Original Investigation

Use of Whole-Exome Sequencing to Determine the Genetic Basis of Multiple Mitochondrial Respiratory Chain Complex Deficiencies

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IMPORTANCE Mitochondrial disorders have emerged as a common cause of inherited disease, but their diagnosis remains challenging. Multiple respiratory chain complex defects are particularly difficult to diagnose at the molecular level because of the massive number of nuclear genes potentially involved in intramitochondrial protein synthesis, with many not yet linked to human disease.

OBJECTIVE To determine the molecular basis of multiple respiratory chain complex deficiencies.

DESIGN, SETTING, AND PARTICIPANTS We studied 53 patients referred to 2 national centers in the United Kingdom and Germany between 2005 and 2012. All had biochemical evidence of multiple respiratory chain complex defects but no primary pathogenic mitochondrial DNA mutation. Whole-exome sequencing was performed using 62-Mb exome enrichment, followed by variant prioritization using bioinformatic prediction tools, variant validation by Sanger sequencing, and segregation of the variant with the disease phenotype in the family.

RESULTS Presumptive causal variants were identified in 28 patients (53%; 95% CI, 39%-67%) and possible causal variants were identified in 4 (8%; 95% CI, 2%-18%). Together these accounted for 32 patients (60% 95% CI, 46%-74%) and involved 18 different genes. These included recurrent mutations in *RMND1, AARS2*, and *MTO1*, each on a haplotype background consistent with a shared founder allele, and potential novel mutations in 4 possible mitochondrial disease genes (*VARS2, GARS, FLAD1*, and *PTCD1*). Distinguishing clinical features included deafness and renal involvement associated with *RMND1* and cardiomyopathy with *AARS2* and *MTO1.* However, atypical clinical features were present in some patients, including normal liver function and Leigh syndrome (subacute necrotizing encephalomyelopathy) seen in association with *TRMU* mutations and no cardiomyopathy with founder *SCO2* mutations. It was not possible to confidently identify the underlying genetic basis in 21 patients (40%; 95% CI, 26%-54%).

CONCLUSIONS AND RELEVANCE Exome sequencing enhances the ability to identify potential nuclear gene mutations in patients with biochemically defined defects affecting multiple mitochondrial respiratory chain complexes. Additional study is required in independent patient populations to determine the utility of this approach in comparison with traditional diagnostic methods.

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Defects of the mitochondrial respiratory chain have emerged as the most common cause of childhood and adult neurometabolic disease, with an estimated prevalence of 1 in 5000 live births.[1] Clinically they can present at any time of life, are often seen in association with neurological impairment, and cause chronic disability and premature death.[2] Major advances in understanding the molecular basis of mitochondrial disease have been mirrored by a complex, expanding phenotypic spectrum. Although some genetic defects appear to be seen in association with specific clinical features, this is not usually the case, and a systematic multidisciplinary approach is required to make a diagnosis.[3] Biochemical and molecular genetic investigations are time consuming, expensive, and highly specialized, often involving a biopsy of an affected tissue or organ. With a growing list of mitochondrial diseases caused by different nuclear gene defects,[4] achieving a comprehensive molecular diagnosis is now more labor-intensive than ever. This can compromise clinical management through protracted and often repeated investigations, impeding reliable genetic counseling and prenatal diagnosis.

Approximately one-third of patients with mitochondrial disease have a biochemical defect involving multiple respiratory chain complexes, suggesting a defect of intramitochondrial protein synthesis.[5] With only a minority having a primary defect involving mitochondrial DNA (mtDNA), the remainder present a particular challenge. The molecular mechanism potentially involves many different gene products affecting mtDNA replication and expression, including ribosomal structural and assembly proteins, aminoacyl transfer RNA (tRNA) synthetases, tRNA modifying and methylating enzymes, and several initiation, elongation, and termination factors of mitochondrial translation.[6] Recent studies have shown that apparently unique genetic defects are common in this group, often involving proteins not previously thought to influence mitochondrial function, nor with clear mitochondrial localization. The objective of this study was to determine whether a whole-exome sequencing approach could be used to define the molecular basis of disease in these patients.

Methods

Patients

Patients with suspected mitochondrial disease referred to 2 nationally accredited diagnostic laboratories (the Highly Specialised Service Mitochondrial Diagnostic Laboratory, Newcastle upon Tyne, England, and the Medical Genetics Centre, Munich, Germany) between 2005 and 2012 and meeting the inclusion criteria were included in this study. The inclusion criteria were (1) histochemical and/or biochemical diagnosis of mitochondrial disease in a clinically affected tissue (skeletal muscle, liver, or heart) confirming decreased activities of multiple respiratory chain complexes based on published criteria (**Table**)[7]; (2) absence of large-scale mtDNA rearrangements, mtDNA depletion, and mtDNA point mutations,[8] with the exception of patients 20, 21, 25, 43, and 45, in whom decreased levels of mtDNA were confirmed in muscle (mtDNA depletion); and (3) exclusion of major nuclear gene rearrangements by comparative genomic hybridization arrays in patients with congenital structural abnormalities. Standardized clinical assessments were performed by the study authors. Clinical phenotypes were defined using local reference ranges for cardiomyopathy on echocardiography, abnormal renal and liver function test results, severe lactic acidosis (blood level >5 mM/L), and clinical neurophysiology for peripheral neuropathy. Informed consent was obtained from all participants in accordance with protocols approved by local institutions and research ethics committees.

