Mutant NDUFS3 subunit of mitochondrial complex I causes Leigh syndrome

P Bénit, A Slama, F Cartault, I Giurgea, D Chretien, S Lebon, C Marsac, A Munnich, A Röting, P Rustin

Respiratory chain complex I deficiency represents a genetically heterogeneous group of diseases resulting from mutations in mitochondrial or nuclear genes. Mutations have been reported in 13 of the 14 subunits encoding the core of complex I (seven mitochondrial and six nuclear genes) and these result in Leigh or Leigh-like syndromes or cardiomyopathy. In this study, a combination of denaturing high performance liquid chromatography and sequence analysis was used to study the NDUFS3 gene in a series of complex I deficient patients. Mutations found in this gene (NADH dehydrogenase iron-sulphur protein 3), coding for the seventh and last subunit of complex I core, were shown to cause late onset Leigh syndrome, optic atrophy, and complex I deficiency. A biochemical diagnosis of complex I deficiency on cultured amniocytes from a later pregnancy was confirmed through the identification of disease causing NDUFS3 mutations in these cells. While mutations in the NDUFS3 gene thus result in Leigh syndrome, a dissimilar clinical phenotype is observed in mutations in the NDUVF2 and NDUFS2 genes, resulting in encephalomyopathy and cardiomyopathy. The reasons for these differences are uncertain.

Reduced nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase (mitochondrial respiratory chain complex I) catalyses electron transfer from NADH to the ubiquinone pool, with simultaneous vential proton pumping across the inner mitochondrial (mt) membrane. Electrons are transferred from NADH to the primary electron acceptor, a non-covalently bound flavin mononucleotide (FMN), and thence, through a series of iron-sulphur clusters, to bound ubiquinone. This multisubunit complex comprises more than 40 subunits that are encoded by either the mtDNA or the nuclear genome.

Methods

Patient 1, a boy, was the first child of healthy unrelated parents in Réunion Island. He was born after an uncomplicated full term pregnancy (birth weight 2880 g, height 50 cm, head circumference 33 cm). At nine months of age, he had a single episode of febrile convulsions. His psychomotor development was normal until nine years of age, but a kyphoscoliosis had been noted.

At nine years he developed a persistent stiff neck. Brain and medullary magnetic resonance imaging (MRI), electroencephalography (EEG), and electromyography (EMG) were normal. He gradually developed severe axial dystonia with oral and pharyngeal motor dysfunction, dysphagia, and a tetraparetic syndrome. Ocular movements were normal, but the retinal fundus showed a mild pallor of the optic discs. Measurements of visual evoked potentials showed a diminution in retroretinal conduction suggestive of optic nerve atrophy.

At 10 years, cerebral MRI detected a high T2 signal intensity in the putamen, the white matter, and the brain stem. At that time, CSF lactate was 2.7 mM (control below 2 mM). A complex I deficiency was identified in a skeletal muscle biopsy.

Two years later, he developed an acute pancreatitis and a severe respiratory insufficiency that required tracheal intubation and respiratory support for six weeks, and then a tracheostomy.

He died one and half years later after rapid multisystem deterioration. Biochemical prenatal diagnosis was offered and revealed complex I deficiency in a third pregnancy (patient 2). A younger brother is healthy.

Polarographic tests and spectrophotometric assays were carried out on skeletal muscle mitochondria and cultured skin fibroblasts as previously described. Total RNAs were extracted from cultured skin fibroblasts using the Rnasin kit (Qiagen, Courtabeuf, France) and reverse transcribed using random hexamer primers (GenAmp RNA PCR core kit, PE
NDUFS3 mutations in Leigh syndrome

Applied Biosystems, Perkin Elmer, France). Reverse transcriptase polymerase chain reaction (RT-PCR) amplification of the NDUFS3 specific RNA was done in two overlapping fragments spanning the entire coding region of the gene. Fragment 1 was amplified using oligonucleotides 5'-GGAA GGTGAAACAGTGGGCC-3' (forward) and 5'-GCAGGG GTAGTAGTCAGACG-3' (reverse). Fragment 2 was amplified using oligonucleotides 5'-ATACAATGTATAATCGAAAGCCG-3' (forward) and 5'-CATATTTATTTCTAGTGACAT-3' (reverse). Amplification products were mixed with control PCR products, denatured for 10 minutes at 96°C and then gradually reannealed by decreasing sample temperature from 96°C to 30°C. The annealed specimens (7 μl) were loaded onto a DNA Sep column (Transgenomic, city, France) by means of a Dynamax automatic sample injector model AI-1A (Transgenomic). Sequencing of the abnormal D-HPLC profiles was undertaken directly using a Big Dye terminator cycle sequencing kit (ABI Prism, PE Applied Biosystems).

