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Sequence analysis of the complete mitochondrial genome in patients with mitochondrial encephaloneuromyopathies lacking the common pathogenic DNA mutations

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Abstract

The purpose of this study was to identify novel mitochondrial deoxyribonucleic acid (mtDNA) mutations in a series of patients with clinical and/or morphological features of mitochondrial dysfunction, but still no genetic diagnosis. A heterogeneous group of clinical disorders is caused by mutations in mtDNA that damage respiratory chain function of cell energy production. We developed a method to systematically screen the entire mitochondrial genome. The sequence-data were obtained with a rapid automated system. In the six mitochondrial genomes analysed we found 20 variants of the revised Cambridge reference sequence [Nat. Genet. 23 (1999) 147]. In skeletal muscle nineteen novel mtDNA variants were homoplasmic, suggesting secondary pathogenicity or co-responsibility in determination of the disease. In one patient we identified a novel heteroplasmic mtDNA mutation which presumably has a pathogenic role. This screening is therefore useful to extend the mtDNA polymorphism database and should facilitate definition of disease-related mutations in human mtDNA.

Keywords: Mitochondrial encephaloneuromyopathies; mtDNA; Mutation; Polymorphism, Heteroplasmic; Homoplasmic

The mitochondrial disorders are a heterogeneous group of diseases typically manifesting in tissues with high-energy demand, such as nerve, and muscle. They may be caused by mutations in the nuclear genome or mtDNA [2]. Mitochondrial DNA is maternally inherited and fixes mutations 10–17 times faster than comparable nuclear DNA [3]. Diagnosis of human mitochondrial disorders relies on a combination of different approaches, including clinical analysis, investigation of lactate metabolism in vivo, biochemical measurement of respiratory chain activities, and morphological analysis. Confirmatory diagnosis is achieved by mutational analysis of mtDNA. Pathogenic mtDNA point mutations have been found either in transfer RNA (mt tRNA) or in structural genes of mtDNA. Over the past 12 years, numerous genetic causes of human mitochondrial disorders have been described [4,5]. The mtDNA mutations were all maternally inherited or originated de novo. Except for the mtDNA mutations causing Leber hereditary optic neuropathy, mitochondrial non-syndromic sensorineural hearing loss, and a form of mitochondrial hypertrophic cardiomyopathy [6–8], most other pathogenic mutations are heteroplasmic (i.e., a mixture of wild-type and mutant mtDNA in the same tissue or cell), and the mtDNA mutations manifest clinically when their percentage exceeds a tissue-specific threshold [9]. Non-pathogenic polymorphisms are normally homoplasmic [10].

However, a recent study showed that the great majority of DNA mutations in patients with mitochondrial

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disorders remained unidentified [11]. The unidentified mutations may occur in the nuclear genes or anywhere in mtDNA. The aim of this study was to explore the complete sequence of mtDNA by sequence analysis for novel mutations in tRNA and/or coding protein genes. Six unrelated patients with clinical and/or morphological features of mitochondrial dysfunction were investigated by this procedure.

Materials and methods

Patients

All patients were recruited in our Department of Neurological and Behavioural Sciences, and had clinical features highly suggestive of classical mitochondrial disorder.

P1. Sixty-eight-year-old woman with severe bilateral progressively worsening palpebral ptosis and dilatative cardiomyopathy. Mild myopathic changes characterized the EMG. Basal serum lactate was normal. A few COX-negative ragged red fibres were evident in muscle biopsy.

P2. Sixty-four-year-old man with progressive chronic ophthalmoplegia and mitochondrial myopathy. At age 54 years, first evidence of dysphagia, dysphonia, and palpebral ptosis, which progressively worsened. Neurological examination confirmed the clinical picture. EMG was myopathic and serum CK levels were elevated (193 U/L; v.n 20–170). Basal serum lactate was normal. Muscle biopsy suggested mitochondrial myopathy.

P3. Sixty-six-year-old man with muscular weakness and limb–girdle hypotrophy. EMG showed mild myopathy. Serum CK levels were elevated. Muscle biopsy suggested mitochondrial myopathy.

