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Combined Enzymatic Complex I and III Deficiency Associated with Mutations in the Nuclear Encoded *NDUFS4* Gene

S. M. S. Budde,* L. P. W. J. van den Heuvel,*,[1] A. J. Janssen,* R. J. P. Smeets,* C. A. F. Buskens,* L. DeMeirleir,† R. Van Coster,‡ M. Baethmann,§ T. Voit,§ J. M. F. Trijbels,* and J. A. M. Smeitink*

**Nijmegen Centre for Mitochondrial Disorders, Department of Paediatrics, University Medical Centre Nijmegen, Nijmegen, The Netherlands; †Department of Paediatric Neurology, University Hospital, Vrije Universiteit Brussel, Brussels, Belgium; ‡Department of Paediatrics, University Hospital, Ghent, Belgium; and §Department of Paediatrics, University of Essen, Essen, Germany*

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Combined OXPHOS-system enzyme deficiencies are observed in approximately 25% of all OXPHOS-system disturbances. Of these, combined complex I and III deficiency is relatively scarce. So far, only mtDNA and thymidine phosphorylase (TP) mutations have been associated with combined OXPHOS-system disturbances. In this report we show, for the first time, that a nuclear gene mutation in a structural, nuclear encoded complex I gene is associated with combined complex I and III deficiency. After our initial report we describe mutations in the NDUFS4 gene of complex I in two additional patients. The first mutation is a deletion of G at position 289 or 290. Amino acid 96 changes from a tryptophan to a stop codon. The mutation was found homozygous in the patient; both parents are heterozygous for the mutation. The second mutation is a transition from C to T at cDNA position 316. Codon is changed from CGA (arginine) to TGA (stop). The patient is homozygous for the mutation; both parents are heterozygous. Both mutations in the *NDUFS4* gene led to a premature stop in Leigh-like patients with an early lethal phenotype. We hypothesise that the structural integrity of the OXPHOS system, in mammal supermolecular structures, may be responsible for the observed biochemical features.

Key Words: **mitochondria; OXPHOS system; NADH: ubiquinone oxidoreductase; NDUFS4; Leigh syndrome.**

[1] To whom correspondence should be addressed at Nijmegen Centre for Mitochondrial Disorders, Department of Paediatrics, UMC Nijmegen, Geert Grooteplein 20, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Fax: NL-24-3616428. E-mail: B.vandeheuvel@ckslkn.azn.nl.

OXPHOS-system disorders belong to the most frequently observed inborn errors of metabolism. The incidence of these devastating disorders is approximately 1:10,000 live births. Both isolated and combined OXPHOS-complex deficiencies have been described. Isolated complex I deficiency is the most frequently detected abnormality in our centre (1). The combined OXPHOS-deficient group contributes 25% of all OXPHOS deficiencies (1). Virtually all kind of combinations are possible with combined complex I and IV deficiency as the main contributor. So far, mtDNA mutations and mutations in the TP gene have been associated with combined OXPHOS deficiencies (2–5). The genetic identity of all other combined deficiencies still have to be elucidated.

Recently, we described the first nuclear encoded gene mutations in complex I (1). Of these genes, the NDUFS4 protein is located in the iron-sulphur protein (IP) fraction of complex I. The IP and the flavoprotein (FP) fraction form the catalytic sector and are located in the peripheral arm of the complex which protrudes into the mitochondrial matrix (6). The human *NDUFS4* cDNA sequence has an open reading frame of 525 bp which codes for an 175 amino acid protein (7). The protein contains a conserved consensus phosphorylation site (RVS) at amino acid positions 171–173 (7–10). The bovine equivalent of NDUFS4, the 18kDa or AQDQ subunit, is phosphorylated by a cAMP-dependent protein kinase (11).

