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SHORT REPORT

An mtDNA mutation, 14453G→A, in the NADH dehydrogenase subunit 6 associated with severe MELAS syndrome

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We report a novel point mutation in the gene for the mitochondrially encoded ND6 subunit of the NADH:ubiquinone oxidoreductase (complex I of the respiratory chain) in a patient with MELAS syndrome. The mutation causes a change from alanine to valine in the most conserved region of the ND6 subunit. The patient was heteroplasmic for the mutation in both muscle and blood, but the mutation was not detected in the patient's mother. A marked reduction of complex I activity was found in the patient's muscular tissue. This is the first report of a mutation in the ND6 subunit causing MELAS. Our data confirm the genetic heterogeneity in mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes syndrome, and confirms that MELAS can be caused by mutation in polypeptide-coding mtDNA genes. *European Journal of Human Genetics* (2001) **9,** 805–809.

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Introduction

Molecular characterization of mitochondrial encephalomyopathies has progressed rapidly, and many pathogenic point mutations as well as deletions in the mitochondrial DNA (mtDNA) have been reported.[1] The mutations can be divided into two groups, one affecting the mitochondrial protein biosynthesis in general (tRNA and rRNA genes), the other causing amino acid substitutions in the enzyme complexes involved in the oxidative phosphorylation (protein-encoding genes).[1,2]

Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) is a genetically heterogeneous disorder. The majority of MELAS cases are associated with the mtDNA 3243A→G point mutation in the gene for $tRNA^{Leu(UUR)}$. In about 10% of the patients other mtDNA mutations are expected to be causal for MELAS. To date, five such mutations in the gene for $tRNA^{Leu(UUR)}$, one in the gene for $tRNA^{Phe}$ and one in the gene for $tRNA^{Val}$ have been identified. Furthermore, five mutations in the mitochondrial protein-coding genes, COXIII (9957T→C), ND5 (13513G→A), and CYB (14787del4) associated with MELAS have been published.[1,3] As a whole, the clinical spectrum of diseases caused by mtDNA mutations is very wide and point mutations in mtDNA protein-coding genes have been found associated with various clinical presentations.[4] The most well known are the point mutations in ND1 (3460G→A),[5] ND4 (11778G→A)[6] and ND6 (14484T→C)[7] causing Leber hereditary optic neuropathy (LHON). Classical LHON is presenting with acute to subacute central vision loss, but pedigrees in which both LHON and dystonia are segregating, due to point mutations in the gene encoding ND6, have been reported.[8,9]

Here we report a 6-year-old patient with the typical clinical features of MELAS and no signs of LHON, who harbours a novel mitochondrial point mutation in the gene encoding

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the ND6 subunit of NADH:ubiquinone oxidoreductase (complex I of the respiratory chain).

Materials and methods

Patients

Our patient is a girl, born in 1994, with normal development until the age of 2 years. Between 2 and 3 years of age she had at least five episodes of vomiting followed by ketotic acidosis. When the girl was around 4 years old she developed myoclonic epilepsy and she had occasional simple partial seizures. At the same time, she developed general weakness and ataxia with intermitting dystonia. She was admitted to a hospital where the first MR-scan showed cerebellar hypoplasia and infarctions at the right hemisphere. A lumbar puncture was performed and it showed lactate of 4.69 mmol/l (normal range: 1.2 – 1.6) and pyruvate of 0.265 mmol/l (normal range: 0.085 – 0.132). Further ultrasound examination of the muscle showed increased echogenicity. A muscle biopsy revealed lipid storage myopathy with some COX-negative fibres. Electron microscopy showed normal mitochondria. The girl developed episodes of lethargy, lactic acidosis and alternating uniparesis and she was hospitalised during one of these episodes under the suspicion of non-convulsive status. This could be approved by EEG, showing some abnormalities at the occipito-temporal region and no normal background activity. A new MR-scan was performed and it now showed multi-infarction in both hemispheres, involving most of cerebral cortex and basal ganglia lesion (symmetrical). In addition to the MR-imaging picture an MR-spectroscopy showed high lactate spikes but N-acetylaspartate was within the normal levels.

Ophthalmological examination revealed no oculomotor anomalies. She had normal fixation but due to her bad general condition no measurements of visual acuity, colour vision or visual fields were obtainable. Ophthalmoscopy revealed optic nerves with a normal colour and no capillary abnormalities of the peripapillary area. Nevertheless, monocular and binocular flash stimulations did not show any VEP response (visual evoked potentials).

The mother was healthy, with no history of a mitochondrial disorder.

