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MtDNA mutations are a common cause of severe disease phenotypes in children with Leigh syndrome

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ABSTRACT

Leigh syndrome is a common clinical manifestation in children with mitochondrial disease and other types of inborn errors of metabolism. We characterised clinical symptoms, prognosis, respiratory chain function and performed extensive genetic analysis of 25 Swedish children suffering from Leigh syndrome with the aim to obtain insights into the molecular pathophysiology and to provide a rationale for genetic counselling. We reviewed the clinical history of all patients and used muscle biopsies in order to perform molecular, biochemical and genetic investigations, including sequencing the entire mitochondrial DNA (mtDNA), the mitochondrial DNA polymerase (*POLGA*) gene and the surfeit locus protein 1 (*SURF1*) gene. Respiratory chain enzyme activity measurements identified five patients with isolated complex I deficiency and five with combined enzyme deficiencies. No patient presented with isolated complex IV deficiency. Seven patients had a decreased ATP production rate. Extensive sequence analysis identified eight patients with pathogenic mtDNA mutations and one patient with mutations in POLGA. Mutations of mtDNA are a common cause of LS and mtDNA analysis should always be included in the diagnosis of LS patients, whereas SURF1 mutations are not a common cause of LS in Sweden. Unexpectedly, age of onset, clinical symptoms and prognosis did not reveal any clear differences in LS patients with mtDNA or nuclear DNA mutations.

1. Introduction

Leigh syndrome (LS) or subacute necrotising encephalopathy (OMIM #256000) is a progressive neurodegenerative disorder of infancy and early childhood [1]. Focal, bilateral lesions in one or more areas of the central nervous system, including the brainstem, thalamus, basal ganglia, cerebellum or spinal cord are observed at autopsy [2]. The lesions typically consist of areas of demyelinization, gliosis, necrosis, spongiosis and capillary proliferation. Definite diagnosis of LS is based on neuropathology, but imaging techniques can be used to support the diagnosis. Typical findings include bilateral hypodensities in the basal ganglia or brainstem on computed tomography (CT) or hyperintense lesions on T2-weighted magnetic resonance imaging (MRI) [3]. Patients with LS have a wide spectrum of clinical presentations including hypotonia, ataxia, dystonia, epilepsy, respiratory abnormalities, nystagmus, optic atrophy and ophthalmoplegia. Failure to thrive or developmental retardation is often an early but unspecific sign of the disease. Stepwise deterioration with some recovery of skills between episodes of regression is common. The severe neurological symptoms are caused by a range of different biochemical and molecular defects interfering with cellular energy production in affected brain regions.

One of the most common causes of LS is a disturbance of the mitochondrial oxidative phosphorylation (OXPHOS) system. The OXPHOS system comprises five enzyme complexes, where an electron transport chain (complexes I to IV) creates a proton gradient across the mitochondrial inner membrane, which, in turn, is used by ATP synthase (complex V) to generate ATP. This process is under dual genetic control, because key subunits of complexes I, III, IV and V are encoded by mtDNA. Mitochondrial diseases in children encompass a wide spectrum of clinical phenotypes and LS is a common disease entity with an estimated minimal incidence of 1 in 34,000 preschool Swedish children (<6 years) [4]. Molecular defects have been identified in both nuclear and mitochondrial encoded respiratory chain (RC) subunits in children with LS and the inheritance can be maternal, X-linked or autosomal recessive. Mutations in nuclear genes

Abbreviations: LS, Leigh syndrome; MAPR, Mitochondrial ATP production rate; SURF1, surfeit locus protein 1; POLGA, the catalytic subunit of mitochondrial DNA polymerase; RC, respiratory chain

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that control the assembly of the RC complexes have also been reported in affected patients. For instance, mutations in the complex IV assembly factor, surfeit locus protein 1 (SURF1), have been shown to be a common cause of LS [5,6]. Additionally, mutations in genes encoding subunits for pyruvate dehydrogenase, such as the X chromosome-linked E1α subunit gene, have been associated with LS. The aetiology of a large proportion of LS cases is still unknown, despite substantial progress in genetic characterisation. A study of 67 patients with definite or probable LS identified the underlying biochemical or molecular defect in ~80% of definitive Leigh cases, and in ~40% of probable cases [7]. A second study involving 66 LS patients identified the biochemical or molecular cause in only ~50% of the patients [8]. We performed a study of 25 Swedish children diagnosed with LS in order to characterise the clinical symptoms, prognosis, respiratory chain function and genetic aetiology and to provide a rationale for genetic counselling for affected families. We assessed the importance of mtDNA mutations as a cause for LS by performing an extensive analysis of mtDNA in all cases. We report here that mtDNA mutations are a frequent cause of LS. Unexpectedly, we find that nuclear and mtDNA mutations cause similar LS disease phenotypes associated with early onset and poor prognosis. We found no nuclear DNA mutations affecting the *SURF1* gene in this cohort of Swedish LS patients.

