LETTER TO JMG

Analysis of mitochondrial DNA sequences in patients with isolated or combined oxidative phosphorylation system deficiency

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Background: Enzyme deficiencies of the oxidative phosphorylation (OXPHOS) system may be caused by mutations in the mitochondrial DNA (mtDNA) or in the nuclear DNA.
Objective: To analyse the sequences of the mtDNA coding region in 25 patients with OXPHOS system deficiency to identify the underlying genetic defect.
Results: Three novel non-synonymous substitutions in protein-coding genes, 4681T→C in MT-ND2, 9891T→C in MT-CO3 and 14122A→G in MT-ND5, and one novel substitution in the 12S rRNA gene, 686A→G, were found. The definitely pathogenic mutation 3460G→A was identified in an 18-year-old woman who had severe isolated complex I deficiency and progressive myopathy.
Conclusions: Bioinformatic analyses suggest a pathogenic role for the novel 4681T→C substitution found in a boy with Leigh's disease. These results show that the clinical phenotype caused by the primary Leber's hereditary optic neuropathy mutation 3460G→A is more variable than has been thought. In the remaining 23 patients, the role of mtDNA mutations as a cause of the OXPHOS system deficiency could be excluded. The deficiency in these children probably originates from mutations in the nuclear genes coding for respiratory enzyme subunits or assembly factors.

Key points

- Enzyme deficiencies of the oxidative phosphorylation (OXPHOS) system may be caused by mutations in the mitochondrial DNA (mtDNA) or in the nuclear DNA. The sequence of mtDNA-coding region was analysed in 25 patients with OXPHOS system deficiency to identify the underlying genetic defect.
- 4681T→C, a novel substitution in MT-ND2, was found in a patient with Leigh's disease. Further analyses suggested a pathogenic role for this substitution.
- 3460G→A, one of the mutations causing Leber's hereditary optic neuropathy, was identified in a patient with progressive myopathy. The finding suggests that the clinical phenotype caused by this mutation is more variable than what has been known.

The oxidative phosphorylation (OXPHOS) system consists of five enzyme complexes composed of >70 subunits encoded by the nuclear genome and 13 subunits encoded by mitochondrial DNA (mtDNA). Both isolated and combined enzyme complex deficiencies have been reported in children with various clinical phenotypes. Defects in the OXPHOS system are common causes of inborn errors in energy metabolism, with an estimated incidence of 1 per 10 000 live births.[1] The inheritance pattern is autosomal recessive in most cases, but autosomal dominant and X-chromosomal inheritance has also been described. Maternal inheritance points to a mutation in mtDNA as the cause of the disease.[2]

More than 2000 human mtDNA-coding region sequences have been reported since 2000, and about half of these sequences are from Europeans.[3–8] The total number of non-synonymous mutations leading to an amino acid replacement in mtDNA of European origin has been estimated to be 1081, but as many as 18 100 sequences should be analysed to identify 95% of these substitutions.[9] Sequencing of the complete mtDNA from patients with an OXPHOS system deficiency will evidently lead to the identification of novel pathogenic mutations. This approach has already yielded several novel mutations in MT-ND genes so far, and some of them—for example, 10191T→C and 14487T→C—may not be uncommon causes of disease.[10 11]

There is a growing need to analyse complete mtDNA sequences with a high throughput and in a cost-efficient manner. We analysed the entire coding region of mtDNA in 28 patients (consisting of children and young adults) with OXPHOS system deficiency using a protocol consisting of conformation-sensitive gel electrophoresis (CSGE) of amplified mtDNA fragments and subsequent sequencing of those fragments that differed in mobility in CSGE. Obtained sequences were compared with previously reported mtDNA sequences to identify haplotype-specific or novel variants, and to detect possible sequencing errors.[12] The quality of the sequences was confirmed by comparison of the sequences obtained using the CSGE protocol with those obtained using direct mtDNA sequencing, and by correct identification of three samples with a known pathogenic mutation. Three novel non-synonymous substitutions and one novel rRNA substitution were detected, and their pathogenic potential was estimated on several criteria.