Critical evaluation of our complex I deficient patient cohort revealed in one of our earlier reported patients with an *NDUFS4* gene mutation a slight reduction of complex III, in cultured skin fibroblasts, a residual activity of 98% (7). Although we initially paid no atten-

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tion to this finding, in a later stage of our ongoing research to elucidate the genetic cause of combined OXPHOS deficiencies, we decided to evaluate two fibroblast cell lines of Leigh-like patients with combined complex I and III deficiency for mutations in this regulatory complex I gene. Quite unexpectedly both patients showed premature stops in this gene, whereas both parents showed heterozygosity. Our findings may contribute to the understanding of the complexity of the OXPHOS-system in health and disease.

MATERIALS AND METHODS

Case report, patient 1. The patient (a girl) was born after an uneventful third pregnancy and delivery as the second child of consanguineous parents. The first pregnancy resulted in a spontaneous abortion at 3 months; an elder sister is without complaints. In retrospect, within one week of age hypotonia, absent eye-contact, lethargy and failure to thrive became obvious. At the age of 3 months she was admitted for further investigations. Physical examination showed besides the above mentioned symptoms a microcephaly (head circumference 36.3 cm, <3rd percentile). Funduscopy was normal. Blood clinical chemistry showed lactic acidaemia (5.5 mmol/L; $N < 2.1$) with an increased lactate/pyruvate ratio (34; N 12–16). Blood alanine concentration was 634 μmol/L (N 100–440). Cerebrospinal fluid lactic acid was 7.6 mmol/L ($N < 2.1$) and total protein 680 mg/L ($N < 550$). Urinary organic acid analysis showed increased concentrations of lactic acid and dicarboxylic acids with relatively high 3-hydroxydicarboxylic acids concentrations. Cerebral computer tomography scan and magnetic resonance imaging revealed bilateral basal ganglia hypodensities. The brainstem exposed no visible abnormalities. Electrophysiological investigations displayed no visual evoked responses, normal sensory evoked potentials and no convulsive activity on the EEG. The echocardiogram showed neither structural defects nor hypertrophy or dilatation of the heart. Under the clinical suspicion of a mitochondriocytopathy biopsies of skin, skeletal muscle and liver were taken for further morphological, histochemical, biochemical and molecular-genetic studies. Despite the supplementation of biotin, riboflavin and L-carnitine the child's condition progressively worsened. She died at the age of 3 months in the course of a progressive respiratory insufficiency.

Mitochondrial DNA analysis in blood showed a normal size and none of the following mutations: MELAS 3243, MERRF 8344, LEBER 11778 and 3460, NARP 8893. PCR-SSCP of mtATPase did not reveal any abnormal pattern.

Case report, patient 2. This patient was the first child of healthy first grade cousins. He was born spontaneously at term following an uncomplicated pregnancy. Postnatally hypospadia was noted, otherwise the patient appeared normal until the age of 7 weeks. At that time muscular hypotonia and lack of visual and auditive attention were observed.

At 3 months of age the patient was admitted to a peripheral pediatric hospital with an upper airway infection, cough and feeding problems. His general condition was poor. Physical examination revealed tachycardia, hepatomegaly (2–3 cm under costal margin) and mild splenomegaly. Spontaneous movements were markedly reduced. The patient required intubation and artificial ventilation. Radiologically pneumonia was diagnosed and successfully treated. In spite of radiological improvement it was impossible to wean the patient off the ventilator.

Laboratory investigation on admission suggested bacterial infection with leucocytosis (12.0/μl, 16% rods) and elevated C-reactive protein (1:32). Acid-base status, glucose, electrolytes, creatine, CK, free and total carnitine, very long chain fatty acids, total protein and transaminases were normal. Urinary organic acid analysis was normal. Thrombocytes were elevated on several measurements with values between 438,000/μl and 743,000/μl. Lactate was within the normal range twice (1.5 and 1.8 mmol/L).

The patient was transferred to our hospital for further work-up. He was artificially ventilated and in a stable condition. On exam there was facial dysmorphia with slightly upslant palpebral fissures and microgenia. His axial and peripheral muscle tone were markedly reduced. Knee and ankle jerks were positive. The liver was palpable 3 cm below the costal margin. There was a 2/6 systolic murmur. No visual fixation could be observed, the reaction to light was equivocal. Funduscopy was normal.