2. Materials and methods

All 25 patients were primarily investigated and diagnosed with LS, by their local paediatrician between the years 1989 and 2006 and then referred to the Centre for Inherited Metabolic Diseases at Karolinska University Hospital in Huddinge, Sweden. Patients 15 and 21 were referred post mortem. All patients were born in central Sweden, with seven children having at least one parent of non-Swedish origin. Patients 14, 15, 20 and 21 were diagnosed at autopsy. Patients 23 and 24 had typical features on CT. The remaining 19 patients had typical findings on MRI. One of us (KN) examined 15 of the patients at different stages of their disease and reviewed the medical records from all 25 patients. Patients 7 and 11 have been reported previously [9]. The study was approved by the regional ethics committee in Stockholm, Sweden.

2.1. Laboratory investigations

Organic acids in urine were analyzed by using gas chromatography combined with mass spectrometry [10]. Lactate levels were measured in blood and cerebrospinal fluid (CSF) by laboratories at the local hospitals.

2.2. Biochemical measurements

All patients, except 15 and 21, were subjected to a percutaneous muscle biopsy using a conchotome as described previously [11]. Patients 1–4, 6, 8, 10–13, 18–20, 22–24 were investigated between 1991 and 2000 to determine mitochondrial adenosine triphosphate (ATP) production rate (MAPR), RC enzyme activities, glutamate dehydrogenase activities and citrate synthase activities, as described previously [12,13]. The results from these patients were compared with a control group aged one month to five years ($n=12$). Patients 5, 7, 9, 14, 16, 17 and 25 were investigated between 2001 and 2006 with an improved set of the same methods [11], which included the determination of complex I activity (NADH-coenzyme Q reductase), except in patients 14 and 16. The results were compared with a control group aged two months to five years ($n=11$). Patient results diverging more than ± 2 standard deviations from the control group were considered pathological. Pyruvate dehydrogenase complex (PDHc) activity was determined with a radioisotope method as described previously [14].

2.3. Morphological analyses

Morphological examinations of skeletal muscle, including electron microscopy and enzyme histochemical staining, were performed as described previously [15].

2.4. DNA sequence analysis

Both DNA strands of all samples were sequenced using BigDye v3.1 terminator technology (Applied Biosystems), run on a 3130XL sequencer (Applied Biosystems) and aligned to the appropriate reference sequences using SeqScape software v2.5 (Applied Biosystems). All primers used in PCR amplifications for sequencing were M13-tagged for ease of sequencing.

The complete mitochondrial genome was amplified from all patients except 7, 11, 16 and 19 who had known pathogenic mutations, and patients 15 and 23, being monozygotic twins of patients 14 and 24, respectively. We used 28 overlapping primer pairs (essentially as in [16] and shown in Supplemental material), and the resulting PCR products were sequenced and aligned to the revised Cambridge reference sequence (AC_000021.2). Sequence changes were further compared to MitoMap [17] and the Human Mitochondrial Genome Database (mtDB) [18] (both accessed June 2007). Mutations were also compared to the mtDNA database from MIGENIX Corp., San Diego, California, USA (Elson J. University of Newcastle, UK, personal communication).

The gene encoding the surfeit locus protein 1 (*SURF1*) was sequenced in all patients except the ones, in which we had found known pathogenic mtDNA mutations (described in [5] and see Supplemental material). The catalytic subunit of mitochondrial DNA polymerase (POLGA) was sequenced in patients 7, 11, 14, 15, 16, 17, 19 and 20 (described previously in [19] and shown in Supplemental material).

2.5. Quantification of mtDNA mutation levels

Mutation levels were quantified by last-hot-cycle RFLP analyses, where PCR amplifications were labelled with [α^{32}P] dCTP (GE Healthcare) in the last cycle, followed by digestion with an appropriate endonuclease (see Supplemental material) and separation by electrophoresis in non-denaturing polyacrylamide gels. Quantification was performed by phosphor-imaging (BioRad), and corrected for GC-content of the wildtype and mutant fragments.

3. Results

3.1. Patients

Patients were diagnosed with LS based on the following, previously used, criteria: (i) a progressive neurological disease with basal ganglia and/or brainstem involvement and either (ii) characteristic neuropathological changes at autopsy or (iii) characteristic findings on cerebral imaging, which had to include symmetrical changes in the basal ganglia and/or brainstem [3]. We identified 25 children with LS, 14 boys and 11 girls, from our database of 1285 patients investigated because of suspected mitochondrial disease. Two pairs of monozygotic twins were among the patients but no other siblings were affected.