PATIENTS AND METHODS

Patients

The total number of patients was 28 , ranging from newborn babies to 18-year-old adults (Department of Pediatrics, Nijmegen Centre for Mitochondrial Disorders, University Medical Centre Nijmegen, Nijmegen, The Netherlands), and included three people with a definitely pathogenic mtDNA mutation (table 1). In all, 11 children had an isolated OXPHOS system deficiency and 17 had a combined OXPHOS system

Abbreviations: CSGE, conformation-sensitive gel electrophoresis; LHON, Leber's hereditary optic neuropathy; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; RFLP, restriction fragment length polymorphism

Table 1 Oxidative phosphorylation system activities in the muscles of 28 patients

Patient	CI	CII	CIII	CIV	CII+CIII
A	91	n	n	n	n
B	30	n	n	10	26
C	70	n	n	n	n
D	71	75	0	92	n
E	76	n	50	n	n
F	<100	n	<100	n	n
G*	n	n	n	n	n
H	26	n	n	n	n
I	77	64	69	n	n
J	96	n	68	n	n
K	86	n	70	n	n
L	74	n	79	n	n
M	99	n	n	54	n
N	n	n	70	n	n
O	94	n	95	n	n
P	60	n	69	24	n
Q	86	n	13	n	n
R	n	n	94	n	n
S	24	n	55	61	n
T	71	n	83	n	n
U	0	n	n	12	n
V	83	n	n	n	n
W	n	n	94	n	n
X	21	n	n	n	n
Y	7	n	n	n	n
m1†	6	n	n	n	n
m2†	n	n	n	n	97
m3†	3	n	10	4	n

CI–CIV, complex I to IV; n, normal.
Oxidative phosphorylation enzyme activities are given as percentages of the lowest value in the controls.
Samples were obtained from the right lateral vastus muscle.
*Decreased pyruvate oxidation and ATP production.
†Samples with definite pathogenic mutations: m1, 3243A→G; m2, 8993T→G; m3, 7497G→A.[14]

deficiency in skeletal muscle.[13] In most cases, mtDNA was isolated from muscle, or in some cases from blood or fibroblasts. All patient samples were obtained after informed consent.

Molecular methods

The CSGE protocol consisted of analysis of the nucleotide sequence in the entire mtDNA coding region (523–16090 bp) by CSGE and subsequent sequencing.[15 16] Primers used for amplification of the fragments for CSGE and those used for subsequent sequencing have been reported previously.[15] The sequence variants identified were compared with those reported in human mitochondrial genome databases (MITOMAP, http://www.mitomap.org; mtDB, http://www.genpat.uu.se/mtDB/; or GiiB-JST mtSNP, http://www.giib.or.jp/mtsnp/index_e.html) or previous publications.[4 5 8] Variants that were found to be compatible with haplogroup polymorphisms were accepted without further analyses, whereas all novel or rare substitutions were confirmed either by sequencing in both directions or by restriction fragment length polymorphism (RFLP; table 2).

The sensitivity of the CSGE protocol for identifying substitutions was tested by analysing three patients with known mtDNA mutations. The samples were included in the set in a blinded fashion and the code was broken only after completion of the sequences. Furthermore, five randomly selected DNA samples were subjected to direct sequencing and the chromatograms analysed by a person who had no access to the CSGE results. The mtDNA sequences were then compared with the corresponding sequences obtained with the CSGE protocol to compare the substitution detection rate between the two methods.