RESULTS
Polarographic assays showed low pyruvate plus malate oxidation in skeletal muscle mitochondria isolated from patient 1 compared with control (table 1). Activity ratios showed that complex I dependent activities were significantly decreased (that is, pyruvate plus malate oxidation, NADH cytochrome c reductase activity): complex IV/I activity ratios showed a 50% deficiency in complex I activity in skeletal muscle and cultured skin fibroblasts of patient 1. A similar figure was obtained in the cultured amniocytes of patient 2 (the fetus), with a 50% decrease in NADH quinone reductase activity (table 1). This partial defect is possibly consistent with a complete inhibition of complex I dependent substrate oxidation in vivo.

RT-PCR amplification and D-HPLC analysis of NDUFS3 mRNAs from cultured skin fibroblasts of patient 1 detected two abnormal patterns of migration (fig 1). Direct sequencing of the abnormal amplification product revealed compound heterozygosity for two distinct mutations (fig 1, panels A and B). A C→T transition in exon 5 at nt 434 changed a rather conserved threonine into isoleucine (T145I) (fig 1, panel C). This base substitution changed a neutral amino acid into a hydrophilic residue. In addition, a C→T transition in exon 6 at nt 595 changed a highly conserved arginine into a tryptophan (R199W) (fig 1, panel D). This base substitution transformed a basic amino acid residue with hydrophilic properties into a polar residue in a putative casein II phosphorylation site. In patient 2, a T transition in exon 5 at nt 206 changed a rather conserved tyrosine into phenylalanine (Y69F) (fig 1, panel E). This base substitution altered a hydrophobic amino acid residue.

DISCUSSION
In this report, we describe mutations of the NDUFS3 gene in a case of late onset Leigh syndrome and complex I deficiency. The first evidence of encephalopathy with pyramidal and extrapyramidal symptoms was noted at nine years of age. Shortly after, MRI imaging detected focal, symmetrical, and necrotic lesions in the thalamus, brain stem, and white matter, suggestive of Leigh syndrome. Optic atrophy was noted as well. This was followed by progressive deterioration and death four years later.

The R199W mutation identified involved a conserved amino acid and was absent from healthy controls. On the other hand, the T145I mutation changed a neutral residue into a polar residue in a putative casein II phosphorylation site and was also absent from healthy individuals, except for one from Reunion Island who was heterozygous for this base change, with no other mutation. These amino acid changes therefore most probably represent disease causing mutations in the NDUFS3 protein.

Up to now, mutations in nuclear genes encoding complex I subunits (NDUFV1, NDUFS1, NDUFS2, NDUFS4, NDUFS7, and NDUFS8) have been shown to cause neurological disease (Leigh or Leigh-like syndrome), while heart involvement has been reported in patients harbouring mutations in two other nuclear genes encoding complex I subunits (NDUFV2 and NDUFS9). To date, no simple explanation accounts for this clinical variability. The NDUFS1, NDUFS2, NDUFS1, NDUFS2, and NDUFS3 genes all encode subunits that are presumably involved in the catalysis of electron transfer from NADH to ubiquinone. The possibility that only part of these mutations results in superoxide overproduction has been proposed to explain specific cardiac involvement, as superoxide overproduction is known to readily trigger cardiomyocyte hypertrophy. On the other hand, differences in residual...

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<tr>
<th>Table 1</th>
<th>Polargraphic and spectrophotometric assays of respiratory chain activity in patients and controls</th>
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<tr>
<td></td>
<td>Pyruvate (plus malate) oxidation</td>
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<tr>
<td><strong>Patient 1</strong></td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle mitochondria</td>
<td>20 (94 ± 48)</td>
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<tr>
<td>Cultured skin fibroblasts</td>
<td>7.7 (3.3 to 6.8)</td>
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<tr>
<td><strong>Patient 2</strong></td>
<td></td>
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<tr>
<td>Fetus</td>
<td>6.9 (7.7 to 9.5)</td>
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<tr>
<td>PCR products, denatured for 10 minutes at 96°C</td>
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Bold letters denote abnormal values. Determinations were done in three different investigation centres using their own reference values, as indicated in the brackets. However, all investigations resulted in a similar conclusion, that there was complex I deficiency causing decreased pyruvate plus malate oxidation.