Despite the common neurological criteria used in this study, the cohort showed a wide variety of clinical presentations (Table 1). For instance, 16 children (64%) developed seizures, occasionally progressing to drug-resistant epilepsy. A majority of the patients had failure to thrive/poor feeding. More than half of the patients suffered from gastrointestinal problems, such as episodic vomiting or signs of gastrointestinal dysmotility. Eight patients (32%) showed signs of

Table 1
Summary of clinical findings

Patient	Sex	Age at onset	Deceased, age at death	Developmental retardation	Hypotonia	Spasticity/ hypertonia	Ataxia	Dystonia/ dyskinesia	Seizures	Optic atrophy
1	M	Birth	9 y	+	+	+	−	+	−	+
2	F	6 mo	7 y 4 mo	+	+	+	−	+	+	−
3	F	Birth		+	−	−	−	−	+	−
4	M	3 mo	4 y 11 mo	+	+	−	+	−	−	−
5	F	3 mo	1 y	+	+	−	−	+	−	−
6	M	7 y		−	−	+	−	+	−	−
7	F	3 mo		+	+	+	−	+	+m	−
8	F	Birth		+	+	+	+	−	+	−
9	M	21 mo		−	+	−	+	+	−	−
10	F	18 mo	9 y 10 mo	+	+	+	+	+	+	−
11	F	Birth		+	+	+	+	+	+	−
12	F	5 mo		+	+	−	+	+	−	−
13	M	3 mo	6 y	+	−	+	−	+	+m	−
14*	M	5 mo	5 mo	−	−	+	−	+	+	−
15*	M	5 mo	5 mo	−	−	+	−	+	+	−
16	M	5 mo	3 y	+	+	−	−	+	+	−
17	M	3 mo	13 mo	+	+	−	+	−	+m	−
18	M	Birth	20 mo	+	+	+	−	−	+	−
19	F	2 mo	1 y	+	+	−	−	−	−	−
20	M	4 mo	1 y	+	+	−	−	+	−	−
21	F	Birth	9 mo	+	+	−	−	+	+	−
22	F	2 y	14 y 10 mo	+	+	+	+	−	+m	+
23*	M	Birth	9 y 7 mo	+	+	−	−	+	+	−
24*	M	Birth	14 y 10 mo	+	+	−	−	+	+	+
25	M	2 y		−	−	−	−	+	−	−

M = male, F = female, mo = months, y = years, + = pathological finding, − = normal finding, m = myoclonus, ND = not determined, * = monozygotic twins. Abnormal eye motility includes strabism, ophtalmoplegia and nystagmus. Gastrointestinal problems include episodic vomiting and signs of gastrointestinal dysmotility, such as episodic abdominal pain, alternating diarrhoea and constipation.

hepatic dysfunction. Patient 1 presented with transient neonatal hyperammonemia, while patient 22 showed transient signs of hepatic dysfunction assumed to be due to medication with valproic acid. The other six patients had a slight to moderate increase in serum transaminases.

3.2. Lactate and organic acids in urine

Lactate levels were normal (<2.3 mmol/l) in four patients, while the remaining 21 patients (84%) showed various degrees of increased lactate levels in blood and/or CSF (Table 2). Ten patients (40%) excreted elevated levels of Krebs cycle intermediates, mostly fumarate and malate, in the urine. Three boys, patients 1, 23 and 24, excreted increased levels of 3-methylglutaconic acid. They all presented with developmental retardation, hypotonia, extrapyramidal symptoms, hearing loss, feeding difficulties and hepatic disorder. Patient 12 excreted methylmalonic acid without responding to treatment with megadoses of vitamin B12. Patient 2 excreted high amounts of α-ketoglutaric acid.

3.3. Muscle morphology

We performed muscle biopsies in 23 patients and 15 of these had normal muscle morphology. Five patients had slightly increased lipid accumulation and three patients had fibre size variation with one patient having a decreased fibre size. None of the subjects had ragged-red fibres or cytochrome *c* oxidase deficiency as determined by COX/SDH double staining (data not shown).

3.4. Biochemistry

Ten patients (43%) had deficiencies in RC enzyme activities. Five patients (22%) presented with an isolated complex I deficiency and five had combined enzyme deficiencies. No patient showed an isolated complex II or complex IV deficiency. MAPR was normal in 14 patients (60%), while seven patients had decreased and two patients increased MAPR (Table 2).

Deficient PDHc activity has been proposed to be involved in approximately 10% of LS patients. Three patients (patients 2, 14 and 21) were subjected to analysis of PDHc and had normal activities. In a further 6 patients (5, 7, 9, 16, 17 and 25; investigated between 2001 and 2006) a PDHc deficiency could be excluded because MAPR in the presence of pyruvate + malate was normal. Prior to 2001 our method for determination of mitochondrial ATP production was less sensitive to assess pyruvate oxidation and it is therefore possible that PDHc deficiency may exist in this group.

