

A novel mutation in *NDUFS4* causes Leigh syndrome in an Ashkenazi Jewish family

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Summary Leigh syndrome is a neurodegenerative disorder of infancy or childhood generally due to mutations in nuclear or mitochondrial genes involved in mitochondrial energy metabolism. We performed linkage analysis in an Ashkenazi Jewish (AJ) family

without consanguinity with three affected children. Linkage to microsatellite markers D5S1969 and D5S407 led to evaluation of the complex I gene *NDUFS4*, in which we identified a novel homozygous c.462delA mutation that disrupts the reading frame. The resulting protein lacks a cAMP-dependent protein kinase phosphorylation site required for activation of mitochondrial respiratory chain complex I. In a random sample of 5000 healthy AJ individuals, the carrier frequency of the *NDUFS4* mutation c.462delA was 1 in 1000, suggesting that it should be considered in all AJ patients with Leigh syndrome.

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Abbreviations

AJ	Ashkenazi Jewish
BCS1L	BCS1-like protein
DLD	dihydrolipoamide dehydrogenase
MRS	magnetic resonance spectroscopy
NDUFs	NADH-ubiquinone oxidoreductase Fe-S proteins and flavoproteins
PDHC	pyruvate dehydrogenase complex
RT-PCR	reverse transcription polymerase chain reaction
SDHA	succinate dehydrogenase complex, subunit A
SURF1	surfeit 1

Introduction

Leigh syndrome (OMIM 256000), or subacute necrotizing encephalomyelopathy, is a devastating, paediatric neurodegenerative disorder characterized by bilateral, symmetric lesions in the brainstem, midbrain,

pons, thalamus, basal ganglia, and cerebellum (Leigh 1951). Individuals with Leigh syndrome present with variable clinical symptoms that include hypotonia, psychomotor retardation or regression, respiratory difficulties, recurrent vomiting, nystagmus, ataxia, peripheral neuropathy, external ophthalmoplegia, loss of vision, impaired hearing, and seizures (Loeffen et al 2000; Pitkanen et al 1996). Laboratory investigations often reveal elevated lactate levels in blood and cerebral spinal fluid. Leigh syndrome is associated with progressive neurological dysfunction and is usually lethal within the first two years of life. Biochemically, Leigh syndrome can result from a number of inherited defects in mitochondrial energy metabolism. Genetically, Leigh syndrome can be autosomal recessive, X linked, or maternally inherited, and can result from mutations in genes encoding subunits of the pyruvate dehydrogenase complex (PDHC) or subunits of respiratory chain complexes I, II, III, IV or V, but predominantly of complexes I and IV (DiMauro and Schon 2001; Kirby et al 1999; Loeffen et al 2000; Robinson 1998). Complex I is a large multiprotein enzyme complex that transports electrons from NADH to ubiquinone as protons are shuttled across the inner mitochondrial membrane into the intermembrane space. Complex I is composed of seven mitochondrial DNA-encoded proteins and 39 nuclear-encoded subunits (Carroll et al 2003). Mutations have been identified in all seven mitochondrial DNA-encoded subunits and in the nuclear genes encoding the NADH-ubiquinone oxidoreductase Fe-S proteins and flavoproteins (NDUFs) *NDUFS1*, *NDUFS2*, *NDUFS4*, *NDUFS7*, *NDUFS8*, *NDUFV1* and *NDUFV2* (Benit et al 2001, 2003a, b; Budde et al 2000; Loeffen et al 1998, 2001; Petruzzella et al 2001; Schuelke et al 1999; Triepels et al 1999; van den Heuvel et al 1998). Mutations in *NDUFS1*, *NDUFS4*, *NDUFS7*, *NDUFS8* and *NDUFV1* have been reported to cause Leigh syndrome or Leigh syndrome-like disease (Benit et al 2001; Budde et al 2000; Loeffen et al 1998; Petruzzella et al 2001; Schuelke et al 1999; Triepels et al 1999; van den Heuvel et al 1998), while mutations in *NDUFS2* and *NDUFV2* have been associated with cardiomyopathy and encephalomyopathy (Benit et al 2003a; Loeffen et al 2001). The identification of mutations causing complex I defects has been hampered by the large number of proteins in this complex and by the limited understanding of the role of each component.

We describe an Ashkenazi Jewish (AJ) family with three affected children with Leigh syndrome and harbouring a homozygous c.462delA mutation in *NDUFS4* (OMIM 602694; GenBank accession no.