P4. Forty-eight-year-old woman with ataxia, dystonia, myoclonus-epilepsy, leucoencephalopathy, severe cognitive, impairment, and a 18-year history of progressively worsening of gait and cognitive condition. Epilepsy first appeared at the age of 15 years. Neurological examination revealed pyramidal features and confirmed dystonia. MRI showed a diffuse leucoencephalopathy and severe axonal damage. Muscle biopsy showed few COX-negative fibres. Other causes of white matter disorder, including metachromatic leucodystrophy, Krabbe, and other neurometabolic diseases were excluded.

P5. Thirty-two-year-old woman with seizures since 13 years of age. Neurological examination also revealed nystagmus and diffuse hypotonia. MRI showed signs of leucoencephalopathy. Basal serum lactate levels were normal. Skeletal muscle histochemistry showed mild mitochondrial abnormalities. The maternal line of the pedigree showed many subjects with diabetes.

P6. Fifty-two-year-old woman with bilateral neurosensory deafness, macular dystrophy, atypical pigmentary retinopathy, and somatic short stature. Serum lactic acid levels were elevated. MRI revealed hypotrophy of the corpus callosum with diffuse abnormalities of white matter. Muscle biopsy showed mild mitochondrial abnormalities.

In all cases, mutational analysis was negative for MELAS, MERRF, NARP mutations, and rearrangements of mitochondrial DNA.

DNA analysis

Total genomic DNA was extracted from skeletal muscle and/or blood according to standard purification protocols (Qiagen, QIAmp DNA Blood Midi Kit and QIAmp DNA Mini Kit). Total DNA underwent standard genetic screening. Because no known mtDNA mutation was found, we developed a strategy for sequencing the entire mtDNA genome. Using a subset of 42 primers for entire mtDNA amplification, mtDNA was amplified in twenty-one overlapping polymerase chain reaction (PCR) fragments of 800 base pairs (bp) to 1200 bp each. Double stranded PCR products were purified directly by the QIAquick PCR Purification Kit (Qiagen) and used for automated DNA sequencing; sequence-data were analysed using Chromas software and compared with the revised Cambridge reference sequence [1]. All substitutions that caused amino acid changes and heteroplasmic mutations were confirmed by PCR/restriction fragment length polymorphism (RFLP) analysis. Mismatch primers were generated when mutations did not gain or lose restriction enzyme sites. Enzyme digestion analysis was extended to all available family relatives and controls. Densitometric analysis was performed to estimate the amount of mutant mtDNA.

Pathogenicity criteria for mtDNA sequence changes

Specific criteria for mtDNA pathogenicity were in order of decreasing importance: (1) cosegregation of mutations with the clinical phenotype; (2) heteroplasmy; (3) absence of mutations in >100 healthy controls; (4) functional impairment in one or more respiratory chain (RC) enzyme complexes; (5) haplogroup divergency of identical mutations in different index patients; and (6) phylogenetic conservation of the affected nucleotide. In the case of homoplasmy, pathogenicity was mainly ruled out by the presence of the identical homoplasmic mtDNA mutation in healthy maternal relatives.

Results and discussion

We sequenced six mitochondrial genomes and identified a total of 20 novel variants (Table 1).

Their sites were distributed in this way: 15 mutations in protein coding regions (three are missense, the others are synonymous), four in ribosomal RNA genes (rRNA), and one in the D-loop region. All mutations were only specifically present in one case and had not been previously described (MITOMAP: http://www.gen.emory.edu/mitomap.html).

In our screening we observed one substitution in the heteroplasmic state, while all others were homoplasmic. PCR-RFLP analysis of mtDNA amplified from muscle and/or blood was only performed for the heteroplasmic mutation and the homoplasmic variants in protein coding regions that caused amino acid changes at sites with high evolutionary conservation.