3.5. Sequence analysis

In order to determine the underlying genetic cause for LS in these patients, we performed extensive mtDNA sequence analysis on 19 patients (see Materials and methods). We identified 213 changes from the Cambridge reference sequence, of which most have been reported to be polymorphisms according to the MitoMap [17] or mtDB human [18] databases. We identified 11 novel mutations considered not to be pathogenic. Eight patients (32%) carried six different mtDNA mutations, previously reported to cause LS (Table 3). Several mutations were quantified in a variety of tissues and, if available, maternal relatives, in order to suggest causality (see Table 2 and Supplemental Fig. 1).

Patient 22 carried three novel mutations at positions 8920, 10,861 and 14,473. The latter two are silent changes, whereas the m.8920G>A transition results in a glycine to serine substitution at position 132 in ATPase6. Additionally, the patient carried the previously described m.9957T>C mutation in COX III [20,44]. Both, the m.8920G>A and the m.9957T>C mutations were present at high levels in samples from the patient and three healthy maternal relatives (Fig. 2B and C). We thus do not expect these mutations to be pathogenic.

We sequenced the POLGA locus in 6 of the 8 patients with suspected hepatic involvement (Table 2; Patient 14 was not included as he had a pathogenic mtDNA mutation and only one of the identical

Abnormal eye motility	Ptosis	Hearing impairment	Respiratory abnormalities	Swallowing difficulties/ dysartria	Failure to thrive/ poor feeding	Gastrointestinal problems	Renal tubulopathy	Hepatic involvement	Congenital adrenal hyperplasia	Consanguinity
−	+	+	+	−	+	+	+	+	−	−
+	+	−	−	−	+	+	−	+	−	−
−	−	−	−	−	+	+	−	−	+	−
+	−	−	+	−	+	−	−	−	−	−
+	−	−	+	−	+	+	+	−	−	+
−	−	−	−	+	−	−	−	−	−	−
+	−	−	−	+	+	+	−	−	−	−
+	−	−	−	−	+	+	ND	+	−	−
−	−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	+	−	−	−	−	+
+	−	−	−	+	+	+	−	−	−	−
+	−	−	−	+	+	−	−	−	−	−
−	−	+	−	−	+	+	ND	−	−	+
−	−	−	+	−	+	+	−	+	−	−
−	−	−	+	−	+	+	−	−	−	−
−	+	+	−	+	−	−	−	−	−	−
+	−	−	−	−	−	+	−	+	−	−
+	−	−	−	−	+	+	−	−	−	+
+	−	−	+	−	+	−	−	−	−	−
−	−	−	−	+	+	−	ND	−	−	−
+	−	−	+	+	+	+	+	−	−	−
−	−	−	−	+	+	+	−	+	−	+
+	−	+	−	−	+	+	−	+	−	−
+	−	+	−	−	+	+	−	+	−	−
+	−	−	−	−	−	−	−	−	−	−

twins, patients 23 and 24, was investigated), and found one patient (patient 17) to be a compound heterozygote for the POLGA substitutions W748S [21] and R852C [22]. This patient also carried the recently described G11D substitution linked to the R852C substitution [24].

Although there were no cases with complex IV deficiency in the 25 LS patients presented here, we nevertheless sequenced the *SURF1* gene from all patients ($n = 17$) that did not have pathogenic mtDNA or POLGA mutations. No pathogenic SURF1 mutations were found in these cases.

Table 2
Summary of biochemical and genetic findings

Patient	P-lactate	CSF-lactate	Organic acids	RC enzyme deficiency	MAPR	Tissue sequenced	mtDNA	Mutation load	POLGA
1	↑	↑↑	++	I + III and II + III	−	Muscle	−	−	−
2	↑↑	↑	++	−	↓	Muscle	−	−	−
3	↑↑	ND	+	I + III	−	Fibroblasts	−	−	ND
4	↑↑	ND	+	−	−	Fibroblasts	−	−	ND
5	↑↑	−	+	I + III	−	Muscle	−	−	ND
6	−	↑	−	−	−	Muscle	m.10197G>A	31% fibroblast, 50% muscle	ND
7	↑↑	↑↑	+	I and I + III	−	Muscle	m.10191T>C	Ref. [9]	ND
8	−	ND	−	−	↓	Fibroblasts	−	−	−
9	↑	−	−	−	−	Muscle	−	−	ND
10	↑	−	−	−	−	Fibroblasts	−	−	ND
11	−	↑	−	I + III	↑	Muscle	m.14487T>C	Ref. [9]	ND
12	↑	↑	+	I + III	−	Muscle	−	−	ND
13	↑↑	↑	−	−	−	Muscle	−	−	ND
14*	↑	↑↑	+	I + III	↓	Muscle	m.14487T>C	Homoplasmic	ND
15*	↑	↑	+	ND	ND	ND	ND	−	ND
16	↑↑	ND	+	I + III and II + III	↓	Muscle	m.8993T>G	96% blood, 86% fibroblast, 95% muscle	ND
17	−	−	−	−	−	Muscle	−	−	W748S/R852C
18	↑↑	−	−	−	−	Muscle	−	−	ND
19	↑↑	↑	+	−	−	Muscle	m.8993T>C	ND	ND
20	↑	↑	++	−	↓	Muscle	m.14459G>A	99% fibroblasts	ND
21	−	−	−	ND	ND	Fibroblasts	−	−	ND
22	↑	−	+	−	↑	Fibroblasts	m.8920G>A; m.9957T>C	Homoplasmic	−
23*	↑	ND	+	I + III and II + III and IV	↓	ND	ND	−	ND
24*	↑↑	ND	+	I + III and II + III and IV	↓	Fibroblasts	−	−	−
25	−	−	−	−	−	Fibroblasts	−	−	ND