NM_002495). We demonstrate that the c.462delA *NDUFS4* mutation represents an AJ founder mutation with a carrier frequency of approximately 1 in 1000. Identification of this AJ founder mutation should facilitate genetic evaluation of Leigh syndrome in AJ families.

Case reports

Case 1

The proband, a male infant, presented at 3.5 months of age with strabismus, difficulty gaining weight and irritability. Neurological examination showed hypotonia, head lag, ptosis and strabismus. MRI of the brain demonstrated bilateral symmetrical T2 signal abnormalities in the cerebral peduncles, red nuclei, pons and medulla. Magnetic resonance spectroscopy (MRS) showed increased lactate and decreased *N*-acetylaspartic acid. MRI and MRS results suggested the diagnosis of Leigh syndrome. His prenatal history was unremarkable. He was the product of a 41-week gestation, born via normal spontaneous vaginal delivery to a 37-year-old G1P0 mother with a birth weight of 3436 g and head circumference of 32 cm. His Apgar scores were 9 and 9 at 1 and 5 minutes. At 6 months of age, he was admitted to the hospital for failure to thrive and received nasogastric tube feeds. During the hospitalization, he was found unresponsive, required intubation, and could not be extubated thereafter. His clinical course was progressive and characterized by failure to thrive and decreased gut motility that required a gastrostomy-jejunostomy feeding tube. His persistent respiratory insufficiency required a tracheostomy and ventilator support. He also developed a hypertrophic cardiomyopathy with moderate concentric hypertrophy and qualitatively good biventricular systolic function. He had recurrent unexplained fevers and sustained hypertension, presumably secondary to autonomic dysfunction. Neurologically, he regressed developmentally and became less responsive and more hypotonic despite treatment with coenzyme Q₁₀, L-carnitine, thiamine and sodium citrate. Multiple electroencephalograms demonstrated mild to moderate diffuse slowing without evidence of seizures, indicating an encephalopathy with diffuse cerebral dysfunction. At 10 months of age, he died at home. Family history at the time was unremarkable. He had a 13-year-old paternal half brother and a 10-year-old paternal half-sister who were both healthy. Both his parents were Ashkenazi Jews. There was no known history of consanguinity. There was no maternal or

Table 1 Activities of mitochondrial enzymes in skeletal muscle biopsy in a 10% muscle homogenate

Enzyme	Complex	Activity ($\mu\text{mol}/\text{min per g}$)	Control activity \pm standard deviation ($\mu\text{mol}/\text{min per g}$)
Cytochrome- <i>c</i> oxidase	IV	3.85	2.80 \pm 0.52
Succinate cytochrome- <i>c</i> reductase	II + III	0.72	0.70 \pm 0.23
NADH cytochrome- <i>c</i> reductase	I + III	0.59	1.02 \pm 0.38
NADH dehydrogenase	I	31.36	35.48 \pm 7.07
Citrate synthase		17.22	9.88 \pm 2.55
Succinate dehydrogenase		1.67	1.00 \pm 0.53

paternal family history of childhood deaths, seizures, mental retardation, stroke, blindness, hearing loss, diabetes or cardiomyopathy.

His metabolic evaluation was significant for a plasma lactate that ranged from 1.0 to 9.5 mmol/L (normal, <2.2) over a 4-month period, plasma lactate: pyruvate ratio of 22 (normal <25) measured simultaneously when the cerebrospinal fluid lactate was 4.5 mmol/L (normal <2.2) and cerebrospinal fluid pyruvate was 0.17 mmol/L (normal <0.13). A muscle biopsy at 5 months of age demonstrated a few atrophic fibres without ragged-red fibres, COX-deficient fibres, or mitochondrial proliferation. Biochemical testing demonstrated mildly decreased activity of complex I, especially when normalized to citrate synthase (Table 1).

Cases 2 and 3

The parents of the proband subsequently had fraternal triplets, two female and one male, conceived with the assistance of clomiphene citrate. The babies were born at 34 weeks of gestation by Caesarean section because of fetal distress in triplet C. The prenatal history was otherwise unremarkable with the exception of multiple gestations. Triplet A was female with a birth weight of 1989 g; triplet B was male with a birth weight of 1932 g, and triplet C was female with a birth weight of 1165 g.