Novel and rare variants were studied for heteroplasmy by RFLP of fragments that had been amplified in the presence of 6.3–12.5 μCi of adenosine triphosphate labelled with sulphur-35 (Perkin-Elmer, Boston, Massachusetts, USA). The labelled polymerase chain reaction product was digested with a compatible restriction enzyme and the digestion products were separated on a 6% or 10% DNA-polyacrylamide gel electrophoresis. The level of heteroplasmy was then estimated by scanning the wild-type and mutant digestion products.[17]

The pathogenic potential of the novel non-synonymous substitutions was estimated using information on the nature of the ensuing amino acid exchange, conservation of the position between species, and the effect of the amino acid exchange on the secondary or tertiary structure of the protein. These predictions were calculated using information deposited in an EMBL database (www.embl-heidelberg.de/predictprotein/predictprotein.html) or in the GiiB-JST mtSNP database (www.giib.or.jp/mtsnp/index_e.html). Conservation indices were calculated as described previously.[18]

Statistical methods

An evolutionary network was constructed based on the mtDNA variations in the 28 patients. A binary data file was created and the calculations for the network were carried out using the reduced median algorithm implemented in the Network 4.106 software (www.fluxus-engineering.com).

Table 2 Amplification primers and restriction enzymes used in the verification of novel variants

Variant	Forward primer	Reverse primer	Annealing temperature (°C)	RFLP
686A→G	L328	H725	59	None*
4233T→C	L3969	H4324	59	*Bcl*I
4681T→C	L4409	H4739	59	None*
6665C→T	L6383	H6778	53	*Xmn*I
7954T→C	L7813	H8203	58	None*
8065G→A	L7813	H8203	58	*HpyCH4 V*
9657C→T	L9485	H9875	61	*Tru*1I
9891T→C	L9772	H10107	57	*Bst*F5I
11629A→G	L11431	H11814	56	*Mbo*I
12924A→G	L12750	H13050	59	*Ava*II
13608T→C	L13462	H13851	51	*Taq*I
13671A→G	L13462	H13851	51	None*
14122A→G	L13914	H14208	46	*Bst*F5I

H, heavy; L, light; RFLP, restriction fragment length polymorphism.
The location of the first nucleotide of the primers in the light (L) and heavy (H) chain is shown. The average primer length is 22 bp.
*The variant was confirmed by sequencing with the same primers as in the amplification.

The number of novel non-synonymous sequence variants among the 25 patients was compared with the results of a permutation analysis. Twenty five sequences were sampled 1000 times with replacements from 617 previously published sequences belonging to the European haplogroups.[4 5] The number of non-synonymous variants that were present in the sampled sequences and not present in the remaining sequences was counted in each permutation, and a 95% confidence interval was derived from these values. The three patients with known pathogenic mutations were excluded from the analysis.

RESULTS

The CSGE protocol was used to determine nucleotide sequence in the mtDNA coding region in the 28 patients with OXPHOS system deficiency. A comparison of the sequences with revised Cambridge reference sequence[16] yielded 93 synonymous substitutions, 44 non-synonymous substitutions, 33 rRNA or tRNA substitutions, two length variants in homopolymeric cytosine tracks spanning between nucleotides 568–573 and 5895–5899, and one single-nucleotide deletion.

The 28 sequences were then imported into an evolutionary network that has been constructed using mtDNA sequence information on 617 Europeans (fig 1). This enabled easy identification of the haplogroup-specific polymorphisms and novel private substitutions, and confirmed that all the patients belonged to one or other of the European haplogroups. All the novel private substitutions were then confirmed by either RFLP or direct sequencing of the respective fragment in both directions. In total, we discovered three novel non-synonymous substitutions, one novel substitution in an rRNA gene and nine novel synonymous substitutions (table 3). All the synonymous substitutions and most of the non-synonymous ones were considered to be polymorphisms.

Two approaches were used to evaluate the quality of the sequences. Firstly, three samples harbouring a known mtDNA mutation had been included in the sample set in a blind fashion. Each was identified correctly by the CSGE protocol. Secondly, the CSGE protocol and direct sequencing were compared in terms of substitution detection rate. Complete mtDNA was sequenced from five randomly selected patients. Direct sequencing yielded the synonymous substitution 5147G→A, which was not detected by CSGE, and the CSGE protocol yielded the novel heteroplasmic substitution 14122A→G, which was not observed in the sequence chromatograms. These results suggested a sensitivity of 98.8% and a specificity of 100% for both methods.