P = plasma, CSF = cerebrospinal fluid, ↑ = increased lactate 2.3–5.0 mmol/l (ref. in plasma and CSF < 2.3 mmol/l), ↑↑ = increased lactate >5.0 mmol/l, + = pathological finding, ++ = highly pathological secretion, − = normal finding, ND = not determined, * = monozygotic twins. I, I + III, II + III, IV refer to the analyses of the respiratory chain enzyme activities of NADH-coenzyme Q reductase, NADH-cytochrome *c* reductase, succinate-cytochrome *c* reductase and cytochrome *c* oxidase, respectively. MAPR = mitochondrial ATP production rate, ↑ = increased MAPR, ↓ = decreased MAPR.

Table 3
Summary of novel and previously reported polymorphisms and pathogenic mtDNA mutations (bold)

Mutation	Gene	Substitution	Patient	Comments and reference
m.291insA	*D-loop*	–	17	Novel, delA reported
m.896A>G	*12S rRNA*	–	2	Novel
m.2709A>G	*16S rRNA*	–	5	Novel
m.6977A>G	*COXI*	Silent	10	Novel
m.8519G>A*	*ATPase8*	E52K	9	Homoplasmic in mother and patient, [40]
m.8920G>A	*ATPase6*	G132S	22	Novel, maternal relatives and patient homoplasmic.
m.8993T>C	*ATPase6*	L156P	19	[41]
m.8993T>G	*ATPase6*	L156R	16	[42]
m.9804G>A	*COXIII*	A200T	21	Associated with LHON [43]
m.9957T>C*	*COXIII*	F251L	22	[20,44]
m.10191T>C#	*ND3*	S45P	7	[45]
m.10197G>A	*ND3*	A47T	6	[26,27]
m.10410T>A	*mt-tRNA*^{Arg}	–	13	Novel, T10410C reported
m.10496A>G	*ND4L*	Silent	25	Novel
m.10861T>C	*ND4*	Silent	22	Novel
m.13148C>T	*ND5*	P271L	5	Novel
m.13528A>G	*ND5*	T291A	8	Not conserved — LHON-like phenotype, [46]
m.14357A>G	*ND6*	V106A	14	Novel
m.14459G>A	*ND6*	A72V	20	[47,48]
m.14487T>C#	*ND6*	M63V	11, 14	[28,29]
m.14473A>G	*ND6*	Silent	22	Novel

= determined by CSGE, confirmed by RFLP, * = reported as polymorphism in this study.

In total, of the 25 patients presented here, 24 (96%) showed signs of disease before or at the age of two years and 20 patients (80%) had onset before six months of age (Fig. 1A). In a few cases onset was dramatic with premature birth, asphyxia at birth or severe hepatic dysfunction. However, more often the patients presented with subtle and unspecific symptoms such as failure to thrive or slightly retarded motor development. Many of these children later developed episodes of sudden deterioration, often in relation to an infection. Median age of onset in the entire cohort was three months, with the group carrying pathogenic mtDNA mutations not differing from the remaining patients. The mean age of disease onset was at 8.7 months for the entire cohort, with the mtDNA mutation group presenting at 13.5 months, in comparison to 6.5 months for the remaining patients. However, this difference in mean age of onset was completely explained by the late onset of disease of patient 6 in the mtDNA mutation group. This patient was referred to a physiotherapist at the age of seven years because of mild motor problems involving balance and coordination. At eight years of age he developed dysartria. Initially, there was a slight progress but at 14 years of age the clinical situation is stable. We identified moderate levels of the m.10197G>A mutation in fibroblasts and skeletal muscle samples from this patient, 31 and 50% mutation load, respectively (Fig. 2).