Molecular Genetics and Bioinformatics

Exome sequencing, bioinformatic analysis, variant confirmation, and segregation analysis were performed in Newcastle upon Tyne. Genomic DNA was isolated from primary cell lines, muscle, or circulating lymphocytes (DNeasy, Qiagen); fragmented and enriched by Illumina TruSeq 62-Mb exome capture; and sequenced (Illumina HiSeq 2000, 100-bp paired-end reads). The in-house bioinformatics pipeline involved the following steps: alignment to the human reference genome (UCSC hg19),[9] removal of duplicate sequence reads (Picard version 1.85; http://picard.sourceforge.net), and variant detection (Varscan version 2.2;[10]; Dindel version 1.01[11]). On-target variant filtering excluded those with minor allele frequency greater than 0.01 in several databases: dbSNP135, 1000 genomes (February 2012 data release); the National Heart, Lung, and Blood Institute Exome Sequencing Project, 6500 exomes; and 238 unrelated in-house controls. We used published and experimentally validated bioinformatic tools to predict mitochondrial localization and probable effect on mitochondrial function.[12,13] Rare homozygous and compound heterozygous variants were defined, and protein altering and/or putative "disease-causing" mutations, along with their functional annotation, were identified using ANNOVAR.[14] Candidate genes were filtered against a list of bioinformatically predicted mitochondrial proteins,[12,13] as well as genes that matched a Gene Ontology term of *mitoch* and prioritized if previously seen in association with a disease phenotype (eTable 1 in the Supplement). Putative pathogenic variants were confirmed by Sanger sequencing using custom-designed primers (http://frodo.wi.mit.edu) on an ABI 3130XL (BigDye, Applied Biosystems) and compared with transcripts available in the Nucleotide database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nuccore), allowing segregation analyses where possible.

Variants were classified into 4 groups, defined a priori: (1) presumptive pathogenic: homozygous or compound heterozygous mutations in genes previously shown to cause multiple respiratory chain complex deficiencies; (2) possible pathogenic: homozygous or compound heterozygous mutations in novel genes predicted to cause a mitochondrial translation defect based on their proposed function and similarity to known disease genes; (3) variants of unknown significance: homozygous or compound heterozygous mutations in novel or known disease genes not known to be associated with mitochondrial

Table. Clinical and Molecular Genetic Characteristics of 53 Patients With Multiple Respiratory Chain Complex Defects[a]