In contrast to earlier measurements this time laboratory investigations revealed elevated lactate levels in blood (3.0 mmol/L) and CSF (3.4 mmol/l). Plasma alanine was elevated (693 μmol/L). Analysis of the mitochondrial DNA excluded a mutation at nt8993 (NARP). Cranial MRI showed hyperintense signals in T2 weighted images in the basal ganglia pedunculi cerebri and the pariaquaductal region resembling Leigh syndrome. Cardial ultrasound disclosed concentric hypertrophy of the left ventricle with hypercontractility. Brain stem auditory evoked potentials were abnormal with no response on the right side and a vaguely visible wave V on the left side. Visual evoked potentials were absent and after somatosensory stimulation only the cervical response could be detected. The contracortical responses were lacking.

A muscle biopsy was performed under the suspicion of a mitochondrial disorder. Histology revealed an irregular intermyofibrillary texture resembling ragged-red fibers. No NADH- or COX-negative fibres were observed. Additionally there was accumulation of lipid droplets in the sarcoplasm.

The patient died soon thereafter from cardiocirculatory insufficiency despite continued mechanical ventilation.

Biochemical analysis. Enzyme activities were measured in fresh muscle homogenate and cultured skin fibroblasts. Skin fibroblasts were cultured according to standard procedures. Enzyme activities were determined with minor modifications as previously described (12, 13).

Mutation detection and restriction enzyme analysis. Total RNA was isolated from cultured skin fibroblasts (14), 5 μg of RNA was transcribed in a RT reaction using Superscript II RNase H^- reverse transcriptase (Life Technologies) and random hexamer primers. The entire coding region of the *NDUFS4* subunit was amplified. Fifty ng of each primers was added to 1μl of cDNA and 0.5 units of *Taq* polymerase (Life Technologies) in a total volume of 25 μl containing 2.5 μl dNTP's (2.5 mM), 2.5 μl 10× PCR buffer and 1.5 mM $MgCl_2$ (Life Technologies). The reaction parameters were 94°C for 4 min, followed by 35 cycles of 1 min 94°C, 1 min 58°C, and 30 s 72°C and finally a 10 min extension at 72°C. The primers used in this PCR reaction were forward primer C118F1N and reverse primer C118R2N (all primer sequences are listed in Table 1). The sequence of the 5′ untranslated region was determined using a 5′ RACE kit according to the manufacturers instructions (Life Technologies). PCR products were sequenced in two parts on an automated ABI 377 sequencer using BigDye terminators (Perkin Elmer) according to the manufacturer's instructions (Perkin Elmer). For sequencing 4 primers were used, outer primers C118F1N and C118R2N and interior located primers C118F2N and C118R1N (Table 1). Comparison of wild type and patients *NDUFS4* sequence was performed with Sequence Navigator software (Perkin Elmer). For direct sequencing on genomic DNA, primers C118d290F and C118d290R were used to create a PCR fragment (PCR parameters are as described above) and to perform DNA sequence analysis.

Restriction enzyme analysis for the first mutation (del290G) was performed by PCR-PIRA on genomic DNA. Primer 290PIRA was used in a PCR reaction together with primer C118R1N. The reaction parameters are as described above. The PCR fragments were di-

TABLE 1

Primer Sequences Used for Sequencing, PCR, and PCR-PIRA of the *NDUFS4* Gene

Primer name	cDNA position	Primer sequence
Forward primers		
C118F1N	−22/−5	5′-CCT GGC GTT TGC CTG CAG-3′
C118F2N	249/269	5′-TCG CAA TAA CAT GCA GTC TGG-3′
C118d290F	230/251	5′-TCA GGA TCT TGT TCC TGC TCG-3′
C118d290PIRA	266/290	5′-CTG GAG TAA ACA ACA CAA AGA ACT G-3′
C118C316TF	234/257	5′-GAT CTT TGT TCC TGC TCG CAA TAA C-3′
Reverse primers		
C118R1N	331/311	5′-AAG GAT TTT CCC ATC GCT CCC-3′
C118R2N	553/532	5′-AAG CAG AGA TAT AGT CAG TGC C-3′
C118d290R	364/353	5′-ATA AGG GAT CAG CCG TTG ATG C-3′
C118C316TPIRA	342/317	5′-CAA CCC ATC AAA GGA TTT TCC CAG C-3′

gested using the restriction enzyme Bsr I (Life Technologies). Primer C118d290PIRA introduced a mutation in the *NDUFS4* fragment, which results in a restriction site in the PCR fragment of the wildtype *NDUFS4* for restriction enzyme Bsr I.