The four novel substitutions detected in the 28 patients were examined further for their pathogenic potential. Interestingly, the definitely pathogenic mutation 3460G→A was found in an 18-year-old woman with severe isolated complex I deficiency (patient Y, table 4), being homoplasmic in the muscle of this patient. This patient had a slowly progressive myopathy, with muscle weakness and exercise intolerance as the most prominent clinical features starting at age around 10 years. Currently she is in her 20s, regularly uses a wheelchair, and has normal mental and intellectual capacities.

The three novel non-synonymous substitutions were 4681T→C in MT-ND2, 9891T→C in MT-CO3 and 14122A→G in MT-ND5. The 4681T→C substitution, in patient C, leads to Leu71Pro in the third transmembrane helix of the ND2 subunit of complex I. The 9891T→C substitution, in patient E, causes a Ser229Pro amino acid exchange in cytochrome c oxidase subunit 3 and abolishes a predicted TSK phosphorylation site. The 14122A→G substitution, in patient G, causes an Ile596Val substitution in the ND5 subunit of complex I. This substitution was heteroplasmic, with a proportion of 87% in fibroblasts. The novel substitution 686A→G was found in MT-RNR1 in patient E.

We also found three variants that have previously been reported in only a single sequence: 6681T→C in MT-CO1, 13630A→G in MT-ND5 and 12188T→C in MT-TH.[5 8] The 6681T→C substitution was found in mtDNA belonging to subhaplogroup J1, identical to that of the previously reported sequence; the 13630A→G substitution was found in a sample belonging to haplogroup U5a, also identical to that of the previously reported sequence; and 12188T→C was found in patient J, who belonged to subhaplogroup U5a, although the variant had previously been found in an African person belonging to subhaplogroup L3d. Interestingly, 12188T→C was heteroplasmic in our patient, with a proportion of 83% in muscle.

The 25 patients harboured seven non-synonymous substitutions (4681T→C, 9181A→G, 9891T→C, 14122A→G, 14325T→C, 15287T→C, 15851A→G) that were not found in the 617 control sequences belonging to European haplogroups. A permutation analysis among the 617 sequences indicated that the mean number of such substitutions in a random sample of 25 sequences was 6.5 (95% confidence interval 2 to 12).

DISCUSSION

About 2000 complete mtDNA coding region sequences from healthy people and patients with various disorders have been reported during the past few years, and with the development of suitable technologies, the analysis of mtDNA sequences has become a feasible diagnostic tool. We analysed the mtDNA-coding region of 28 patients with OXPHOS deficiency, and found one 18-year-old with the 3460G→A mutation in MT-ND1, two children with the novel non-synonymous substitutions 4681T→C in MT-ND2 or 14122A→G in MT-ND5, and one child with the novel substitutions 9891T→C in MT-CO3 and 686A→G in MT-RNR1. The 3460G→A mutation is one of the three primary causes of Leber's hereditary optic neuropathy (LHON) and 4681T→C is suggested to be a pathogenic mutation.[19]

The quality of mtDNA sequences has been a subject of debate during the past few years.[12 20 21] We used CSGE to screen patient mtDNA for the presence of sequence changes relative to a reference sample, and subsequent sequencing to identify nucleotide changes. We then compared the resulting mtDNA sequences with the published mtDNA sequences of people of European origin.[4 5] Analysis of these sequence data enabled an easy distinction between haplotype-specific polymorphisms and substitutions that needed further evaluation. In addition, we established the sensitivity of the CSGE protocol by two approaches. Firstly, we were able to correctly identify three patients with known mtDNA mutations who were included in the sample set. Secondly, the mtDNA-coding region in five patients was analysed both by the CSGE protocol and by direct sequencing, and comparison of the results suggested a high sensitivity and specificity for the two methods. Furthermore, previous data have suggested that the limit of detection of heteroplasmy in various heteroduplex screening assays is $<10\%$.[22]