Patient No. (Sex)	Country of Origin	Family History	Age at Onset/ Age at Last Follow-up	Clinical Presentation				Genetic Analysis	
				Muscle	CNS	Heart	Liver	Gene	Variant
Presumptive pathogenic									
1 (M)	British Pakistani	C	6 mo/4 y	+	–	–	–	*RMND1*	Hom c.1349G>C: p.*450Serext*32
2 (M)[b]	British Pakistani	C	3 mo/1 y[c]	+	+	–	–	*RMND1*	Hom c.1349G>C: p.*450Serext*32
3 (F)	British Pakistani	C	18 mo/5 y	+	+	+	–	*RMND1*	Hom c.1349G>C: p.*450Serext*32
4 (F)[b]	British Pakistani	C	6 mo/10 y[c]	+	–	+	–	*RMND1*	Hom c.1349G>C: p.*450Serext*32
5 (F)	British Pakistani	C	<1 mo/18 mo	+	–	–	–	*RMND1*	Hom c.1349G>C: p.*450Serext*32
6 (M)	British	N	18 mo/5 y[c]	+	–	–	–	*RMND1*	c.713A>G: p.Asn238Ser c.829_830 + 2delGAGT: p.Glu277Glyfs*2
7 (M)[b]	British	N	Birth/6 wk[c]	+	+	+	–	*AARS2*	c.1774C>T: p.Arg592Trp c.2882C>T: p.Ala961Val
8 (M)	German	N	Birth/1 mo[c]	+	+	+	–	*AARS2*	c.1616A>G: p.Tyr539Cys c.1774C>T: p.Arg592Trp
9 (F)[b]	German	C	3 wk/2 mo[c]	+	–	+	–	*AARS2*	Hom c.1774C>T: p.Arg592Trp
10 (F)	British	N	Birth/3 mo[c]	–	–	+	–	*AARS2*	c.647_648insG: p.Cys218Leufs*6 c.1774C>T: p.Arg592Trp
11 (F)	British	N	6 mo/11 mo[c]	–	–	+	–	*AARS2*	Hom c.1774C>T: p.Arg592Trp
12 (F)[b]	Croatian	1 Sib	Birth/1 mo	+	+	+	–	*MTO1*	c.631_631delG: p.Gly211Aspfs*3 c.1282G>A: p.Ala428Thr
13 (M)[b]	British Pakistani	C	Birth/1 y[c]	+	–	+	–	*MTO1*	Hom c.1232C>T: p.Thr411Ile
14 (M)[b]	British Pakistani	C	1 y/3 y[c]	+	+	+	+	*MTO1*	Hom c.1232C>T: p.Thr411Ile
15 (M)	British	N	<1 y/2 y	+	–	–	–	*MTO1*	c.122T>G: p.Val41Gly c.767A>G: p.His256Arg c.1282G>A: p.Ala428Thr
16 (M)[b]	Turkish	N	Birth/3 mo[c]	+	–	–	+	*EARS2*	Hom c.193A>G: p.Lys65Glu
17 (M)[b]	British	N	2 mo/6 mo[c]	+	+	–	+	*EARS2*	c.322C>T: p.Arg108Trp c.814G>A: p.Ala272Thr
18 (F)[b]	German	1 Sib	3 y/16 y	+	–	–	–	*MTFMT*	c.452C>T: p.Pro151Leu c.994C>T: p.Arg332*
19 (F)	British	N	Birth/20 y	+	+	+	–	*MTFMT*	c.626C>T: p.Ser209Leu c.1100_1101delTT: p.Phe367Serfs*22
20 (M)	British	N	2 y/2.5 y[c]	–	–	+	–	*MGME1*	c.532C>T: p.Arg178Trp c.794C>T: p.Thr265Ile
21 (F)	Irish	1 Sib	2.5 y/13 y	+	+	–	–	*C12orf65*	Hom c.96_99dupATCC: p.Pro34Ilefs*25
22 (M)[b]	Lebanese	C	14 y/37 y[c]	+	–	–	–	*YARS2*	Hom c.137G>A: p.Gly46Asp
23 (F)	Turkish	C	4 y/17 y	+	+	–	–	*PUS1*	Hom c. 426C>A: p.Cys142*
24 (M)	British Pakistani	C	Birth/1 mo[c]	+	+	+	–	*TRMU*	Hom c.287A>G: p.Asn96Ser
25 (F)	British	C	Birth/<1 mo[c]	+	+	–	–	*TK2*	Hom c.1A>G: p.Met1Val
26 (F)	Polish	N	7 mo/18 mo[c]	+	+	–	–	*SCO2*	Hom c.418G>A: p.Glu140Lys
27 (F)[b]	German	N	Birth/3 wk[c]	+	–	+	–	*ELAC2*	c.1478C>T: p.Pro493Leu c.1621G>A: p.Ala541Thr
28 (M)[b]	Turkish	1 Sib	4 y/7 y	+	+	–	–	*ETHE1*	Hom c.3G>T: p.Met1Ile
Possible pathogenic									
29 (M)	British	N	<1 y/10 y	+	+	–	–	*VARS2*	c.1135G>A: p.Ala379Thr c.1877C>A: p.Ala626Asp
30 (M)[b]	Turkish	C	4 mo/8 mo[c]	+	–	–	–	*FLAD1*	Hom c.397_400 delTTCT: p.Phe134Cysfs*8
31 (F)	Turkish	C, 2 Sibs	Birth/1 mo[c]	+	–	+	–	*GARS*	Hom c.2065C>T: p.Arg689Cys
32 (F)[b]	British	N	4 mo/8 mo[c]	–	–	+	–	*PTCD1*	c.337C>T: p.Arg113Trp c.388C>T: p.Arg130* c.550G>A: p.Gly184Arg

(continued)

Table. Clinical and Molecular Genetic Characteristics of 53 Patients With Multiple Respiratory Chain Complex Defects[a] (continued)