Isolation of DNA

Total genomic DNA from blood, muscle, and filter paper (Guthrie card) blood spots was isolated according to standard procedures.

mtDNA amplification and sequence analysis

The mitochondrial genome was PCR amplified in two segments using Expand™ Long Template PCR System (Boehringer, Mannnheim) as described.[10] Sequencing was performed using BigDye sequencing kit on an ABI 377 DNA sequencer, both from Applied Biosystems. The entire mtDNA was sequenced, except part of the D-loop. (Primer sequences are available on request). The obtained sequences were compared with the revised Cambridge reference sequence (AC J01415).[11,12]

Restriction analysis

The classical MELAS mutation 3243A→G was tested for by restriction analysis using the enzyme *Apa*I. The mutation 14453G→A does not introduce or destroy any useful restriction site. We designed a mismatch L-strand primer, corresponding to the mtDNA position 14421-14452 (5′-CTGACCCCCATGCCTCAGGATACTCCTCAGTA-3′) and a 20-mer H-strand primer corresponding to positions 14580 – 14561. The mismatch primer creates a *Mae*III restriction site when the mutation is present.

After amplification with the mismatch primer system, the PCR products were incubated with *Mae*III and subjected to electrophoresis in a 1.5% agarose gel subsequently stained with ethidium bromide.

Quantification of mutated mtDNA (heteroplasmy analyses)

The relative proportions of mutant and wild type mtDNA (degree of heteroplasmy) were determined by solid phase minisequencing, as previously described.[13]

Biochemical analysis

Mitochondrial enzyme activities were measured in post-nuclear supernatants of frozen skeletal muscle tissue as described[14–16] with some modifications. The activities of respiratory chain complexes I-IV are expressed relative to the matrix enzyme citrate synthase to account for different mitochondrial contents.

Results

The routinely assayed MELAS mutation 3243A→G was absent. When the result from the enzyme analysis showed a decreased activity of complex I in muscle (Table 1), a comprehensive sequence analysis of the patient's mtDNA was performed. We sequenced the entire mtDNA, except part of the D-loop, using DNA isolated from a muscle biopsy.

The sequence analysis of the patient's muscle mtDNA revealed homoplasmy for three transitions and heteroplasmy for one transition when compared to the Cambridge reference sequence (Table 2).[11,12]

Heteroplasmy for the missense mutation 14453G→A was found in both muscle (Figure 1) and blood. The presence of the mutation was confirmed by *Mae*III restriction analysis (Figure 2). The mutation was undetectable in the mother's blood and was not found in 50 randomly chosen healthy controls either. For a more conclusive determination of the relative proportions of mutant and wild-type mtDNA, we performed solid-phase minisequencing, and found that the mutation was present in 82% of mtDNA of the patient's

Table 1 Activities* of mitochondrial enzymes in muscle

	Patient	*Controls* (n=10) *Median*	*Range*
Complex I	0.094	0.333	0.289–0.567
Complex II	0.303	0.292	0.244–0.407
Complex III	1.300	1.690	1.080–2.230
Complex IV	4.780	3.250	2.260–4.570
Citrate synthase	108	155	65–224

*Values are expressed as mU/mU citrate synthase, except for citrate synthase activity, which is given in mU/mg protein

Table 2 Results from the sequencing of muscle mtDNA

Mutation	*Gene/region*	*Heteroplasmy*	*Amino acid change*	*Reference*
549C→T	control region	no		[1]
5628T→C	MTTA	no		this study
13535A→G	ND5	no	N400S	this study
14453 G→A	**ND6**	**yes**	**A74V**	**this study**

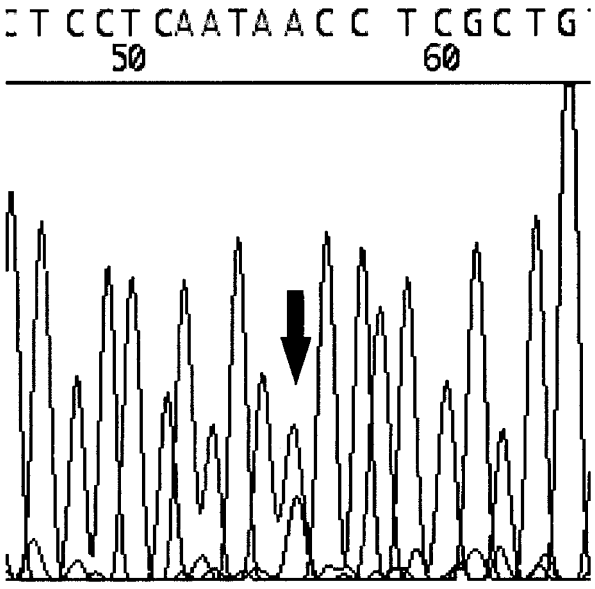

Figure 1 Sequence of part of the patient's ND6 gene (muscle). Arrow indicates the nucleotide 14453.

muscle, 78% in blood, and 75% in blood extracted from the Guthrie card.