Sequence analysis of muscle mtDNA in P5 revealed heteroplasmy for a C–T transition at nucleotide position 3340 (Fig. 1A) in the ND1 gene, which encodes subunit 1 of respiratory complex I (NADH dehydrogenase), and homoplasmy for two novel transitions. RFLP analysis of PCR-amplified fragments encompassing the mutant region confirmed that the mutation was heteroplasmic, with 30% mutant mtDNA in muscle and blood; blood from the patient's mother and sister had 30% and 40% mutant mtDNA, respectively (Fig. 1B). The mutation was absent from 207 unrelated patients affected with various encephalomyopathies. Biochemical analysis of the patient's skeletal muscle showed normal activity of all respiratory chain complexes (data not shown).

Table 1
Twenty novel substitutions in muscle mtDNA of six patients with mitochondrial encephalomyopathy [a]

Patient	Mutation detected	Gene affected	Amino Acid change
P1	1530 A–G (H)	12S rRNA	
	2294 A–G (H)	16S rRNA	
	5498 A–G (H)	ND2	Syn
	6518 T–C (H)	Cox I	Syn
	9266 G–A (H)	Cox III	Syn
P2	8014 A–T (H)	Cox II	Syn
	13098 A–G (H)	ND5	Syn
	14082 C–G (H)	ND5	Syn
P3	2849 G–A (H)	16S rRNA	
P4	12633 C–T (H)	ND5	Syn
	15295 C–T (H)	Cytb	Syn
P5	3158 ins T	16S rRNA	
	3340 C–T (h)	ND1	Pro–Ser
	6719 T–C (H)	Cox I	Syn
	8803 A–T (H)	ATPase6	Thr–Ser
P6	524 C del	D-Loop	
	8137 C–T (H)	Cox II	Syn
	8684 C–T (H)	ATPase6	Thr–Ile
	10142 C–T (H)	ND3	Syn
	13500 T–C (H)	ND5	Syn

For each variant we indicate homoplasmic (H) or heteroplasmic (h) state, mtDNA-region and whether it causes amino acid changes or is anonymous (syn).

[a] Found sequence variants include the following known polymorphisms. Patient 1 (750, A–G; 1438, A–G; 1811, A–G; 2706, A–G; 3423, G–T; 4703, T–C; 4769, A–G; 8860, A–G; 10506, A–G; 11467, A–G; 11719, G–A; 12308, A–G; 12372, G–A; 13934, C–T; 14139 A–G, 14766 C–T, 15326 A–G, 15454 T–C), patient 2 (709, G–A; 750, A–G; 1438, A–G; 2706, A–G; 3423, G–T; 4769, A–G; 7028, C–T; 8860, A–G; 15218, A–G; 15326, A–G; 16067, C–T; 16129, G–A), patient 3 (750, A–G; 1719, G–A; 3423, G–T; 4529, A–T; 4769, A–G; 8860, A–G; 10034, T–C; 10238, T–C; 10398, A–G; 11719, G–A; 12501, G–A; 12705, C–T; 13395, A–G; 13780, A–G; 14783, T–C; 15043, G–A; 15326, A–G; 15924, A–G; 16086, C–T; 16129, G–A), patient 4 (1438, A–G; 1888, G–A; 3423, G–T; 4216, T–C; 4769, A–G; 4917, A–G; 7028, C–T; 7853, G–A; 8697, G–A; 8860, A–G; 10463, T–C; 11251, A–G; 13368, G–A; 14766, C–T; 14905, G–A; 15110, G–A; 15326, A–G; 15452, C–A; 15607, A–G; 15928, G–A; 16126, T–C; 16163, A–G; 16186, C–T; 16189, T–C), patient 5 (519, del A; 520, del C; 750, A–G; 1438, A–G; 2218, C–T; 2706, A–G; 3591, G–A; 4769, A–G; 4985, G–A; 4991, G–A; 7028, C–T; 7581, T–C; 8860, A–G; 9027, C–G; 11467, A–G; 11719, G–A; 12308, A–G; 12372, G–A; 12879, T–C; 13104, A–G; 13422, A–G; 14070, A–G; 14364, G–A; 15148, G–A; 15954, A–C; 16038, A–G; 16163, A–G; 16183, A–G; 16188, C–T), patient 6 (523, del A; 1438, A–G; 1811, A–G; 2706, A–G; 4769, A–G; 8860, A–G; 10084, T–C; 11467, A–G; 11719, G–A; 12308, A–G; 12372, G–A; 13395, A–G; and 14569, G–A).