Patient No. (Sex)	Country of Origin	Family History	Age at Onset/ Age at Last Follow-up	Clinical Presentation				Genetic Analysis	
				Muscle	CNS	Heart	Liver	Gene	Variant
Variants of unknown significance									
33 (F)[b]	Turkish	C	2 y/6 y	+	+	−	−	*SLC25A12* *METAP1D*	Hom c.1333G>A: p.Ala445Thr Hom c.497 + 2T>A
34 (M)	British	N	17 y/20 y	−	+	−	−	*ACSM5*	c.1157A>C: p.Lys386Thr c.1273C>A: p.Pro425Thr
35 (F)	Georgian	C	7 y/10 y	+	+	−	−	*PERP* *MEF2A* *ACSM5*	Hom c.206T>C: p.Met69Thr c.1262A>C: p.Gln421Pro c.1265A>C: p.Gln422Pro c.68A>G: p.His23Arg c.73A>C:p.Lys25Gln
36 (M)	German	C	10 y/14 y	+	−	−	−	*HKDC1* *ETFA* *IREB2* *SMCR7*	Hom c.1276C>T: p.Arg426Cys Hom c.20C>T: p.Pro7Leu Hom c.2393C>T: p.Thr798Ile Hom c.241C>T: p.Gln81*
37 (F)	British	N	<1 mo/5 mo	+	+	−	−	*PC*	c.1876C>T: p.Arg626Trp c.1892G>A: p.Arg631Gln
38 (M)	Turkish	C	4 y/6 y	+	+	−	+	*TPO*	Hom c.443C>T: p.Ala148Val
39 (M)	British	N	<1 y/5 y	+	+	−	−	*HERC2*	c.6448C>G: p.Leu2150Val c.9979G>A: p.Val3327Met
40 (M)[b]	Turkish	C	2 wk/3 wk[c]	+	−	−	−	*MAGI1* *NDRG3* *TPX2* *TAF9*	Hom c.2290A>C: p.Thr764Pro Hom c.469G>A: p.Gly157Ser Hom c.505C>T: p.Pro169Ser Hom c.406G>C: p.Glu136Gln
41 (M)[b]	Croatian	N	Birth/9 mo[c]	+	+	+	−	*SLC25A43* *FAAH2*	c.493C>T: p.Arg165* (X-linked) c.368T>C: p.Phe123Ser (X-linked)
42 (M)	Hungarian	N	6 y/14 y	+	−	−	−	*DLAT* *SDHD* *POLRMT* *ARHGEF5*	c.55G>C: p.Glu19Gln c.626A>G: p.Gln209Arg c.34G>A: p.Gly12Ser c.386T>C: p.Leu129Ser c.112C>T: p.Pro38Ser c.232G>A: p.Val78Met c.1738G>T: p.Gly580Cys c.4066A>G: p.Asn1356Asp
43 (F)	British	N	Birth/<1 mo[c]	+	−	−	−	*TYMP* *ACSM2A* *LRPPRC* *HTRA2* *ALDH1L1* *BCKDHB* *SLC25A4*	c.242G>A: p.Arg81Gln c.1003G>A: p.Val335Ile c.4132A>G: p.Ser1378Gly c.1210C>T: p.Arg404Trp c.2143G>C: p.Glu715Gln c.23C>T: p.Ala8Val c.239G>A: p.Arg80His
44 (M)[b]	Turkish	1 Sib	18 mo/2 y	+	−	−	+	*PPL* *SLC5A10*	c.263A>G: p.Asp88Gly c.1003C>A: p.Leu335Met c.674A>G: p.Glu225Gly c.1799T>C: p.Leu600Pro
45 (F)	German	Healthy dizygotic twin	1 mo/6 mo[c]	+	+	−	+	*FASN* *FNDC1*	c.1850C>T: p.Pro617Leu c.2657T>C: p.Phe886Ser c.4429A>G: p.Thr1477Ala c.4547C>A: p.Thr1516Asn
46 (M)	German	N	6 y/21 y	+	−	−	−	*PDPR* *LONP1*	c.616A>G: p.Ile206Val c.1774A>G: p.Thr592Ala c.79G>C: p.Ala27Pro c.2485G>A: p.Ala829Thr
47 (M)	Egyptian	C	2 mo/2 y[c]	+	+	−	+	*MIPEP* *STARD13*	Hom c.671A>G: p.Asn224Ser Hom c.1186C>T: p.His396Tyr
Unresolved									
48 (M)	Turkish	C	4 mo/1 y	+	+	+	+		No candidate variants detected
49 (F)[b]	German	N	1 y/3 y	+	+	−	−		No candidate variants detected
50 (F)[b]	British Pakistani	C	Birth/4 d[c]	−	−	+	−		No candidate variants detected
51 (F)	British Pakistani	C	Adult onset	+	−	−	−		No candidate variants detected
52 (M)	German	N	2 y/5 y[c]	+	+	−	+		No candidate variants detected
53 (F)	German	N	Birth/4 y	+	+	−	−		No candidate variants detected

Abbreviations: CNS, central nervous system; C, consanguinity; Hom, homozygous; N, no family history.

[a] Patients were categorized into 4 groups based on the molecular genetic results defined a priori (see Methods). The complete data set is shown in eTable 2 in the Supplement, including the results of biochemical analyses.

[b] Included in Kemp et al.[5]

[c] Age at death.

pathology; and (4) unresolved: cases in which a single plausible genetic cause could not be identified.

Binomial confidence intervals were calculated using the Clopper-Pearson method.

Results

Clinical Presentation

The clinical presentation and laboratory findings of the 53 unrelated patients are summarized in the Table and in eTable 2 in the Supplement. The majority (51/53 [96%; 95% CI, 87%-99%]) of the patients presented in childhood (<15 years old) and most (35/53 [66%; 95% CI, 52%-78%]) developed symptoms within the first year of life. Parental consanguinity was apparent in 24 cases, and 3 cases had an additional affected sibling. The most frequent clinical feature was muscle weakness with hypotonia (47/53 [89%; 95% CI, 77%-96%]), followed by clinical or imaging features of central neurological disease (28/53 [53%; 95% CI, 39%-67%]), cardiomyopathy (19/53 [36%; 95% CI, 23%-50%]), and abnormal liver function (9/53 [17%; 95% CI, 8%-30%]); a combination of these symptoms was present in most cases (34/53 [64%; 95% CI, 50%-77%]). Severe lactic acidosis was observed in half of the patients (27/53 [51%; 95% CI, 37%-65%]). The presence of deafness (8/53 [15%; 95% CI, 7%-28%]), ptosis or progressive external ophthalmoplegia (4/53 [8%; 95% CI, 2%-18%]), renal impairment (5/53 [9%; 95% CI, 3%-21%]), axonal neuropathy (3/53 [6%; 95% CI, 1%-16%]), sideroblastic anemia (2/53 [4%; 95% CI, 0%-13%]), immune deficiency (1/53 [2%; 95% CI, 0%-10%]), and optic atrophy (2/53 [4%; 95% CI, 0%-13%]) was also noted.