Triplet C (case 2) had gastrointestinal bleeding immediately after birth but otherwise remained in the neonatal intensive care unit for 6 weeks solely to gain weight. At 5 months of age, she presented with

exotropia and irritability and was hypotonic. MRI of the brain demonstrated symmetric areas of signal hyperintensity on T2-weighted images in the cerebral peduncles, periaqueductal regions of the midbrain, dorsal pontomedullary junction, corpus callosum, and medial globus pallidus. At 6 months of age, a gastrostomy tube was placed to assist with feeding, and she died two days later with lactic acidosis.

Triplet B (case 3) presented at 6 months of age with exotropia, hypotonia and poor head control. Unlike his previously two affected siblings, by 5–6 months of age he was able to roll over and he was more active. He had been hospitalized for three weeks after birth for prematurity and required intubation for two days immediately after birth. He developed progressive irritability and difficulty feeding. At 7 $\frac{1}{2}$ months of age, his weight was at the 5th centile. At that time, he had bilateral ptosis, alternating exotropia, and severe hypotonia with normal reflexes. His metabolic evaluation was significant for a lactate of 3.4 mmol/L and pyruvate of 0.17 mmol/L. He was treated with coenzyme Q₁₀, riboflavin, thiamine and vitamin E, but died at 10 months of age at home.

Triplet A remains healthy at 4 years of age.

Materials and methods

Subjects and population studied

Blood samples from the three affected children, one unaffected child, and the parents were collected with

Table 2 Genotypes of three loci linked to Leigh syndrome in this family. Linkage analysis was performed on DNA from the mother (M), father (F), three affected children (cases 1–3) and the normal sibling

Marker (location)	M	F	Case #1	Case #2	Case #3	Normal sibling
D2S364 (2q32)	3,5	3,4	3,5	3,5	3,5	3,3
D2S118 (2q32)	3,2	2,4	2,2	2,2	2,2	2,3
D5S1969 (5q11)	2,3	3,4	3,3	3,3	3,3	3,4
D5S407 (5q11)	3,3	1,4	1,3	1,3	1,3	3,4
D7S640 (7q33)	2,3	4,1	4,2	4,2	4,2	1,3

the approval of the institutional review boards at Fordham University and Columbia University Medical Center. Blood samples from 5000 anonymous individuals of AJ descent were obtained from the Dor Yeshorim screening programme (Ekstein and Katzenstein 2001).

DNA purification

DNA was purified from the blood of the proband and his affected siblings, family members, and anonymous

donors participating in the Dor Yeshorim genetic testing programme using the QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's directions.

Haplotype analysis

To determine genotypes, linkage analysis was performed using the ABI PRISM Linkage Mapping Set v2.5, which comprises 811 fluorescently labelled PCR primer pairs that define a ~5 centimorgan (cM)-