Seventeen patients are no longer alive (68%) and of those ten patients died before the age of five years (40%), and the remaining seven patients before 15 years of age (Fig. 1B). The mean age of death for the patients with mtDNA mutations was 14 months whereas the remaining group had a mean age of death of 78.3 months (mean value of the entire cohort was 58 months). Of the 20 patients with onset before six months of age, 15 (75%) are no longer living (Table 1). Disease progression differed greatly among the cohort, ranging from only several weeks between initial symptoms and death (patients 14 and 15) to several years (e.g. patients 1 and 24). The average time between initial symptoms and death was close to 10 months ($n=5$; S.D. = 12.6) within the group with pathological mtDNA mutations and 74.3 months ($n=12$; S.D. = 54.4) for the remaining patients. Remarkably, the oldest patient in our material is a 22 year old female (patient 8), who had onset of disease at birth manifested as hypotonia, developmental retardation, epilepsy and poor feeding.

4. Discussion

We demonstrate here that pathogenic mutations of mtDNA are a frequent cause of LS in children. Eight of 25 investigated patients (32%) carried pathogenic mtDNA mutations and one patient was a compound heterozygote for pathogenic POLGA mutations. The extensive analysis of mtDNA, with more than 6.4×10^5 bases sequenced, makes it very likely that the remaining 16 patients have yet undiagnosed nuclear mutations. The diagnosis of LS is in majority of the patients based on the combination of typically localized findings on neuroimaging and clinical symptoms derived from these lesions. Nine of the 23 investigated patients showed normal mitochondrial function, a common diagnostic problem for a subset of patients with suspected mitochondrial disorders. In three of these patients we could detect a pathogenic mutation in mtDNA or in POLGA. The majority of the remaining six patients had very typical clinical features combined with biochemical abnormalities such as increased levels of lactate or abnormal excretion of organic acids, making it very likely that they are/were suffering from a respiratory chain disorder. However, a few children remain with a more uncertain diagnosis and even if the principal aetiology behind LS is a primary respiratory chain disorder, it can in exceptional cases also be caused by other rare neurological or neurometabolic disorders. Excluding all potential aetiologies was not possible in this retrospective study.

One patient (patient 17) was compound heterozygous for three previously described mutations in POLGA. Patients that are compound

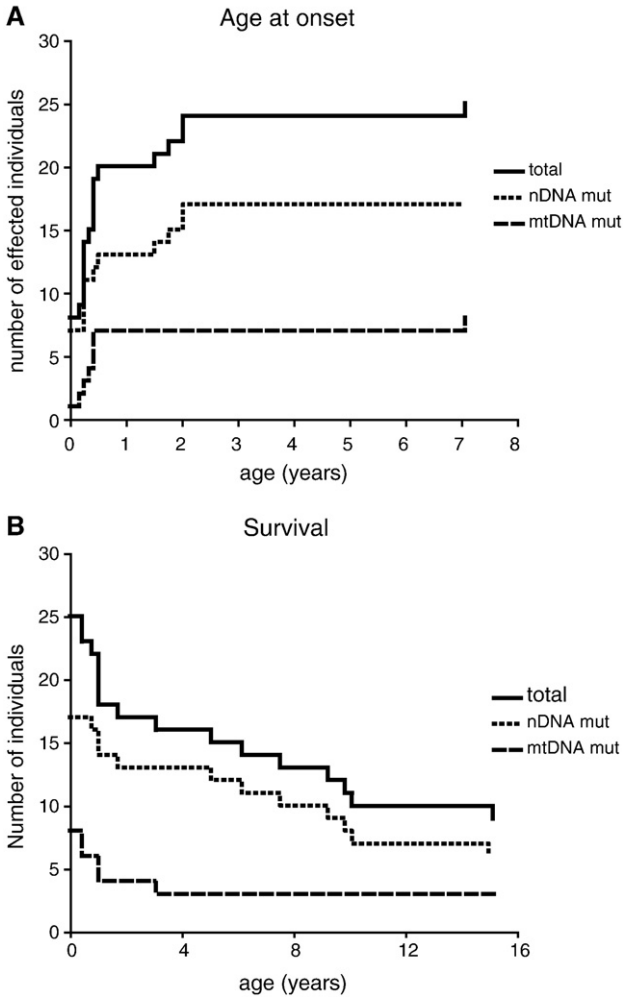

Fig. 1. Similar disease onset (A) and prognosis (B) in patients with mtDNA mutations (lines) or nuclear mutations (dots). Combined data is shown as a solid graph. Age is presented in years, data presented as numbers of total.