Whole-Exome Sequencing

The mean per base depth of coverage for the exome consensus coding sequence was 79-fold, with 88.6% of bases covered more than 20-fold. Coverage and depth statistics for each patient are shown in eTable 3 in the Supplement. The results of the bioinformatic analysis are shown in eTable 1 in the Supplement, which also includes the allele frequency data. Confirmed variants are shown in the Table. Presumptive pathogenic variants were found in 28 patients (53%; 95% CI, 39%-67%) and possible pathogenic variants in 4 patients (8%; 95% CI, 2%-18%) for a combined result of 32 of 53 (60%; 95% CI, 46%-74%). The underlying genetic basis of disease was not confirmed in 21 patients (40%; 95% CI, 26%-54%). Variants of uncertain significance were found in 15 patients (28%; 95% CI, 17%-42%) and 6 cases remained unresolved (11%; 95% CI, 11%-34%).

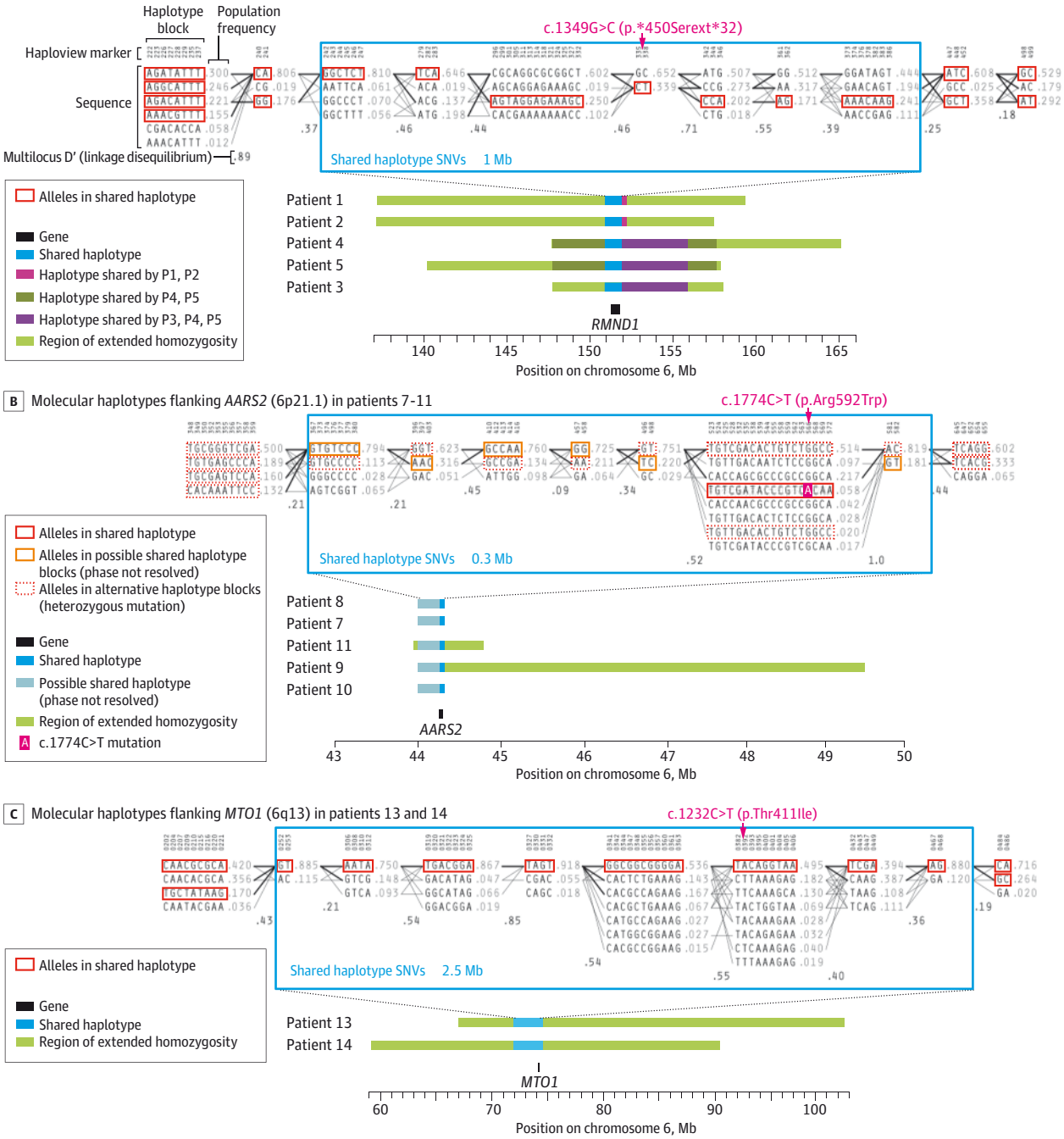
Presumptive Pathogenic Group

A single, novel homozygous c.1349G>C (p.*450Serext*32) *RMND1* (NM_017909.3)[15,16] stop codon mutation was identified in 5 independent patients. In each patient, the phenotype was severe, affecting different organs but including myopathy, profound deafness, and renal involvement. The 5 homozygotes were from consanguineous families of Pakistani origin. A founder effect was supported by the presence of a homogeneous haplotype flanking the mutation (**Figure 1**A). One other patient had different compound heterozygous mutations in *RMND1*. Mutations in *AARS2* (NM_020745.3)[17] were the second most frequently identified defect (5 patients). All presented with severe infantile cardiomyopathy, with additional muscle (3 patients) and central neurological features (2 patients) in a subgroup. Interestingly, despite being from different European ethnic backgrounds, all carried the previously reported c.1774C>T (p.Arg592Trp) mutation on at least 1 allele (Figure 1B).[17] Mutations in *MTO1* (NM_012123.3)[18] were identified in 4 patients. All had muscle weakness on presentation with lactic acidosis, 2 had central neurological features, and, unlike a previous report,[18] 1 did not have cardiomyopathy; 2 patients were homozygous for a p.Thr411Ile *MTO1* mutation recently shown to cause a severe respiratory phenotype in a yeast model[19] (Figure 1C). Homozygous or compound heterozygous mutations were detected in previously characterized mitochondrial translation genes, including 2 patients with *EARS2* (NM_001083614.1)[20] mutations (1 having leukoencephalopathy and no corpus callosum),[21] 2 patients with *MTFMT* (NM_139242.3) mutations,[22] and 1 patient with *C12orf65* (NM_152269) mutations.