LHON is a maternally inherited mitochondrial disease, causing blindness in young men. Most pedigrees harbour one of the three primary mtDNA mutations 3460G→A, 11778G→A or 14484T→C.[23] Here, the 3460G→A mutation was discovered in a young adult (patient Y), with isolated complex I deficiency and progressive myopathy as the sole manifestations of her mitochondrial disease (de Vries *et al*, in preparation), whereas her younger brother recently developed classical LHON. Interestingly, the mtDNA sequence of this patient was identical to that reported for a Dutch patient (patient S016) who had clinically typical

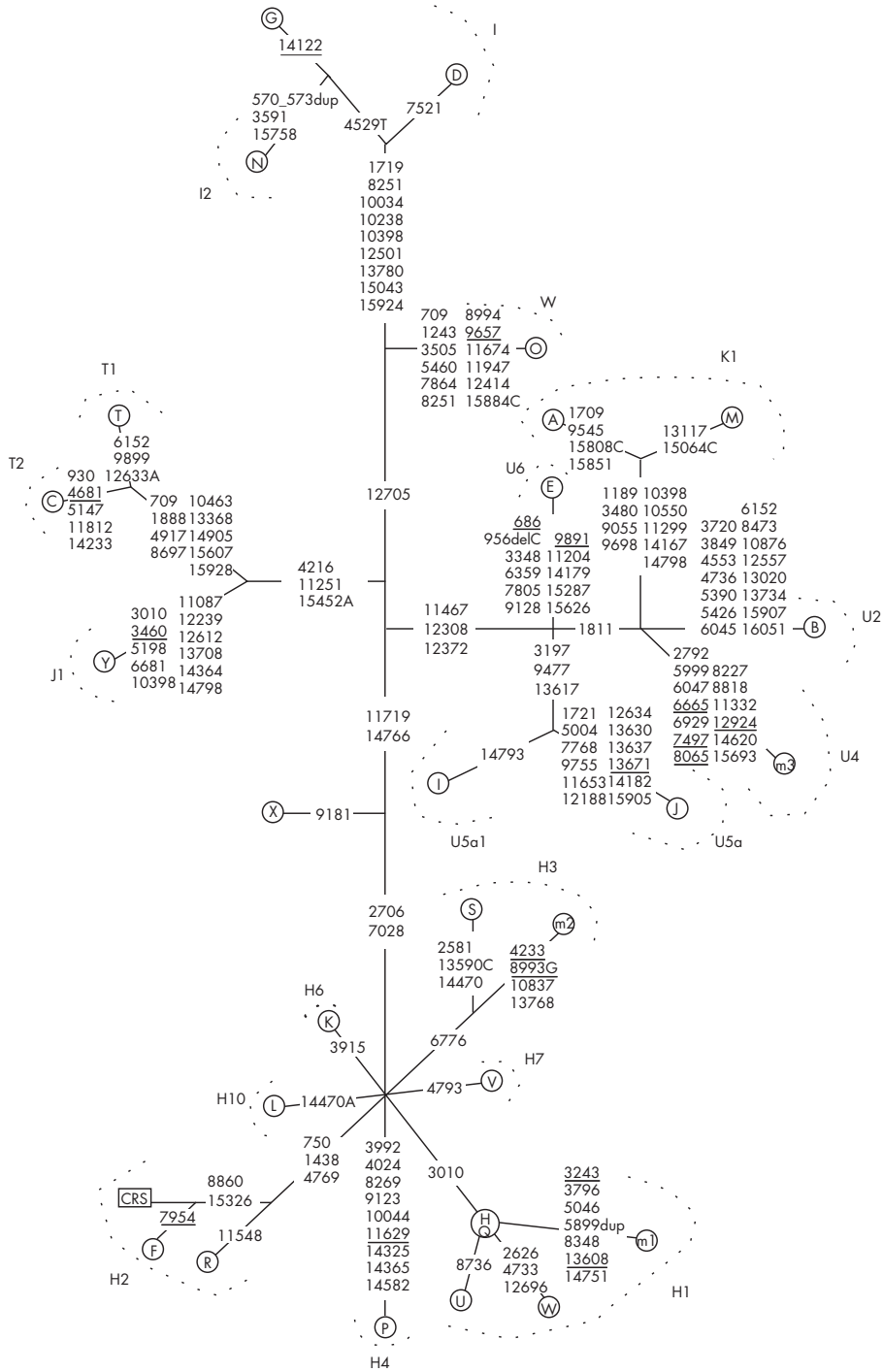

Figure 1 Mitochondrial DNA coding region sequences in 28 Dutch patients and young adults, shown in the form of a phylogenetic network. All the patients belonged to European haplogroups. The letters inside the nodes denote samples (table 1). Transversions are marked with the letter after the nucleotide number; otherwise the variants shown on the lines connecting the nodes are transitions. Haplogroups are marked with a dashed line. New or pathogenic variants are underlined. dup, duplication; del, deletion.

LHON.[24 25] There is a high probability that our patient Y and patient S016 belong to the same pedigree carrying the 3460G→A LHON mutation. Another primary LHON mutation, 11778G→A, has been reported recently in two siblings with complex I deficiency and encephalomyopathy, hearing impairment, optic atrophy and cardiac involvement.[11] Data on these siblings and on our patient Y suggest that the clinical phenotype caused by the primary LHON mutation is more variable than has been thought.

The pathogenic potential of the seven novel or rare variants detected in our series was estimated. The 4681T→C mutation in MT-ND2 was found in patient C, with isolated complex I deficiency and Leigh's disease. This patient had progressive encephalomyopathy and died from a respiratory failure at age 10 years. The genetic defect was thus compatible with the biochemical defect, providing strong evidence for pathogenicity. The 4681T→C mutation causes a replacement of the hydrophobic Leu71 by proline in the third transmembrane

Table 3 Novel and previously known pathogenic mitochondrial DNA variants found in 28 Dutch children and young adults

