATK's UnifiedGenotyper). The major problem was the identification of a complex insertion deletion in a *SURF1*-positive control sample, reflecting current limitations in the alignment and variant detection tools for indels. Nevertheless, the deletion was partially identified and thus it would have guided follow-up confirmation on Sanger sequencing.

In summary, despite the limitations in discovering the mutations in all patients examined, a targeted NGS approach is likely to be the only solution for many mitochondrial disorders with

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different and various genetic etiologies. The nuclear mutations in RCC catalytic units or assembly factors may be not as common as previously suspected in patients with mitochondrial RCC deficiency. This study demonstrates that the clinical spectrum of mitochondrial disease is much broader than is currently thought and therefore many patients remain undiagnosed and may not be receiving proper treatment. Technical advancements will continue to drive down the cost of NGS and will help reduce the need for invasive muscle biopsies and determine the appropriate treatment in many patients.

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References

1 Elliott H, Samuels D, Eden J, Relton C, Chinnery P. Pathogenic mitochondrial DNA mutations are common in the general population. *Am. J. Hum. Genet.* 2008; **83**: 254–60.
2 Gellerich FN, Mayr JA, Reuter S, Sperl W, Zierz S. The problem of interlab variation in methods for mitochondrial disease diagnosis: Enzymatic measurement of respiratory chain complexes. *Mitochondrion* 2004; **4**: 427–39.
3 Chen X, Thorburn DR, Wong LJ *et al*. Quality improvement of mitochondrial respiratory chain complex enzyme assays using Caenorhabditis elegans. *Genet. Med.* 2011; **13**: 794–9.
4 Bernier FP, Boneh A, Dennett X, Chow CW, Cleary MA, Thorburn DR. Diagnostic criteria for respiratory chain disorders in adults and children. *Neurology* 2002; **59**: 1406–11.
5 Oglesbee D, Freedenberg D, Kramer KA, Anderson BD, Hahn SH. Normal muscle respiratory chain enzymes can complicate mitochondrial disease diagnosis. *Pediatr. Neurol.* 2006; **35**: 289–92.
6 Scharfe C, Lu H, Neuenburg J *et al*. Mapping gene associations in human mitochondria using clinical disease phenotypes. *PLoS Comput. Biol.* 2009; **5**: e1000374.
7 Calvo SE, Mootha VK. The mitochondrial proteome and human disease. *Annu. Rev. Genomics Hum. Genet.* 2010; **11**: 25–44.
8 Biesecker LG, Mullikin JC, Facio FM *et al*. The ClinSeq project: Piloting large-scale genome sequencing for research in genomic medicine. *Genome Res.* 2009; **19**: 1665–74.
9 Ng SB, Nickerson DA, Bamshad MJ, Shendure J. Massively parallel sequencing and rare disease. *Hum. Mol. Genet.* 2010; **19**: R119–24.
10 Tsurusaki Y, Osaka H, Hamanoue H *et al*. Rapid detection of a mutation causing X-linked leucoencephalopathy by exome sequencing. *J. Med. Genet.* 2011; **48**: 606–9.
11 Meder B, Haas J, Keller A *et al*. Targeted next-generation sequencing for the molecular genetic diagnostics of cardiomyopathies. *Circ. Cardiovasc. Genet.* 2011; **4**: 110–122.
12 Amstutz U, Andrey-Zurcher G, Suciu D, Jaggi R, Haberle J, Largiader CR. Sequence capture and next-generation resequencing of multiple tagged nucleic acid samples for mutation screening of urea cycle disorders. *Clin. Chem.* 2011; **57**: 102–11.
13 Bell CJ, Dinwiddie DL, Miller NA *et al*. Carrier testing for severe childhood recessive diseases by next-generation sequencing. *Sci. Transl. Med.* 2011; **3**: 65ra64.
14 Vasta V, Ng S, Turner E, Shendure J, Hahn S. Next generation sequence analysis for mitochondrial disorders. *Genome Med.* 2009; **1**: 100–110.