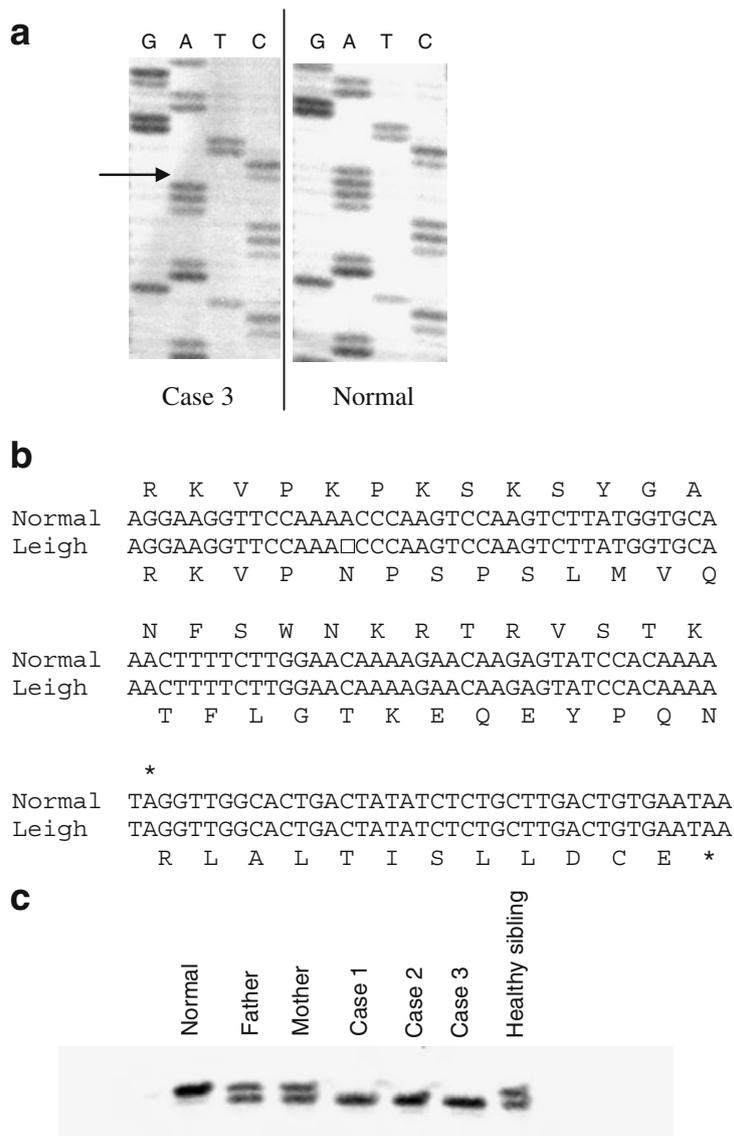


Fig. 1 A single base deletion in the *NDUFS4* transcript. **(a)** Sequence analysis of *NDUFS4* cDNA from patient 3 and a normal control. The location of a single base deletion of an adenine is denoted with an arrow. **(b)** Alignment of normal and patient 3 *NDUFS4* cDNA and the predicted amino acid sequence. Alignment of a portion of the normal and mutated *NDUFS4* cDNAs demonstrates that the deletion results in a frameshift, generating a protein in which the last 22 amino acids of the native protein are replaced with 34 novel amino acids. The location of the 'A' deletion is indicated by a □. Termination codons are indicated by an asterisk. **(c)** PCR-based size assay to detect the presence of the c.462delA allele. Primers, one of which was radiolabelled, were used to amplify genomic DNA and products were run on a 6% denaturing polyacrylamide gel, as described in the Materials and Methods. Autoradiography demonstrates products of 143 bp from normal alleles and 142 bp from alleles with the 'A' deletion

resolution human index map. The PCR products generated were analysed using an ABI 3700 capillary sequencer. The inheritance of microsatellite markers around loci linked to Leigh syndrome was examined.

RT-PCR analysis on whole blood-derived RNA

To screen for mutations in candidate genes in regions previously linked to Leigh syndrome, RNA was isolated from whole blood from patient 3, from the child's parents and from a normal control using the PAXgene Blood RNA Kit (Qiagen). To generate fragments for sequence analysis, we employed RT-PCR using primers to the cDNA sequences of the *BCSIL* (BCS1-like protein), *NDUFS4* and *DLD* (dihydrolipoamide dehydrogenase) transcripts designed to generate products 200–300 bp in length. RT-PCR was performed in 10 µl reactions using the One-Step RT-PCR Kit (Qiagen) under the following cycling conditions: 50°C × 30 s, 95°C × 15 min, followed by 40 cycles of 94°C × 15 s, 57°C × 30 s, 72°C × 30 s.

DNA sequencing

DNA sequences were determined by the dideoxy chain termination method using the AmpliCycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

PCR detection of the c.462del mutation

For detection of the c.462del mutation, genomic DNA was amplified by PCR (94°C × 5 min, then 40 cycles of 94°C × 30 s, 58°C × 30 s, 72°C × 30 s) with a γ -[³³P]ATP end-labelled primer (5'-TTCACAGTCAAG CAGAGA-3') and an unlabelled primer (5'-CCTAG GATGGAGCTATGACA-3'). The amplified DNA was run on a 6% denaturing polyacrylamide gel.

Results

Mutations in genes involved in mitochondrial energy metabolism, including the nuclear genes encoding *NDUFS7*, *NDUFV1*, *NDUFS8*, *NDUFS3*, *COX15*, *SURF1*, *DLD*, *NDUFS4*, *SDHA* and *BCSIL*, have all been associated with Leigh syndrome. Given the family history, we assumed an autosomal recessive mode of inheritance and analysed the data for linkage of the microsatellite markers with genes previously associated with Leigh syndrome. We observed that the microsatellite markers D2S364 and D2S118, which

map to 2q33 (36.6 and 28 cM from *BCSIL*), microsatellite markers D5S1969 and D5S407 on 5q11 (0.25 and 3 cM from *NDUFS4*), and microsatellite marker D7S640 on 7q31 (24.9 cM from *DLD*), were linked with the Leigh syndrome phenotype (Table 2).