[23] Single patients with a clinical presentation resembling previously described cases carried homozygous or compound heterozygous mutations in *YARS2* (NM_001040436.2),[24] *PUS1* (NM_025215.5),[25] *MGME1* (NM_052865.2),[26] *ETHE1* (NM_014297.3),[27] *ELAC2* (NM_018127.6),[28] and *TK2* (NM_004614.3),[29] the latter case seen in association with severe loss of mtDNA copy number due to mutation (c.1A>G, p.Met1Val) of the initiating methionine codon. Atypical presentations included a patient with a homozygous *TRMU* (NM_018006.4)[30] mutation seen in association with heart, central nervous system, and muscle involvement but no liver involvement, and a subclinical, mild anemia in patient 23 carrying a homozygous nonsense mutation in *PUS1*. In addition, patient 26 had typical features of Leigh syndrome and multiple respiratory chain complex defects at the time of biopsy but was homozygous for the p.Glu140Lys *SCO2* (NM_001169111.1)[31] founder mutation, which is usually seen in association with an isolated complex IV defect and cardiomyopathy, features not present in this patient.

Possible Pathogenic Group

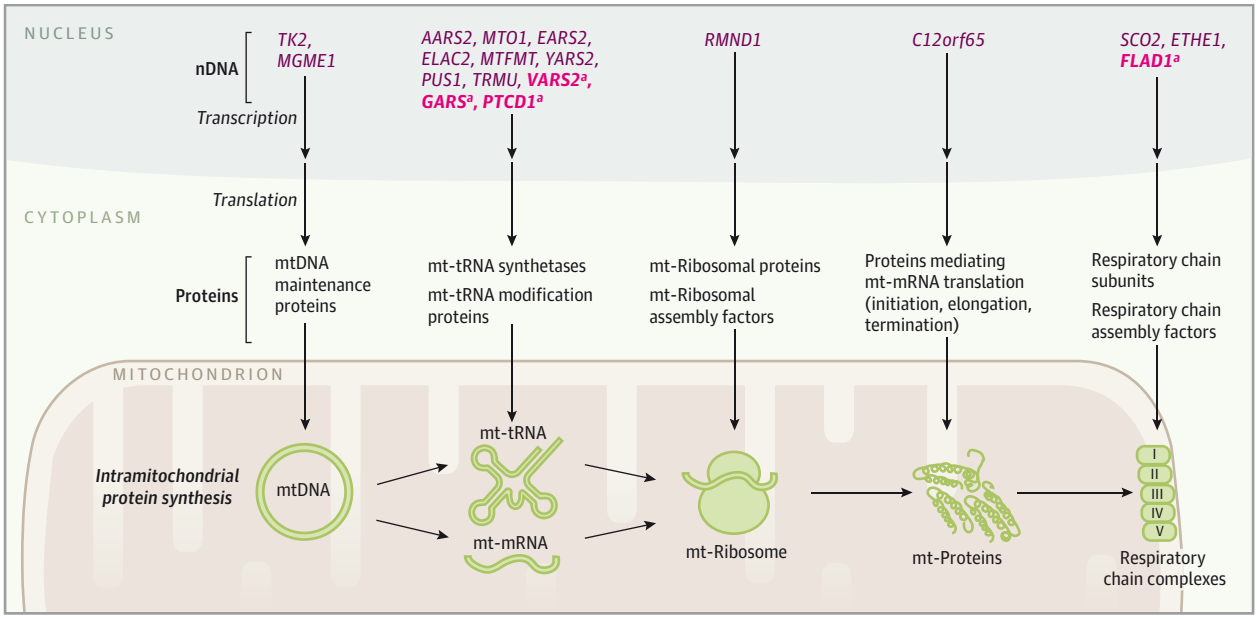
Possible disease-causing variants were identified in novel mitochondrial disease genes in 4 patients, each predicted to affect mitochondrial protein synthesis. *VARS2* (NM_001167734.1) and *GARS* (NM_002047.2) encode mitochondrial aminoacyl-tRNA synthetase genes. *FLAD1* (NM_025207.4) encodes a key factor of the riboflavin metabolism, and *PTCD1* (NM_015545.3) is a gene encoding a mitochondrially targeted pentatricopeptide reported to be involved in mitochondrial RNA metabolism.[32] *In silico* predictions supported a pathogenic role in each case, but given that they were identified only in single patients, further evidence is required before these variants can be considered definitively pathogenic; where familial samples were available, identified mutations were shown to segregate with disease (Table).