Synonymous substitutions		Non-synonymous substitutions	Substitutions in rRNA or tRNA genes
Novel variants			
4233T→C	11629A→G	4681T→C	686A→G
6665C→T	12924A→G	9891T→C	
7954T→C	13608T→C	14122A→G	
8065G→A	13671A→G		
9657C→T			
Pathogenic variants			
NA		3460G→A	3243A→G*
		8993T→G*	7497G→A*

NA, not applicable.
*Three children with definite pathogenic mutations were included in the sample set to examine the reliability of the conformation-sensitive gel electrophoresis protocol.

helix of the ND2 subunit of complex I, and although the conservation index of this amino acid replacement is only 36%, the position is highly conserved for hydrophobic amino acids. This suggests that the replacement within the α-helix could cause changes in the tertiary structure of the ND2 subunit, and consequently in the hydrophobic part of the complex.

The pathogenicity of the 4681T→C mutation has been proved by two methods. Firstly, sequencing of the entire coding region of mtDNA did not yield any other putative pathogenic mutation and, secondly, transfer of patient mitochondria into rho-zero cells transferred a decreased complex I activity. Furthermore, analysis of mitochondria from this patient yielded defective assembly of complex I, with a concomitant decrease in its quantity (Ugalde *et al*, in preparation).[26]

Interestingly, the mtDNA sequence of patient C differed from that of the Dutch patient S089 with LHON but without any of the primary LHON mutations only in two nucleotide positions.[24] Patient C harboured 4681T→C, whereas patient S089 had 3338T→C, which causes the substitution Val11Ala in the ND1 subunit. The 3338T→C substitution is a rather rare non-synonymous variant that has previously been reported in only eight other mtDNA sequences. The haplogroups of these eight sequences are U, D, G and N and subhaplogroup L2a, whereas patients C and S089 both belonged to haplogroup T. The high similarity between these two mtDNA sequences suggests that they have a recent common ancestor. Interestingly that both patients have a clinical phenotype associated with a mitochondrial disorder, and it is tempting to speculate that the combination of the mtDNA variants in the founder sequence may contribute to the OXPHOS system defect in these two patients.