Discussion

Molecular analysis of the mtDNA from a 7-year-old girl with MELAS syndrome, and an isolated deficiency of respiratory chain complex I in muscle, revealed heteroplasmy for an ND6 mutation, 14453G→A, which we strongly believe is the pathogenic mutation in this patient. The mutation changes codon 74 in the ND6 gene from an alanine to a valine codon (A74V). Seven other point mutations involving ND6 (Table 3) have been reported as pathogenic, all associated with LHON, LHON/dystonia or LHON/encephalopathy.[6,8,17–20] The ND6 is one of the seven mitochondrially encoded subunits of complex I. Studies on cell lines that are homoplasmic for a frameshift mutation in ND6 have given evidence for a role for this subunit in the assembly of complex I.[21] The pathogeni-

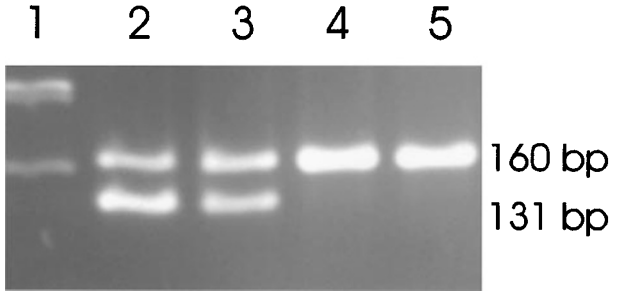

Figure 2 *Mae*III restriction digest for the 14453G→A mutation in the patient, her mother and a normal control. Normal (wt) sequence lacks the *Mae*III site (160 bp). Mutant sequence introduces a *Mae*III site (131 bp and 29 bp). Lane 1: MW marker; lane 2: patient (muscle); lane 3: patient (blood); lane 4: the mother (blood); lane 5: control (blood).

Table 3 Pathogenic mtDNA mutations involving the ND6 subunit gene

Nucleotide position and change	*Amino acid changes in ND6*	*Phenotype*	*Reference*
14484T→C	M64V	LHON	[7]
14568C→T	G36S	LHON	[18]
14498T→C	Y59C	LHON	[18]
14482C→T	M64I	LHON	[18]
14484T→C (ND1: 4160T→C/4136A→G)*	M64V	LHON/encephalopathy	[19]
14459G→A	A72V	LHON/dystonia	[8]
14596A→G (ND4: 11696G→A)*	I26M	LHON/dystonia	[17]
14495A→G	L60S	LHON	[20]
14453G→A	**A74V**	**MELAS**	**this study**

*Secondary mutations.

city of the previously reported 14484T→C and 14459G→A mutations is widely recognised and the effect of these mutations on the actitivity of complex I has been extensively studied.[22,23] The 14484T→C (M64V) mutation significantly increases the sensitivity of complex I to inhibitors binding to the ubiquinone site[22] and studies of transmitochondrial cybrids have shown that the 14459G→A mutation causes a drastic reduction in the actitivity of complex I.[23] The isolated decrease in complex I activity observed in our patient strongly suggests that the 14453G→A (A74V) mutation has the same effect on the ND6 polypeptide as the 14459G→A mutation (A72V), considering the identical amino acid changes and the close proximity of the two altered amino acids.

It is striking that all pathogenic ND6 mutations affect a stretch of amino acids near the N-terminus of this subunit. This region is the most conserved region in the ND6 polypeptide and is believed to be part of a single membrane-spanning domain now considered as a hot spot for mutations.[20] All previously reported ND6 mutations are associated with the LHON phenotype alone or as a LHON/dystonia syndrome. Our patient did not reveal any sign of atrophy of the optic nerve and is severely affected, with biochemical signs typical for a respiratory chain defect such as lactic acidosis. Patients with one of the three common LHON mutations are usually not presenting with a respiratory chain abnormality, although decreased activitiy of complex I can be demonstrated *in vitro*.[24]

In our patient the percentage of mutated mtDNA is high and only slightly lower in blood than in muscle. LHON patients are usually homoplasmic in blood. This is in contrast to most cases with the MELAS mutation 3243A→G, in whom the load of mutated mitochondria is almost invariably much lower in blood than in muscle. In LHON the optic nerves are normally involved at a later age and the ophthalmological findings in our patient do not preclude LHON to appear at a later time. The abolished VEP might be explained by the multi-infarctions of the cerebral hemispheres.

It is difficult to explain why the A74V mutation should cause such a severe phenotype, compared to the cases with LHON with or without dystonia described for the A72V mutation. One possible explanation is that one or more of the other mutations found in our patient's mtDNA act synergistically with 14453G→A in producing the pathological phenotype comparable to what has been proposed for the ND6/14484T→C plus the ND1/4160T→C for LHON/encephalopathy, and also for the ND6/14596A→G plus the ND4/11696G→A for LHON/spastic dystonia.[17,18] In addition to the 14453G→A mutation we found two other novel mutations in the mtDNA of our patient, 5628 T→C ($tRNA^{Ala}$) and 13535A→G (ND5), which might contribute to the decrease in activity of complex I.

In conclusion we believe that the 14453G→A mutation is the pathogenic mutation in our patient. This is in line with the idea that the helix C region in the ND6 polypeptide is a hot spot for pathogenic mutations, and that MELAS can be due to mutations in the protein-coding mtDNA genes.

It still remains to be explained how different mutations in the same gene can cause such completely different phenotypes as LHON and MELAS.

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