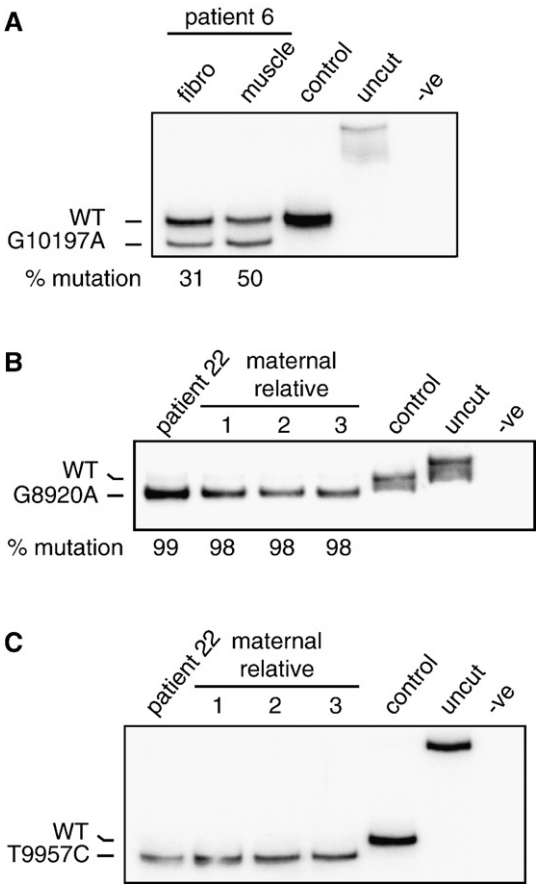

Fig. 2. Last-hot-cycle RFLP analysis of various mtDNA changes. (A) The mutation levels of the m.10197G>A (using the restriction endonuclease *Bcc I*) were determined in fibroblasts (31%) and muscle (50%) from patient 6. (B) The novel m.8920G>A mutation (using the restriction endonuclease *Tat I*) and (C) the m.9957T>C mutation (using the restriction endonuclease *Mbo II*) were both at or close to homoplasmic levels in fibroblasts from patient 22 as well as in blood samples from the maternal uncle (1), aunt (2) and grandmother (3). All mutation levels are calculated as % of the wildtype fragment ±2%. -ve indicates amplification without DNA.

heterozygous for W748S and either L244P, G848S or Y1210fs1216stop develop Alper's syndrome, whereas patients that are compound heterozygous for W748S and either Q497H, L304R, or A467T develop spino-cerebellar ataxia and epilepsy [23]. Recently, a patient carrying the W748S mutation in trans with both, the R852C and G11D, was reported with ataxia, neuropathy and failure to thrive [24]. The patient presented here appeared hypotonic with retarded motor development at three months of age and developed epilepsy with intractable myoclonic seizures at six months of age. His disease state gradually progressed and he died at age 13 months. A subtle hepatic involvement was noted once, with slightly increased transaminases and possible enlarged liver. The patient did not develop microcephaly and an MRI scan at eight months of age showed high signalling lesions in subcortical areas. A follow-up scan at one year of age revealed a progressive decline with high signalling areas of thalamus bilaterally and widespread changes of the white matter, not typically observed in Alper's syndrome (OMIM #203700). The patient had signs of brainstem dysfunction and thus was included in this cohort of LS patients, although presenting with an Alper's-like phenotype. The G11D mutation has recently been found in trans with the R852C mutation, in a 15 year-old patient with symptoms compatible with Alper's syndrome [25]. However, it is not clear yet in what way the G11D mutation influences the disease phenotype and progression.

The group of patients with mtDNA mutations did not differ from the group of patients without demonstrated mutations, with regard to the clinical phenotype. They all had severe neurological symptoms, reflecting injuries of very central parts of the brain, as expected from the inclusion criteria. Seizures were an equally frequent finding in both groups (62.5% and 65%, respectively). Non-neurological symptoms were present in both groups.

Three children (patients 6, 9 and 25) presented with less severe phenotypes and delayed onset of disease. Patient 9 developed an encephalitis-like disease at the age of 21 months, from which he partially recovered after several weeks, but still had a coordination disorder with slight choreatetotic movements. In this patient we found the m.8519G>A transition mutation, which has previously been reported in conjunction with MELAS, seizures, autistic behaviour and developmental delay [17]. However, we found this mutation to be homoplasmic in both the patient and his mother. Additionally, the Uppsala mtDNA database reports one sequence with this transition among its 2704 sequences from healthy subjects [18]. We therefore propose that this mutation is a rare polymorphism.

Patient 25 presented with a squint at two years of age and developed painful dystonias in his lower extremities at age four years. His development was normal. We were unable to identify a genetic cause in this patient. However, patient 6, who carries the m.10197G>A mutation, was referred to a hospital due to mild motor problems at the age of seven years. This mutation in ND3 has recently been described to cause LS and dystonia [26] or an unspecified encephalopathy with basal ganglia lesions [27]. Unlike the previous cases, where high levels of the m.10197G>A mutation caused a complex I deficiency and a severe clinical phenotype, patient 6 of this study had much lower mutation levels and a milder clinical phenotype without RC deficiency in skeletal muscle, suggesting that a more gradual threshold might be associated with this mutation.