15 Calvo SE, Tucker EJ, Compton AG *et al*. High-throughput, pooled sequencing identifies mutations in NUBPL and FOXRED1 in human complex I deficiency. *Nat. Genet.* 2010; **42**: 851–8.
16 Wang W, Shen P, Thiyagarajan S *et al*. Identification of rare DNA variants in mitochondrial disorders with improved array-based sequencing. *Nucleic Acids Res.* 2011; **39**: 44–58.
17 Musunuru K, Pirruccello JP, Do R *et al*. Exome sequencing, ANGPTL3 mutations, and familial combined hypolipidemia. *N. Engl. J. Med.* 2010; **363**: 2220–27.
18 Albers CA, Lunter G, MacArthur DG, McVean G, Ouwehand WH, Durbin R. Dindel: Accurate indel calls from short-read data. *Genome Res.* 2011; **21**: 961–73.
19 Stenson P, Mort M, Ball E *et al*. The human gene mutation database: 2008 update. *Genome Med.* 2009; **1**: 13.
20 Adzhubei IA, Schmidt S, Peshkin L *et al*. A method and server for predicting damaging missense mutations. *Nat. Methods* 2010; **7**: 248–9.
21 Ashley N, O'Rourke A, Smith C *et al*. Depletion of mitochondrial DNA in fibroblast cultures from patients with POLG1 mutations is a consequence of catalytic mutations. *Hum. Mol. Genet.* 2008; **17**: 2496–506.
22 Traverso M, Gazzerro E, Assereto S *et al*. Caveolin-3 T78M and T78K missense mutations lead to different phenotypes in vivo and in vitro. *Lab. Invest.* 2008; **88**: 275–83.
23 Vatta M, Ackerman MJ, Ye B *et al*. Mutant caveolin-3 induces persistent late sodium current and is associated with long-QT syndrome. *Circulation* 2006; **114**: 2104–12.
24 Lossin C. A catalog of SCN1A variants. *Brain Dev.* 2009; **31**: 114–30.
25 Perren A, Barghorn A, Schmid S *et al*. Absence of somatic SDHD mutations in sporadic neuroendocrine tumors and detection of two germline variants in paraganglioma patients. *Oncogene* 2002; **21**: 7605–8.
26 Ni Y, Zbuk KM, Sadler T *et al*. Germline mutations and variants in the succinate dehydrogenase genes in Cowden and Cowden-like syndromes. *Am. J. Hum. Genet.* 2008; **83**: 261–8.
27 Biery BJ, Stein DE, Morton DH, Goodman SI. Gene structure and mutations of glutaryl-coenzyme A dehydrogenase: Impaired association of enzyme subunits that is due to an A421V substitution causes glutaric acidemia type I in the Amish. *Am. J. Hum. Genet.* 1996; **59**: 1006–11.
28 Kocaefe YC, Erdem S, Ozguc M, Tan E. Four novel thymidine phosphorylase gene mutations in mitochondrial neurogastrointestinal encephalomyopathy syndrome (MNGIE) patients. *Eur. J. Hum. Genet.* 2003; **11**: 102–4.
29 McCorquodale DS 3rd, Montenegro G, Peguero A *et al*. Mutation screening of mitofusin 2 in Charcot-Marie-Tooth disease type 2. *J. Neurol.* 2011; **258**: 1234–9.
30 Engelfried K, Vorgerd M, Hagedorn M *et al*. Charcot-Marie-Tooth neuropathy type 2A: Novel mutations in the mitofusin 2 gene (MFN2). *BMC Med. Genet.* 2006; **7**: 53.
31 Braathen GJ, Sand JC, Lobato A, Hoyer H, Russell MB. MFN2 point mutations occur in 3.4% of Charcot-Marie-Tooth families. An investigation of 232 Norwegian CMT families. *BMC Med. Genet.* 2010; **11**: 48.
32 Oglesbee D, He M, Majumder N *et al*. Development of a newborn screening follow-up algorithm for the diagnosis of isobutyryl-CoA dehydrogenase deficiency. *Genet. Med.* 2007; **9**: 108–16.
33 McKinney JT, Longo N, Hahn SH *et al*. Rapid, comprehensive screening of the human medium chain acyl-CoA dehydrogenase gene. *Mol. Genet. Metab.* 2004; **82**: 112–20.
34 Park WD, O'Brien JF, Lundquist PA *et al*. Identification of 58 novel mutations in Niemann-Pick disease type C: Correlation with biochemical phenotype and importance of PTC1-like domains in NPC1. *Hum. Mutat.* 2003; **22**: 313–25.
35 Bennett RR, Schneider HE, Estrella E *et al*. Automated DNA mutation detection using universal conditions direct sequencing:

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Application to ten muscular dystrophy genes. *BMC Genet.* 2009; **10**: 66.
36 Schimmenti LA, Crombez EA, Schwahn BC *et al*. Expanded newborn screening identifies maternal primary carnitine deficiency. *Mol. Genet. Metab.* 2007; **90**: 441–5.
37 Tiranti V, Hoertnagel K, Carrozzo R *et al*. Mutations of SURF-1 in Leigh disease associated with cytochrome c oxidase deficiency. *Am. J. Hum. Genet.* 1998; **63**: 1609–21.
38 Spiekerkoetter U, Sun B, Zytkovicz T, Wanders R, Strauss AW, Wendel U. MS/MS-based newborn and family screening detects asymptomatic patients with very-long-chain acyl-CoA dehydrogenase deficiency. *J. Pediatr.* 2003; **143**: 335–42.
39 Loudianos G, Dessi V, Lovicu M *et al*. Mutation analysis in patients of Mediterranean descent with Wilson disease: Identification of 19 novel mutations. *J. Med. Genet.* 1999; **36**: 833–6.
40 Coughlin EM, Christensen E, Kunz PL *et al*. Molecular analysis and prenatal diagnosis of human fumarase deficiency. *Mol. Genet. Metab.* 1998; **63**: 254–62.
41 Cruz DN, Shaer AJ, Bia MJ, Lifton RP, Simon DB. Gitelman's syndrome revisited: An evaluation of symptoms and health-related quality of life. *Kidney Int.* 2001; **59**: 710–17.
42 Sibbing D, Pfeufer A, Perisic T *et al*. Mutations in the mitochondrial thioredoxin reductase gene TXNRD2 cause dilated cardiomyopathy. *Eur. Heart J.* 2011; **32**: 1121–33.
43 Nishino I, Spinazzola A, Papadimitriou A *et al*. Mitochondrial neurogastrointestinal encephalomyopathy: An autosomal recessive disorder due to thymidine phosphorylase mutations. *Ann. Neurol.* 2000; **47**: 792–800.
44 Matsuda J, Ito M, Naito E, Yokota I, Kuroda Y. DNA diagnosis of pyruvate dehydrogenase deficiency in female patients with congenital lactic acidaemia. *J. Inherit. Metab. Dis.* 1995; **18**: 534–46.
45 Blish KR, Ibdah JA. Maternal heterozygosity for a mitochondrial trifunctional protein mutation as a cause for liver disease in pregnancy. *Med. Hypotheses* 2005; **64**: 96–100.
46 Ijlst L, Wanders RJ, Ushikubo S, Kamijo T, Hashimoto T. Molecular basis of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency: Identification of the major disease-causing mutation in the alpha-subunit of the mitochondrial trifunctional protein. *Biochim. Biophys. Acta* 1994; **1215**: 347–50.
47 Oberkanins C, Moritz A, de Villiers JN, Kotze MJ, Kury F. A reverse-hybridization assay for the rapid and simultaneous detection of nine HFE gene mutations. *Genet. Test.* 2000; **4**: 121–4.
48 Purdue PE, Takada Y, Danpure CJ. Identification of mutations associated with peroxisome-to-mitochondrion mistargeting of alanine/glyoxylate aminotransferase in primary hyperoxaluria type 1. *J. Cell Biol.* 1990; **111**: 2341–51.
49 Gobin S, Thuillier L, Jogl G *et al*. Functional and structural basis of carnitine palmitoyltransferase 1A deficiency. *J. Biol. Chem.* 2003; **278**: 50 428–34.
50 Freisinger P, Futterer N, Lankes E *et al*. Hepatocerebral mitochondrial DNA depletion syndrome caused by deoxyguanosine kinase (DGUOK) mutations. *Arch. Neurol.* 2006; **63**: 1129–34.
51 Hsiao PJ, Tsai KB, Shin SJ *et al*. A novel mutation of transferrin receptor 2 in a Taiwanese woman with type 3 hemochromatosis. *J. Hepatol.* 2007; **47**: 303–6.