Figure 1. Molecular Haplotypes Flanking *RMND1*, *AARS2*, and *MTO1* in Selected Study Patients



Haplotype blocks were generated from selected markers using exomes from 62 in-house controls and from the patients found to harbor mutations in *RMND1*, *AARS2*, and *MTO1*. Population frequencies are shown next to each haplotype; thicker connecting lines show more common crossings than thinner lines. Multilocus D', a measure of the linkage disequilibrium between 2 blocks, is shown. The closer the value is to 0, the greater the amount of historical recombination. SNV indicates single nucleotide variant. A, Molecular haplotypes flanking *RMND1* in patients 1-5. In addition to the main haplotype that includes the *RMND1* mutation, patients 1 and 2 and patients 4 and 5 shared one of 2 different haplotypes situated 3' to the *RMND1* gene. Patients 3, 4, and 5 also shared a haplotype. The *RMND1* mutation, c.1349G>C (p.*450Serext*32), is indicated between Haploview markers 335 and 338. The mutation is not included in the Haploview analysis because all patients were homozygous for the mutation. B, Molecular haplotype flanking *AARS2* in patients 7-11. A shared haplotype spanning exons 10-22, including the c.1774C>T (p.Arg592Trp) mutation at Haploview marker 566, was identified. Six additional haplotype blocks appeared to be shared between p.Arg592Trp *AARS2* mutation carriers; however, for the carriers of the discrete heterozygous *AARS2* mutations (patients 7, 8, and 10), it was not possible to resolve the phase of these blocks. Alternative haplotype blocks in which the mutation was heterozygous were identified in patients 7, 8, and 10. C, Molecular haplotype flanking *MTO1* in patients 13 and 14 show the shared haplotype defining a founder allele. The homozygous *MTO1* mutation, c.1232C>T (p.Thr411Ile), is located between Haploview markers 382 and 392.

Figure 2. Nuclear Genes Associated With Multiple Mitochondrial Respiratory Chain Complex Defects



Genes present within the cell nucleus encode proteins critical for intramitochondrial protein synthesis. These proteins are transported through the double mitochondrial membrane into the mitochondrial matrix. Nuclear genes associated with multiple mitochondrial respiratory chain complex defects are shown at the top. nDNA indicates nuclear DNA; mtDNA, mitochondrial DNA; mt-tRNA, mitochondrial transfer RNA; mt-mRNA, mitochondrial messenger RNA; mt, mitochondrial.

[a] Newly identified nuclear gene with possible pathogenic variants.

Variants of Unknown Significance

In 15 patients we identified 1 or more variants in genes predicted to encode mitochondrial proteins (Table) where there were several plausible candidate disease genes. Most of the gene defects were detected in single patients only (except for *ACSM5* [NM_017888.2] mutations in patients 34 and 36). The current lack of functional data directly linking these genes to multiple respiratory chain complex defects led to their classification as possible and not probable causative variants (**Figure 2**). Identification of additional patients with mutations in these genes and/or functional work is required to validate these findings.

Unresolved

Exome sequencing did not identify any candidate pathogenic variants in 6 patients.

Discussion

In the pre-exome era, the systematic biochemical characterization of 53 patients with multiple respiratory chain complex defects led to detection of the underlying genetic basis in only 1 patient.[5] The work presented herein demonstrates the effect of whole-exome sequencing in this context, which has defined the genetic etiology in 32 of 53 patients (60%, including 28 presumptive and 4 probable causative mutations) with a confirmed biochemical defect consistent with a generalized decrease in mitochondrial translation. The detection rate was even higher in children with onset at younger than 1 year (24/35 [69%]). In 20 patients with prominent cardiac disease, we detected the causative mutation in 15 (80%), while the detection rate was much lower in patients with liver disease (3/9 [33%]). Our findings contrast with large-scale candidate gene analysis using conventional[33] and next-generation sequencing approaches,[34] both of which had a lower diagnostic yield (10%-13%) and by definition did not discover new potential disease genes. A more ambitious approach involving exon capture and sequencing of all predicted mitochondrial genes (the "mitoexome") has delivered a greater diagnostic yield (22%-28%),[12,35] although in cohorts with predominantly isolated respiratory chain complex defects that are less challenging to define at the genetic level.[36]