We identified two potentially disease causing mutations in patient 22. The novel m.8920G>A transition leads to a glycine to serine substitution in ATP6, while the m.9957T>C mutation has been previously reported in a boy with MELAS [20] and a man with non-arteritic ischemic optic neuropathy (NAION) [44]. The latter mutation results in a phenylalanine to leucine substitution in COX III. Neither the previously described cases, nor patient 22 in this report had complex IV deficiency. Additionally, both of these mutations were carried at very high (likely homoplasmic) levels in the patient and three healthy maternal relatives. We therefore suggest that both of these mutations are rare polymorphisms.

The m.14487T>C mutation in ND6 seems to affect complex I stability and has previously been reported in LS patients [28,29]. We identified the mutation in two unrelated families. Interestingly, despite the high mutation levels in patients 11 (previously described [9]) and 14, the clinical and biochemical presentation was very different. Patients 14 and 15 were twin brothers with sudden irritability, hypertonus and hypoventilation at five months of age. They both deteriorated rapidly and died within three weeks after admission to a hospital. In contrast, patient 11 has a milder disease phenotype and is alive at 16 years of age with a severe and slightly progressive handicap. Patient 14 carried the m.14487T>C mutation at very high levels (Fig. 2C) and also carried a novel m.14357A>G transition. This second mutation results in a valine to alanine substitution at position 106 of ND6 and has previously not been described. It is possible that this additional mutation may aggravate the clinical consequences of the m.14487T>C mutation.

Mutations in mitochondrial tRNAs (mt-tRNA) are surprisingly rare in LS [30,31], despite the observation that mt-tRNA mutations comprise of the largest group of mtDNA mutations associated with mitochondrial diseases [32,33]. We found no previously reported mt-tRNA mutations in the presented cohort of 25 LS patients. However, we identified an m.10410T>A transversion in mt-tRNA-Arg, which could increase the stability of the ACC-stem of mt-tRNA-Arg. No primates seem to carry an adenine at this position, and a pyrimidine–pyrimidine interaction is conserved at this position in humans, chimpanzees, bonobos and gorillas. More diverse primates, however, do carry a

pyrimidine–purine base pairing at this position, and it is therefore not clear whether a weak pyrimidine bond at this position is required for a functional tRNA in humans. We have not quantified the levels of this mutation in patient 13, nor in any of his maternal relatives, and it is therefore not possible to asses whether the mutation load correlates with the disease. Three of the patients in our cohort had similar findings as described previously in patients with a syndrome consisting of 3-methylglutaconic aciduria, sensorineural deafness, encephalopathy and Leigh-like syndrome (MEGDEL) [34]. No underlying molecular defect has been identified in MEGDEL patients [34].

Increased levels of lactate in plasma or CSF are often found in LS patients. However, this is not always the case and cannot be used as a criterion for LS, as previously suggested [7]. Four of the patients in this study had normal lactate levels, among them were the boy with POLGA-mutation and a girl with LS diagnosed at autopsy. We found abnormal excretion of organic acids in the urine of 15 patients (60%), suggesting that the measurement of organic acid excretion can be an important diagnostic tool.

Previous studies have reported that 13% and 26% of LS patients have a complex IV deficiency [7,8]. SURF1 is an assembly factor for complex IV and mutations of the *SURF1* gene are frequently found in complex IV-deficient LS patients [35–37]. In order to use an independent method to confirm the absence of complex IV deficiency in our cohort of LS patients, we sequenced the *SURF1* gene. We failed to detect any pathological mutations in SURF1 in 17 analyzed cases, demonstrating that the prevalence of SURF1 mutations is very low in Swedish LS patients.

It can be concluded that mtDNA mutations are common among patients with LS and do not give rise to a milder phenotype. We therefore suggest that sequencing of the entire mtDNA should be considered in all patients with LS, irrespective of disease severity.

Mitochondrial dysfunction is found to play an important role in neuronal death following an acute brain injury, caused by, for example, stroke, trauma or hypoxia and ischemia after perinatal events in the newborn brain [38]. These findings suggest that neurons with mitochondrial dysfunction are prone to degenerate in response to exogenous stress. Consistent with this hypothesis, experimental studies have shown that RC deficient neurons of conditional knockout mice are hypersensitive to excitotoxic stress and display massive cell death after induction of epileptic seizures [39]. Therefore, different neuroprotective treatment strategies that currently are being developed to treat a variety of neurodegenerative diseases should also be considered as treatment of mitochondrial disease in a future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2008.11.014.

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