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ARTICLE

LHON/MELAS overlap syndrome associated with a mitochondrial *MTND1* gene mutation

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Pathogenic point mutations in the mitochondrial *MTND1* gene have previously been described in association with two distinct clinical phenotypes – Leber hereditary optic neuropathy (LHON) and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS). Here we report the first heteroplasmic mitochondrial DNA (mtDNA) point mutation (3376G > A) in the *MTND1* gene associated with an overlap syndrome comprising the clinical features of both LHON and MELAS. Muscle histochemistry revealed subtle mitochondrial abnormalities, while biochemical analysis showed an isolated complex I deficiency. Our findings serve to highlight the growing importance of mutations in mitochondrial complex I structural genes in MELAS and its associated overlap syndromes.
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Introduction

The human mitochondrial genome (mtDNA) is subject to an extensive range of molecular defects that are associated with a wide range of clinical presentations.[1] Many patients present with particular neurological syndromes due to a specific mtDNA mutation, but there is widespread clinical and genetic heterogeneity.[2] One of the most common mitochondrial syndromes is mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) in which approximately 80% of cases harbour a 3243A > G point mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene (MTTL1).[3] Rarer mutations in *MTTL1* and other transfer RNA genes also cause MELAS, as do mutations in the mitochondrial *MTND* genes encoding structural subunits of respiratory chain complex I, most notably in *MTND5* (13513G > A and 13514A > G),[4–6] *MTND6*[7] and more recently, *MTND1*.[8]

Recently, the clinical spectrum of mitochondrial disorders has broadened with the identification of overlap syndromes in which patients present with the cardinal features of more than one recognised phenotype, such as MELAS/Leigh's[9] and MELAS/Leber's hereditary optic neuropathy (LHON).[10] Surprisingly, mutations causing MELAS/LHON syndrome are predominantly found in *MTND5* and have not been described in any of the three *MTND* genes (*MTND1*, *MTND4* and *MTND6*) that harbour primary LHON mutations. Here we describe a patient with a MELAS/LHON overlap syndrome and isolated complex I deficiency in skeletal muscle due to a novel, heteroplasmic mutation in the *MTND1* gene.

Case report

The patient is a 43-year-old woman (II-3 in Figure 1a), who presented with migraine with aura and convulsions at the

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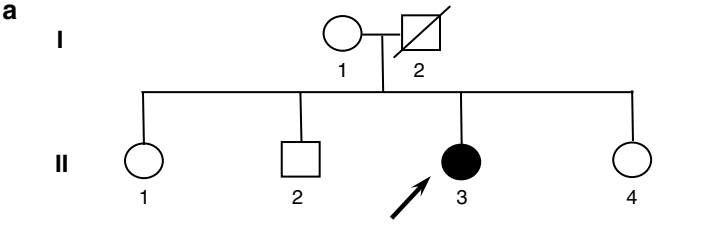
age of 20 years. Status epilepticus at 25 years of age was complicated by cardiorespiratory arrest, and following resuscitation a left hemiparesis and 'tunnel vision' were noted. Worsening of her left hemiparesis was noted at age 30 years, along with incoordination and ataxia. There continued to be partial seizures and episodes of migraine, and she developed deafness and myoclonus. Between the ages of 41 and 43 years, her visual acuity deteriorated from 6/24 on the right and 6/18 on the left to hand movement and counting fingers, respectively. She has bilaterally pale optic discs, left side hemiparesis, occasional myoclonus, mild bilateral finger–nose ataxia and requires a wheelchair for mobilisation outdoors. There is no family history of neuromuscular disease, but her father, older brother and older sister have had or have migraine (Figure 1a). Stroke was diagnosed in old age in her deceased father and paternal grandfather. The father (I-2) had migraine, cerebrovascular disease, intermittent claudication, an aortic aneurysm and ischaemic heart disease, consistent with generalised atherosclerosis. The older sister (II-1) suffered from migraine in childhood, as did the brother (II-2) whose own son has epilepsy and learning disability.

Urinary screening for thymidine was negative. Postexercise lactate was elevated in blood (5.1 mmol/l, reference range 0.6–2.4) and cerebrospinal fluid (CSF) (3.3 mmol/l, reference range 1.2–2.1). Brain magnetic resonance imaging (MRI) revealed areas of high T2/FLAIR signal involving both occipital lobes and the thalami (Figure 1). Visual evoked potentials currently show bilateral delay (P100 latencies are 161 ms on the right and 143 ms on the left) with normal amplitudes (5.67 and 4.13 μV, respectively). Electroretinography was essentially normal. A quadriceps muscle biopsy was suggestive of mitochondrial myopathy, with mild increased red staining on the modified Gomori trichrome apparent in some fibres, but succinate dehydrogenase histochemistry failed to demonstrate significant subsarcolemmal enzyme activity. Cytochrome *c* oxidase (COX) histochemistry was unremarkable.