There are a number of reasons why our approach had a more than 2-fold higher diagnostic yield than previous studies, despite the known difficulty in making a molecular diagnosis in patients with multiple respiratory chain complex deficiencies. First, not being based on any prior assumptions about known candidate disease genes, the whole-exome approach detected 4 new genes that may be responsible for the underlying mitochondrial disorder. Further arguments supporting the pathogenic role of mutations in these new genes come from similarities in function to known mitochondrial genes. For example, *VARS2* and *GARS* encode tRNA synthase genes with products known to enter mitochondria. Recessive mutations in other mitochondrial tRNA synthetase genes responsible for charging tRNAs with different specific cognate amino acids during protein synthesis have been shown to cause identical biochemical defects and similar clinical phenotypes.[17,20,24] Compound heterozygous mutations in *GARS* have recently

been reported in a single family with a multisystemic mitochondrial disease with cardiomyopathy.[37] These findings endorse our approach and support the pathogenic role of the *GARS* mutations in our patient who developed a fatal cardiomyopathy, but the lack of functional data means that mutations in this gene should remain in the possible pathogenic group (Table). Like *GARS, FLAD1* was not originally considered to encode a mitochondrial protein but ultimately was found to encode alternatively spliced cytoplasmic and mitochondrial transcripts and thus is a plausible candidate gene.[38] Although we have not shown proof of the causal link, *in silico* predictions of the deleterious effect of the mutations in genes showing strong functional similarities with known disease genes supports a causal association with the mutations. Second, the unbiased exome approach has the potential to reveal unexpected results. This was the case for 3 additional unreported patients who also met the selection criteria for this study. One patient presenting with neonatal myopathy, encephalopathy, and lactic acidosis with a complex I defect and a less prominent complex IV defect was found to have novel homozygous mutations in the complex I subunit gene *NDUFS6* (c.317_320delAAAC: p.Glu106fs*21). In this context, the complex IV defect was presumably secondary, perhaps mediated through the disruption of respiratory chain supercomplexes. Similarly, 1 child carrying the homozygous common *SCO2* mutation also presented with severe combined complex I and IV defects. These results also illustrate the difficulties in interpreting respiratory chain enzyme analysis, which, even in skilled hands, can misdirect a candidate gene approach. The higher diagnostic yield could in part reflect the growing inventory of genes known to cause mitochondrial diseases and the relatively high proportion of familial and consanguineous cases in our study cohort.

Our findings implicate 18 different genes and 33 possible candidates in 53 patients, underscoring the genetic heterogeneity of this group of mitochondrial disorders (Figure 2). Given this complexity, how should diagnosis be approached in a clinical setting? Despite the relatively small number of individuals with any single mutation, our observations show the phenotypic diversity in patients with multiple respiratory chain complex defects; emerging clinical subgroups do appear to be seen in association with specific genetic defects. For example, *AARS2* and *MTO1* mutations were preferentially seen in association with cardiomyopathy, mutations in *TRMU* presented with liver failure that could improve spontaneously, *YARS2* and *PUS1* with sideroblastic anemia and myopathy, and *RMND1* with deafness, myopathy, renal involvement, and a severe biochemical defect (eTable 2 in the Supplement). However, not all patients fit neatly into these subgroups, including a patient with a *TRMU* mutation and normal liver function, one with a *PUS1* mutation and only subclinical anemia, and a patient with a *SCO2* mutation with no cardiomyopathy. This heterogeneity is typical for mitochondrial diseases and supports the use of next-generation sequencing early in the diagnostic approach. Although a molecular diagnosis is unlikely to lead to specific drug treatments at present, defining the genetic etiology may enable accurate genetic counseling and prenatal diagnosis and personalized disease surveillance for genotype-specific complications.

Given that our case ascertainment was determined by clinical referral and not influenced or biased by the study team, our findings may have broader relevance. However, our study has several limitations. First, we could not identify a potential candidate mutation in 6 patients (11%), possibly because the causative mutations lay in deep intronic regions or may involve deletions or duplications (copy number variations) missed by the exome capture and bioinformatic analysis. It is possible that whole-genome sequencing will identify the pathogenic variants in some or all of these 6 patients. Second, our work was carried out on carefully phenotyped patients defined by a biochemical defect measured in 2 specialist centers, and a high proportion of patients were from consanguineous families. It will be important to replicate our findings in similar patient cohorts investigated in other centers from other parts of the world, where the spectrum of nuclear gene defects may be different. The 53 patients we studied account for approximately 20% of the referrals to our centers with any form of biochemical defect of the respiratory chain determined by a spectrophotometric assay of enzyme activity, and the patients with multiple respiratory chain complex defects account for approximately 60% of patients with a biochemical defect with no known molecular diagnosis. Therefore, the role of exome sequencing in unselected patients with a clinical diagnosis of suspected multiple respiratory chain complex defects remains to be determined, and the effect of exome sequencing in patients with a general diagnosis of suspected mitochondrial disease is not clear. However, applying a whole-exome approach to a group of patients with multiple respiratory chain complex defects that are difficult to diagnose has delivered a high diagnostic yield. Curiously, most of the genes appear to be involved in intramitochondrial gene translation. This explains the phenotypic and biochemical overlap, but it is not clear why apparently subtle differences in function should lead to discrete clinical phenotypes. These discrete phenotypes may point to a few candidate genes, but in our large cohort, this became obvious only after performing this study. It is possible that next-generation sequencing will revolutionize the investigation of mitochondrial diseases, and its early application may provide a rapid diagnosis at a relatively low cost, particularly in patients with multiple respiratory chain complex defects.

Conclusions

Exome sequencing enhances the ability to identify the underlying nuclear gene mutations in patients with multiple mitochondrial respiratory chain complex defects. Additional study is required to determine the utility of this approach compared with traditional diagnostic methods in independent patient populations.

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