*Values obtained after treatment of cultured skin fibroblasts with digitonin and Percoll, as recently described.
complex I activity compromising cell specific functions should be also considered.

With this mutation report in the NDUFS3 gene, disease causing mutations have now been described in all the 14 genes (seven mitochondrial and seven nuclear) encoding the core subunits of complex I and considered essential for catalysing electron transfer from NADH to ubiquinone, and for the generation of the proton motive force. These genes are the most conserved across species, from mammals to bacteria, and the encoded proteins contain all the known redox centres of the enzyme, as well as substrate binding sites. These 14 subunits have been defined as the "minimal enzyme," while other subunits are often referred to as "supernumerary." Among the "supernumerary" subunits, only the NDUFS4 gene has been found mutated in humans. The NDUFS4 subunit is involved in the regulation of complex I activity, with phosphorylation at a cAMP dependent protein kinase consensus site that was found to stimulate complex I activity. The function of the remaining 28 "supernumerary" subunits is unknown so far. These latter subunits may play various different roles, including protection of the complex against oxidative injury, regulation of activity, and assembly or stabilisation of the complex, but alternative functions remain conceivable.

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Figure 1 Molecular analysis of the NDUFS3 gene in patient 1 and control fibroblasts. (A) D-HPLC pattern of NDUFS3 fragment 1 of control fibroblasts (a) and patient fibroblasts (b). (B) D-HPLC pattern of NDUFS3 fragment 2 of control fibroblasts (a) and patient fibroblasts (b). (C, D) Sequence analysis done on fibroblast cDNA from patient 1. (E, F) Sequence alignment of the NDUFS3 proteins from various species. Boxes indicate conserved amino acids. Arrows show the mutated amino acids T145I in fragment 1 (E) and R199W in fragment 2 (F).

Authors’ affiliations

P Bénit, I Giurgea, D Chretien, S Lebon, A Munnich, A Rötig, P Rustin, Unité de Recherche sur les Handicaps Génétiques de l’Enfant (INSERM U393) and Département de Génétique, Hôpital Necker-Enfants Malades, Paris Cedex 15, France
A Slama, Laboratoire de Biochimie 1, AP-HP Hôpital de Bicêtre, Le Kremlin Bicêtre Cedex, France
F Cartault, Centre Hospitalier Départemental Félix Guyon, Service Génétique Bellepierre, Saint-Denis Cedex, La Réunion, France
C Marsac, CERTO Faculté Necker-Enfants Malades, Paris Cedex 15

Correspondence to: Dr Pierre Rustin, INSERM U393, Hôpital Necker-Enfants Malades, 149 rue de Sèvres, 75015 Paris, France; rustin@necker.fr

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Growing up abolishes a genetic protection against asthma

Researchers have discovered that the CCR5Δ32 mutation loses its protective effect against asthma as children mature. This may explain conflicting results and means that future association and linkage studies must take account of age as a confounder.

The researchers went back to a population of schoolchildren aged 8–12 years they studied in 1989 and followed up all those available 10 years later—627 of the original 3406 sample—to determine whether as adults their asthma, wheezing, and atopy status had changed according to their mutation status.

Carrying the deletion significantly reduced the risk of having current asthma in childhood (odds ratio (OR) 0.31 (95% confidence interval 0.14 to 0.72)) but not in adulthood (OR 0.88 (95% CI 0.52 to 1.48)), according to logistic regression corrected for atopy (in 1989) and sex. CCR5Δ32 was not associated with current atopy in either study. Wheezing in childhood had the lowest frequency of heterozygous or homozygous carriers whereas wheezing developing in adults or persisting from childhood had a much higher frequency compared with controls (odds ratio (OR) 0.31 (95% confidence interval 0.14 to 0.72)) but not in adulthood (OR 0.88) according to their mutation status.

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The researchers had shown that children aged 5–15 years with CCR5Δ32 mutation were protected from asthma. Contradictory findings from other studies led them to test whether protection might be age dependent. The deletion stops expression of a chemokine receptor on the surface of T helper 1 cells.

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