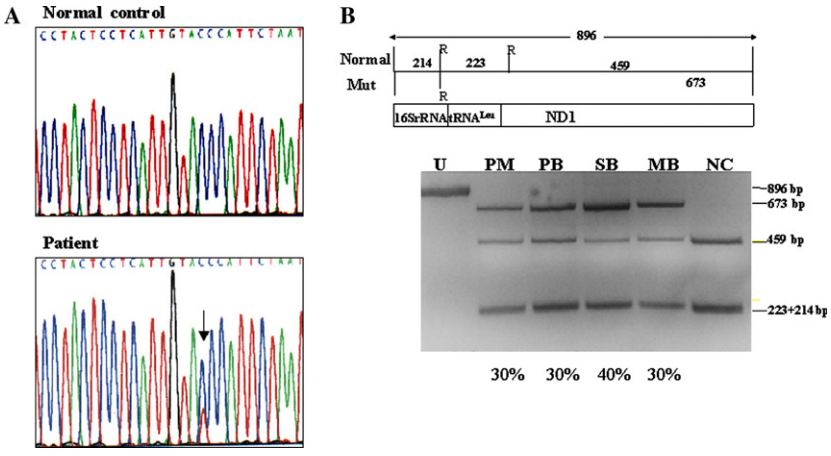

Fig. 1. (A) Direct sequencing of PCR-amplified muscle mtDNA, including ND1 gene, from a normal control and patient 5. Electropherograms of nucleotide positions 3324–3350 showing the heteroplasmic C–T transition at position 3340-nt in muscle DNA (arrow). (B) Restriction fragment length polymorphism analysis using *Rsa*I digestion to detect the nt-3340 mutation. (Top panel) The 896 bp region of mtDNA amplified by PCR; digestion with *Rsa*I (R) produced fragments with the indicated sizes (in bp). (Bottom panel) Electrophoresis on 3% MS agarose gel visualized under ultraviolet (UV) light after ethidium bromide staining. The wild-type amplified 896-bp fragment, including the ND1 gene, is cleaved by *Rsa*I into three fragments of 459, 223, and 214 bp. The C3340T mutation abolished one restriction site; so the mutant fragments contain only one cleavage site (673 and 214 bp). Expected sizes (in bp) are shown on the right. The percentages of mutant mtDNA are indicated below the picture. U, uncut; PM, patient's muscle; PB, patient's blood; SB, sister's blood; MB, mother's blood; and NC, normal control.

The pathogenic relevance of mutation C3340T is difficult to establish. This mutation satisfied several pathogenicity criteria, namely heteroplasmy in both muscle and blood, a Pro → Ser change at conserved amino acid position (P12S) in subunit ND1 (Table 2) and absence in >100 controls. On the other hand, it did not cosegregate with the clinical phenotype because it occurs in a heteroplasmic state with a similar percentage of mutant genome in all available tissues of the proband and in blood of her asymptomatic mother and sister. It is therefore classified as a variation "of unknown pathogenic relevance." However, our results suggest that it does not belong to common European polymorphisms [12]. The mutation load in brain tissue may exceed a tissue-specific threshold and manifest clinically. However, our findings show that this mutation is necessary but not sufficient for manifestation of the clinical abnormalities. As in other reported cases, environmental factors and/or nuclear genes may contribute to the pathogenic effect of the mutation. For example, multiple lines of evidence suggest that pathogenic expression of homoplasmic mtDNA has a two-locus genetic model, involving a primary mitochondrial mutation, and a nuclear modifier, such as a common functional polymorphism in a tissue-specific protein, possibly with mitochondrial location [6]. In many other cases, secondary aetiological factors, genetic or environmental, are involved, as for the 1555 point mutation in 12S mitochondrial ribosomal RNA (rRNA) responsible for up to 27% of cases of non-syndromic sensorineural deafness [13] and explaining the incomplete penetrance of primary Leber's hereditary optic neuropathy (LHON) [14].