Before further molecular analyses, the pathogenic potential of the novel mtDNA variants should be estimated. The probability of the variant being pathogenic increases if the nucleotide change is heteroplasmic, if it is absent in controls, if it is phylogenetically highly conserved or if it cosegregates with the disease in a maternal lineage.[27] In addition, various bioinformatic analyses may be used to predict changes caused by the replacement in the secondary and tertiary structures of the protein. The pathogenic role of heteroplasmic 14122A→G in MT-ND5 was excluded because of the low conservation of the amino acid position and because of an exchange between the non-polar amino acids isoleucine and valine. A rare 13630A→G substitution was found in a patient with combined complex I and III deficiency, but the nucleotide at position 13630 is not conserved and the A→G substitution has previously been found in the same mtDNA subhaplogroup, indicating that it is a rare haplotype-specific polymorphism.[5]

Two rare, highly conserved substitutions were identified in genes encoding cytochrome c oxidase subunits 1 and 3. The 6681T→C substitution in patient Y, who also harboured the 3460G→A LHON mutation, was considered to be a rare polymorphism, as it has previously been found in a sample belonging to the same subhaplogroup. The novel substitution 9891T→C was predicted to abolish a TSK phosphorylation site in cytochrome c oxidase subunit 3, but this defect did not correlate with the decreased complex I activity seen in patient E, and therefore the variant was not considered to be pathogenic. The remaining novel or rare variants were in rRNA or tRNA genes, and we would expect a generalised defect in the OXPHOS system if they were pathogenic. On the other hand, 686A→G and 12188T→C were found in patients with either isolated complex I or combined complex I and III deficiency, suggesting that these substitutions were polymorphisms. The total sequence variation caused by novel non-synonymous variants was estimated to be similar among the 25 patients and among the controls.

In conclusion, we resolved the cause of the OXPHOS system deficiency in 2 of the 25 patients. The 3460G→A mutation in MT-ND1 was discovered in a young adult with isolated complex I deficiency, and a novel 4681T→C mutation in MT-ND2 was found in a child with isolated complex I deficiency causing Leigh's disease. We were not able to find any evidence of pathogenicity in the remaining six novel or rare substitutions. These data exclude the role of mtDNA mutations as a cause of the OXPHOS system deficiency in the remaining 23 patients and suggest that it probably originated from mutations in the nuclear genes coding for respiratory enzyme subunits or assembly factors.

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Table 4 Novel and rare mitochondrial DNA variants found in patients with oxidative phosphorylation system deficiency

Patient	Tissue studied	Nucleotide variant	Haplogroup	Gene	Amino acid change	OXPHOS defect	Conservation index (%)
E	B	686A→G	U6	MT-RNR1	NA	I	NA
E	B	9891T→C	U6	MT-CO3	Ser229Pro	I	90
J	M	12188T→C	U5a	MT-TH	NA	I+III	NA
J	M	13630A→G	U5a	MT-ND5	Thr432Ala	I+III	28
C	F	4681T→C	T2	MT-ND2	Leu71Pro	I	36
G	F	14122A→G	I	MT-ND5	Ile596Val	Other*	26
Y	M	3460G→A	J1	MT-ND1	Ala52Thr	I	90
Y	M	6681T→C	J1	MT-CO1	Tyr260His	I	82

B, blood; F, fibroblasts; M, muscle; NA, not applicable; OXPHOS, oxidative phosphorylation.
*Decreased pyruvate oxidation and adenosine triphosphate production.

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