Methods

The activities of the individual respiratory chain complexes were measured in a post 600 g_{av} supernatant as previously described and expressed relative to the activity of the matrix marker enzyme citrate synthase.[11] Total DNA was extracted from muscle, circulating lymphocytes and urinary cell sediments and screened for the 3243A>G mutation as previously described.[12] The coding sequence of the mitochondrial genome was amplified in overlapping fragments using a series of M13-tailed oligonucleotide primer pairs with DNA isolated from skeletal muscle as template.[13] PCR products were sequenced using Wellred Quickstart dye terminator cycle sequencing chemistries on a CEQ 8000 Genetic Analyser (Beckman Coulter, High Wycombe, UK), and directly compared to the revised



Family member	Clinical data	3376G>A mutation load		
		Muscle	*Blood*	*Urine*
I-1	-	ND	0%	0%
I-2	Migraine, stroke, aortic aneurysm, ischaemic heart disease	ND	ND	ND
II-1	Childhood migraine	ND	0%	0%
II-2	Migraine	ND	0%	0%
II-3	**LHON/MELAS**	**98%**	**18%**	**67%**
II-4	-	ND	0%	0%

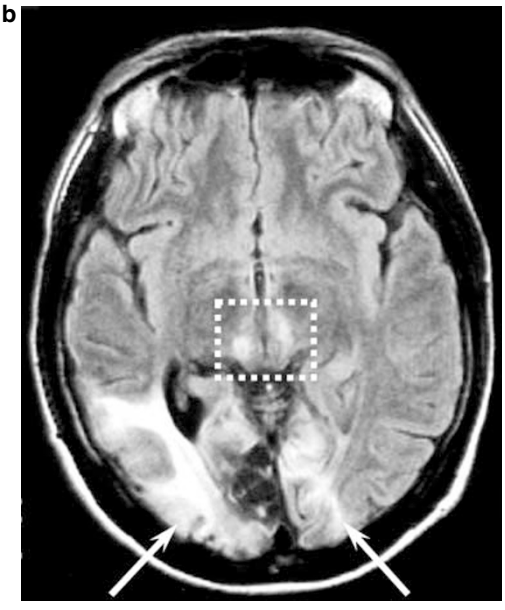


Figure 1 (**a**) Pedigree highlighting the clinical presentation in the family and the level of the 3376G>A mutation in family members available for testing; the proband is shown by an arrow. ND = no determined. (**b**) Brain magnetic resonance imaging (MRI) showing an axial FLAIR section. There is predominantly high signal involving both occipital lobes (arrows), with the right worse than the left; on the right this is associated with prominent encephalomalacia. High signal is also noted at the junction of the cerebral peduncles and both thalami (boxed region).

Cambridge reference sequence (rCRS) using CEQ 8000 Sequence Analysis and CEQuence Investigator software (Beckman Coulter).

Given that the 3376G>A mutation ablates a recognition site for the restriction endonuclease *Bsi*EI, mtDNA heteroplasmy was assessed using PCR-RFLP analysis. A 130 bp PCR product spanning the mutation site was amplified using a forward primer 5′ TTTACAGTCAGAGGTTCAATTCCTC 3′ (nt 3274–3295) and a reverse mismatch primer 5′ GTT GTATATAGCCTAGAATTTTTCG**G**T 3′ (nt 3403–3377; mismatch nucleotide shown in bold). Prior to the last cycle of PCR, 5 μCi [α-^{32}P]dCTP (3000 Ci/mmol) was added. Labelled products were precipitated, digested with 10 U *Bsi*EI, separated through a 12% nondenaturing polyacrylamide gel, and the radioactivity in each fragment was quantified using the ImageQuant software (Molecular Dynamics). In wild-type mtDNA, the 130 bp amplimer is cut to two smaller products of 105 and 25 bp, whereas the 3376G>A mutation abolishes this site. To ensure complete digestion by the *Bsi*EI restriction endonuclease, each sample to be digested was supplemented with a 594 bp ^{32}P-labelled PCR product containing a single restriction site for this enzyme to serve as an internal control; *Bsi*EI cuts this 594 bp product into two smaller fragments of 425 and 169 bp.