The second mutation (C316T) was also confirmed by PCR-PIRA on genomic DNA. Primer C118c316tPIRA creates a restriction site for Hha I (Life technologies) in the wildtype sequence. The forward primer used in this PCR-PIRA was C118c316tF. Besides a modification of the annealing temperature (57°C), the reaction conditions for this PCR were as described before.

RESULTS

Morphological and Biochemical Examinations

In patient 1 muscle pathological studies showed atrophy of some of the fibres without other abnormalities. Liver light-microscopic architecture was normal. Electron microscopy of both tissues was not available. Biochemical studies in isolated muscle tissue of patient 1, measured in another centre, revealed a complex I deficiency (15.7 nmol/mg/min; reference range: 25–45). Due to lack of material complex III activity in muscle tissue wasn't measured in this patient. Biochemical examinations in muscle tissue of patient 2 revealed a severe deficiency of complex I activity (Table 2). The residual activity amounted to 14% of the lowest control value. The activity of complex III was also decreased although to a lower extent (residual activity was 57%). This is also reflected by the slightly decreased activity of succinate:cyt *c* oxidoreductase.

In cultured skin fibroblasts of both patients a complex I deficiency has been established but the residual activity in patient 2 was considerable higher as compared to muscle tissue (60% versus 14%). Fibroblast of patient 1 revealed a residual complex I activity of 47%. Complex III activity in fibroblasts of both patients was also decreased (residual activities were 87% and 67%, respectively for patients 1 and 2) (Table 2).

Molecular-Genetic Results

A 24 base pair sequence of the 5′ untranslated region (5′ATCCTGGCGTTTGCCTGCAGCAAG3′) was re-

TABLE 2

Enzyme Activities in Skeletal Muscle and Fibroblasts

	Enzyme activities					
	Cultured fibroblasts			Skeletal muscle tissue		
	Patient 1	Patient 2	Control	Patient 1	Patient 2	Control
NADH:Q_1 oxydoreductase (CI)	0.047[a]	0.060[a]	0.10–0.31[a]	15.7[c]	10[a]	70–250[a]
Cytochrome *c* oxidase (CIV)	0.79[a]	0.90[a]	0.68–1.19[a]	NM	880[a]	810–3120[a]
Citrate synthase (CS)	321[b]	234[b]	144–257[b]	NM	79.2[b]	37–162[b]
Succinate:cyt *c* oxidoreductase	0.18[a]	0.25[a]	0.16–0.44[a]	NM	291[a]	300–970[a]
Decylubiquinol:cyt *c* oxidoreductase (CIII)	1.11[a]	0.85[a]	1.27–2.62[a]	NM	1430[a]	2500–6610[a]

Note. NM, not measured.
[a] In mU per mU CS.
[b] In mU per mg protein.
[c] In nmol/mg/min; reference range: 25–45.