52 Dursun A, Henneke M, Ozgul K *et al*. Maple syrup urine disease: Mutation analysis in Turkish patients. *J. Inherit. Metab. Dis.* 2002; **25**: 89–97.
53 Foroud T, Uniacke SK, Liu L *et al*. Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. *Neurology* 2003; **60**: 796–801.
54 Kay DM, Moran D, Moses L *et al*. Heterozygous parkin point mutations are as common in control subjects as in Parkinson's patients. *Ann. Neurol.* 2007; **61**: 47–54.
55 Tucker EJ, Mimaki M, Compton AG, McKenzie M, Ryan MT, Thorburn DR. Next-generation sequencing in molecular diagnosis: NUBPL mutations highlight the challenges of variant detection and interpretation. *Hum. Mutat.* 2012; **33**: 411–18.
56 Stewart JD, Tennant S, Powell H *et al*. Novel POLG1 mutations associated with neuromuscular and liver phenotypes in adults and children. *J. Med. Genet.* 2009; **46**: 209–14.
57 Wong L, Naviaux R, Brunetti-Pierri N *et al*. Molecular and clinical genetics of mitochondrial diseases due to POLG mutations. *Hum. Mutat.* 2008; **29**: E150–72.
58 Chan SS, Longley MJ, Copeland WC. Modulation of the W748S mutation in DNA polymerase gamma by the E1143G polymorphism in mitochondrial disorders. *Hum. Mol. Genet.* 2006; **15**: 3473–83.
59 de Vries MC, Rodenburg RJ, Morava E *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.
60 Nguyen KV, Ostergaard E, Ravn SH *et al*. POLG mutations in Alpers syndrome. *Neurology* 2005; **65**: 1493–5.
61 Quinzii C, López L, Naini A, DiMauro S, Hirano M. Human CoQ10 deficiencies. *Biofactors* 2008; **32**: 113–18.
62 Vladutiu GD. Biochemical and molecular correlations in carnitine palmitoyltransferase II deficiency. *Muscle Nerve* 1999; **22**: 949–51.
63 Vladutiu GD, Bennett MJ, Smail D, Wong LJ, Taggart RT, Lindsley HB. A variable myopathy associated with heterozygosity for the R503C mutation in the carnitine palmitoyltransferase II gene. *Mol. Genet. Metab.* 2000; **70**: 134–41.
64 Su H, Fan W, Coskun PE *et al*. Mitochondrial dysfunction in CA1 hippocampal neurons of the UBE3A deficient mouse model for Angelman syndrome. *Neurosci. Lett.* 2011; **487**: 129–33.
65 Molinari F. Mitochondria and neonatal epileptic encephalopathies with suppression burst. *J. Bioenerg. Biomembr.* 2010; **42**: 467–71.
66 Colby G, Wu M, Tzagoloff A. MTO1 codes for a mitochondrial protein required for respiration in paromomycin-resistant mutants of Saccharomyces cerevisiae. *J. Biol. Chem.* 1998; **273**: 27 945–52.
67 Wang X, Yan Q, Guan MX. Mutation in MTO1 involved in tRNA modification impairs mitochondrial RNA metabolism in the yeast Saccharomyces cerevisiae. *Mitochondrion* 2009; **9**: 180–85.
68 Nouws J, Nijtmans LG, Smeitink JA, Vogel RO. Assembly factors as a new class of disease genes for mitochondrial complex I deficiency: Cause, pathology and treatment options. *Brain* 2012; **135**: 12–22.
69 Saneto RP, Naviaux RK. Polymerase gamma disease through the ages. *Dev. Disabil. Res. Rev.* 2010; **16**: 163–74.
70 Tarnopolsky MA. The mitochondrial cocktail: Rationale for combined nutraceutical therapy in mitochondrial cytopathies. *Adv. Drug Deliv. Rev.* 2008; **60**: 1561–7.

Supporting Information

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

Table S1. List of targeted genes.

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