Results

Although routine histology and histochemistry of the muscle biopsy was uninformative, subsequent biochemical investigations revealed an isolated defect of complex I activity in skeletal muscle with normal activities of both complexes II and IV (Table 1), prompting extensive mtDNA studies. Having excluded the 3243A>G MELAS mutation in muscle by PCR-RFLP analysis, we determined the sequence of the entire mtDNA coding region. This identified a number of documented polymorphic variants,[14] together with a silent change (15817A>G) in the *MTCYB* gene and two unreported sequence changes (3376G>A and 3865A>G) in the *MTND1* gene that would be consistent with the observed complex I deficiency and therefore potentially pathogenic. The 3865A>G change predicts a moderate amino-acid substitution (I187V) at a residue that is moderately conserved between species and was present at homoplasmic levels in blood DNA of both the index case and her clinically unaffected mother (not shown). The 3376G>A mutation (Figure 2a) predicts an amino-acid substitution (E24K) from a negatively charge residue to a positively charged residue at a highly conserved site of the ND1 protein (Figure 2b)[15] and on this basis, we chose to further investigate the 3376G>A *MTND1* gene mutation as the likely causative mutation.

Table 1 Respiratory chain activities in muscle homogenate

Complex	Controls (mean ± SD) $n = 20$	Patient
Complex I/citrate synthase	0.168 ± 0.025	0.060 (36%)
Complex II/citrate synthase	0.232 ± 0.035	0.207
Complex IV/citrate synthase	1.65 ± 0.78	1.69
Complex I/complex II	0.724	0.290 (40%)

The % residual complex I activity, expressed relative to the activity of either citrate synthase or complex II, is shown in brackets. Enzyme activities are expressed as nmol NADH oxidised min^{-1} unit citrate synthase^{-1} for complex I, nmol DCPIP reduced min^{-1} unit citrate synthase^{-1} for complex II (succinate:ubiquinone-1 reductase) and the apparent first-order rate constant sec^{-1} unit citrate synthase^{-1} for complex IV ($\times 10^3$). DCPIP = 2,6-dichlorophenol–indophenol; SD = standard deviation.

Last-cycle hot PCR-RFLP analysis of patient tissues showed that the 3376G>A mutation was heteroplasmic and present in skeletal muscle, an affected tissue, at very high levels (98%), while mitotic (blood and urinary epithelial) cells harboured lower levels (18 and 67% respectively), a consistent finding in many patients with mitochondrial genetic defects (Figure 2c). Interestingly, we could not detect the mutation in urinary epithelial cells from the patient's clinically unaffected mother, and while there may be very low but detectable (<1%) levels in her blood (Figure 2c, lane 7), the 3376G>A mutation was not apparent in either blood or urine of the patient's three siblings (Figure 1a), suggesting that the 3376G>A mutation arose sporadically in our patient, similar to the recently described *MTND1* mutations in patients with MELAS.[8] Additionally, the 3376G>A mutation was not present in over 200 of our own control sequences or represented in a large database of >1000 human mtDNA sequences (http://www.genpat.uu.se/mtDB/index.html).

Discussion

An underlying mitochondrial respiratory chain disorder was suspected on account of the patient's clinical phenotype, and confirmed by biochemical studies. Sequencing of the mitochondrial genome revealed a 3376G>A transition in the *MTND1* gene that was heteroplasmic, present at highest levels in skeletal muscle and not evident in the control population. Together these data fulfil the accepted criteria for a novel mutation to be recognised as pathogenic[16] and as such we believe that the 3376G>A mutation is responsible for the clinical presentation in this patient as a result of the biochemical defect in complex I activity.

Several interesting points are worthy of further comment. The first relates to the histochemical findings which revealed only very subtle mitochondrial changes, and important observation in relation to the diagnosis of patients with mitochondrial disease. Our own experience