Our screening detected 19 novel homoplasmic nucleotide changes without primary pathogenic contribution. Two missense mutations were found in the ATPase gene at np 8803 (A to T) and at np 8864 (C to T), in the patients 5 and 6, respectively. The mutation at np 8803 changes a highly conserved threonine residue into serine (Table 2), but this mutation was found in the asymptomatic family members of the patient. The other mutation alters a moderately conserved threonine to isoleucine (Table 2). These and all other homoplasmic variations were classified as neutral polymorphisms by the pathogenicity criteria (Methods) and are not in the MITOMAP database. The significance of homoplasmic variations in mtDNA remains uncertain, though several homoplasmic mutations have been implied in disease processes. The high degree of polymorphic variability is a major problem in assessing the pathogenicity of a new base change. In addition, polymorphisms may be relatively rare themselves and cosegregate with disease, confounding identification of the pathogenic mutation. There are exceptions to the rules of classical pathogenic mtDNA mutations, which are defined as a category of human, maternally inherited disorders characterized by a homoplasmic mtDNA pathogenic mutation with variable penetrance and stereotypical clinical expression, usually restricted to a single tissue. These disorders include LHON, mitochondrial non-syndromic sensorineural hearing loss, and a form of mitochondrial hypertrophic cardiomyopathy [6]. As well a non-synonymous homoplasmic mtDNA mutation was recently associated with severe COX deficiency, multiple neonatal deaths, and Leigh syndrome [15]. It is also possible that homoplasmic mt-tRNA mutations are considerably under-reported as causes of mitochondrial disorders, and they may indeed play a greater role in the development of cardiomyopathy than previously thought [8].

Given the clinical and/or muscle biopsy features and the absence of mtDNA deletions and heteroplasmic point mutations of the individuals studied so far, the novel mtDNA variants which we have identified may well play a direct or indirect role in determining disease. Two or more variants in the same patient may also have a role explaining phenotype diversity in humans. In addition, we found many other known polymorphisms (Table 1) and deduced the frequency of these polymorphisms in our patients. Some variants are more frequent than others; in particular, we found the 8860 G in 100% of the cases, the 1438 G and 4769 G in the 83% of the cases, the 750 G, 2706 G, 3423 T, and 15326 G in the 66% of the cases, while some known polymorphisms are present only in one patient. There is evidently a different genetic mitochondrial background which can further determine the phenotype, or, as previously discussed for the novel C3340 T mtDNA mutation, environmental factors could interact with genetic factors (mitochondrial or nuclear or both), amplifying their effect [16,17]. All these findings may have useful implications for genetic counselling.

In conclusion, complete sequencing of the mitochondrial genome allowed us to detect both novel and already described mtDNA variants, some of which are candidates for further studies to try to establish the relationship between their incidence and their role in determining disease. Our findings also suggest that it would be worthwhile investigating hot spot nuclear genes associated to mitochondrial disease.

Table 2
Evolutionary conservation of novel aminoacid changes in the patients

	3340 ND1	8803 ATPase6	8684 ATPase6
Patient	LLIV **S** ILIA	PHSF **S** PTTQ	LIKL **I** SKQM
Human	LLIV **P** ILIA	PHSF **T** PTTQ	LIKL **T** SKQM
Gorilla	LLIV **P** ILIA	PHSF **T** PTTQ	LIQL **T** SKQM
Chimpanzee	LLIV **P** ILIA	PHSF **T** PTTQ	LIQL **T** SKQM
Mouse	TLLV **P** ILIA	PHTF **T** PTTQ	LVKL **I** IKQM
Cow	MLII **P** ILLA	PHSF **T** PTTQ	MLQL **V** SKQM
Equus	LLIV **P** ILLA	PHSF **T** PTTQ	LVQL **T** SKQM
Chicken	SYIL **P** ILIA	PYTF **T** PTTQ	FTHL **I** TKQL
Xenopus	LYIA **P** ILIA	PYTF **T** PTTQ	FLHN **F** TTIF
Fruit fly	LLIV **C** VLVS	PYIF **T** STSQ	----------

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