Using overlapping sets of primers and RNA from whole blood from patient 3 and a normal control, reverse transcription-PCR (RT-PCR) was used to amplify 200–300 bp fragments from *BCSIL*, *NDUFS4* and *DLD* transcripts, which were then sequenced. Sequence analysis of *NDUFS4* PCR products identified the deletion of one of four consecutive As at nucleotide 462 (c.462delA) (Fig. 1a). Deletion of this nucleotide alters the reading frame and generates a protein in which the last 22 amino acids of the native protein are replaced with 34 novel amino acids (Fig. 1b).

To determine the genotypes of other family members, we performed PCR amplification of genomic DNA from the parents, three probands, the healthy sibling, and a control subject using primers flanking the deletion. The PCR products were electrophoresed on a 6% denaturing polyacrylamide gel. Both parents and the healthy sibling were heterozygous for c.462delA while all three affected probands were homozygous for c.462delA (Fig. 1c).

The carrier frequency of c.462delA was then analyzed in a random sample of 5000 healthy individuals of AJ descent and found to be 1 in 1000.

Discussion

Human diseases associated with disorders in mitochondrial energy metabolism occur with an estimated incidence of 1 in 10 000 live births. Complex I (NADH-ubiquinone reductase) deficiency is one of the most frequent of these disorders (Smeitink and van den Heuvel 1999). Approximately 50% of complex I-deficient individuals exhibit Leigh syndrome or Leigh syndrome-like symptoms (Loeffen et al 2000; Robinson 1998). Complex I is composed of both mitochondrial and nuclear-encoded gene products (Carroll et al 2003). *NDUFS4*, which encodes a nuclear component of complex I, generates a transcript that encodes a protein 175 amino acids long. This protein contains a leader sequence, which is removed after import into the mitochondria, leaving a mature protein of 133 amino acids. *NDUFS4* protein contains two consensus phosphorylation sites. One is located in the leader sequence and the second is a canonical cAMP-dependent protein kinase phosphorylation consensus site (RVS) at amino acids 129–131 of the mature

protein, in which the serine residue is phosphorylated (van den Heuvel et al 1998). Phosphorylation by a cAMP-dependent protein kinase of this site leads to activation of complex I (Papa et al 2001, 2002).

To date, five recessive mutations in *NDUFS4* have been reported to cause Leigh syndrome or Leigh syndrome-like disease. These include a 5 bp duplication (dupl466–470AAGTC), which generates a frameshift at codon K158 and results in a protein 14 amino acids longer than the wild-type protein (van den Heuvel et al 1998); two nonsense mutations (C316T (Budde et al 2000) and G44A (Petruzzella et al 2001)); a deletion (del289G) in the coding region of the transcript resulting in a prematurely terminated protein; and one mutation in the splice acceptor of intron 1 (IVS1nt-1,G>A (Benit et al 2003b)) that results in the skipping of exon 2 and the generation of a transcript encoding a truncated protein of only 39 amino acids. All of the Leigh syndrome/Leigh syndrome-like disease-causing mutations identified in *NDUFS4* to date result in the loss of the conserved phosphorylation site in the mature protein

In this study, we present the genetic basis of Leigh syndrome in a family of AJ descent in which three of four siblings died of Leigh syndrome. Biochemical analyses in the muscle biopsy from one of the probands indicated a mild non-diagnostic deficiency in complex I activity, but analysis of the *NDUFS4* transcript in peripheral blood and of the genomic DNA sequence encoding *NDUFS4* revealed the deletion of one in a series of four consecutive adenines at nucleotides 459–462 of *NDUFS4* cDNA. The loss of this nucleotide shifts the reading frame of the encoded transcript, resulting in the loss of the *NDUFS4* cAMP-dependent protein kinase phosphorylation consensus site. The clinical impact of this mutation is consistent with the previously identified disease-causing mutations in *NDUFS4*, which also result in the loss of this phosphorylation site.

It has been suggested that the existence of several different mutations in *NDUFS4* makes this gene a relatively common genetic cause of complex I deficiency (Petruzzella and Papa 2002). The discovery of this mutation will allow the establishment of a genetic test to evaluate patients of AJ descent with Leigh syndrome or Leigh syndrome-like symptoms and to allow population-based AJ preconception carrier screening and prenatal testing for carrier couples. In fact, we have already successfully used prenatal diagnosis in this family to produce a healthy, unaffected baby boy. Together with the lipamide dehydroge-

nase mutations G229C and 105insA (Shaag et al 1999), this mutation should be tested in AJ patients with Leigh syndrome or Leigh syndrome-like symptoms.

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