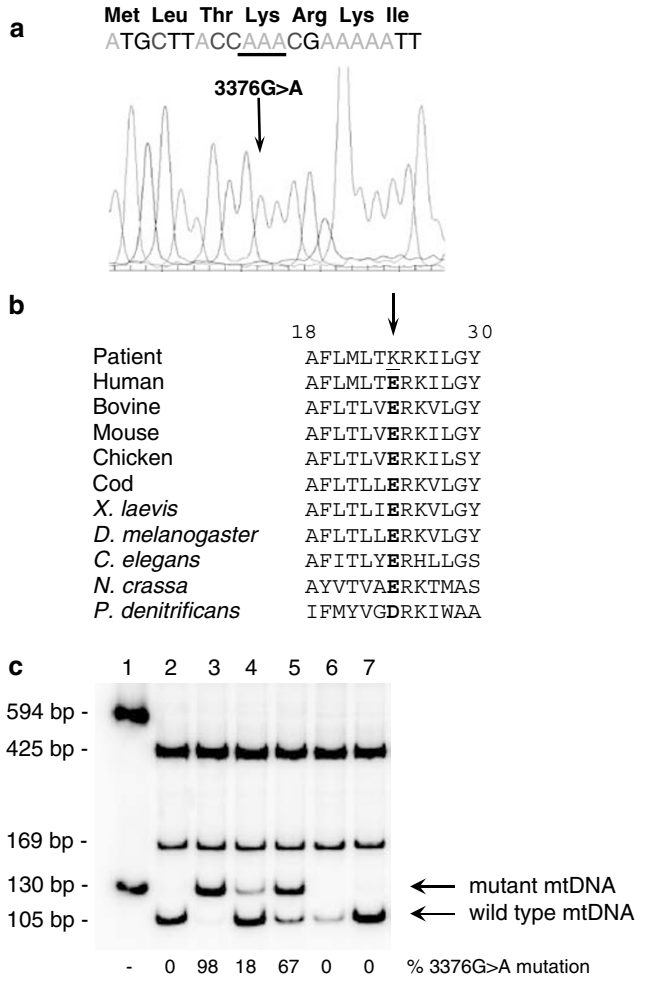


Figure 2 Molecular genetic analysis of the 3376G > A *MTND1* mutation. (**a**) Electropherogram of the *MTND1* gene showing the sequence change in the patient and the predicted alteration to the amino-acid sequence. (**b**) Multiple sequence alignment of the ND1 protein (amino acids 18–30) highlighting the evolutionary conservation of a negatively charged amino-acid at position 24 (shown in bold). (**c**) Quantification of the relative amounts of mutant and wild-type mtDNA in patient tissues by last hot cycle PCR-RFLP analysis. The 3376G > A mutation abolishes a *Bsi*EI restriction site that normally cuts a 130 bp PCR product into fragments of 105 and 25 bp (not shown) in wild-type mtDNA. As this assay relies on complete digestion, samples were supplemented with a 594 bp region of mtDNA containing an additional *Bsi*EI site; complete digestion of this to fragments of 425 and 169 bp served as an internal control. Lane 1, uncut sample; lane 2, control; lane 3, patient's skeletal muscle; lane 4, patient's blood; lane 5, patient's urinary epithelial cells; lane 6, mother's urinary epithelial cells; lane 7, mother's blood.

of investigating adults with pathogenic mtDNA abnormalities suggests that the histochemical assessment of mitochondrial enzyme activity in muscle is the most important diagnostic tool in the screening of patients with mtDNA disease.[17] Although there are no reliable cytochemical assays of complex I activity, some patients with *MTND* gene mutations exhibit significant subsarcolemmal mitochondrial accumulation as 'ragged red' fibres.[4] These have been described in a patient with an intragenic inversion within the *MTND1* gene[18] and one of the patients with MELAS described by Kirby *et al*,[8] though is not a consistent finding for all mutations described in this protein. Indeed, the most common *MTND1* gene mutation, the 3460G > A LHON mutation, does not show abnormal mitochondrial histochemistry.

Second, all previously reported *MTND1* mutations including the 3460G > A LHON mutation result in amino-acid changes in the extramembrane loops of the ND1 protein that face the mitochondrial matrix.[8,18] The amino-acid substitution predicted by the 3376G > A mutation in our patient occurs at a similar position at the junction between the first transmembrane helix and the subsequent hydrophilic loop (see Kirby *et al*[8] for illustration).

Finally, the rapid and progressive visual loss exhibited by our patient is compatible with the optic nerve changes seen in LHON patients. Optic nerve disease in this case has been supported by clinical and electrophysiological data. Comparable findings have been reported in other patients with overlap syndromes harbouring mutations in the *MTND5* gene,[6,10] though most mutations causing isolated LHON are found in other (*MTND1*, *MTND4* and *MTND6*) genes.[19]

In conclusion, these data indicate that this mutation is causative and further illustrates both the genetic and phenotypic diversity of mtDNA disorders and the role of complex I dysfunction in both MELAS and LHON. Histochemical findings may be unremarkable in some cases, with extensive sequencing of the mtDNA coding region required to determine the precise causative defect.

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