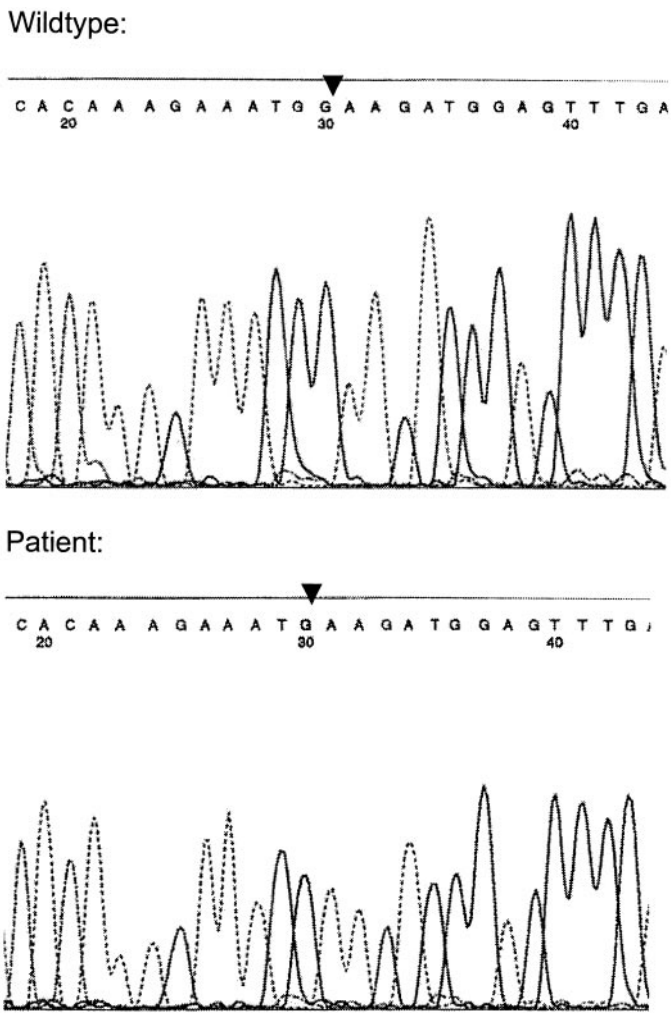

FIG. 1. cDNA sequence of *NDUFS4* fragment. Sequence analysis of patient 1. In the upper figure the wildtype sequence is shown; the lower figure shows the patients sequence. The mutation d290G (or d289G) in the patient results in a premature stop codon at amino acid 96.

vealed using 5′ RACE. This sequence was used to create a forward primer to amplify the complete ORF of the *NDUFS4* (primer C118F1N). Direct sequencing of the PCR fragment of patient 1 obtained by primers C118F1N, C118F2N, C118R1N and C118R2N showed a homozygous deletion of 1 bp at position 289 or 290 (Fig. 1). This deletion of a G causes codon 96 to change from TGG (Trp) to TGA (stop). The mutation destroys the conserved phosphorylation consensus sequence.

Sequencing of nDNA of parents and sibs showed that all were heterozygous for the mutation. The results were sustained by PCR-PIRA on genomic DNA (data not shown).

Sequencing of the cDNA fragment of patient 2 revealed a transition of C to T at cDNA position 316. This mutation causes amino acid 106 to change from arginine (CGA) to a stop (TGA). As a result the protein is truncated at that position. Again the mutation results in the loss of the conserved phosphorylation site. Restriction analysis of the PCR-PIRA fragment with enzyme Hha I confirmed that the patient is homozygous for the mutation. Both parents and a brother of the patient are heterozygous for the mutation (Fig. 2).

DISCUSSION

In the original NDUFS4 mutated complex I deficient patients complex III activity had a residual activity of 98% in cultured skin fibroblasts (expressed on cytochrome c oxidase) (7). When expressing the complex III activity in mitochondrial enriched fraction from fibroblasts on the mitochondrial matrix reference enzyme citrate synthase this gives a CIII activity of 74% of the lowest control value. Based on this finding we decided to investigate two combined complex I and III deficient patients for mutations in the *NDUFS4* gene. Enzyme activities in fibroblasts are usually expressed in mU/mU COX because citrate synthase can be artificially decreased in the enrichment process of the mitochondria. Both patients described in this report show reproducible normal or increased citrate synthase activities. Complex I and III activities of these patients were therefor expressed in mU/mU citrate synthase.

cDNA of the patients was PCR-amplified with primers targeted outside the open reading frame. Direct sequencing of the *NDUFS4* cDNA demonstrated a homozygous deletion of a G at cDNA position 289 or 290

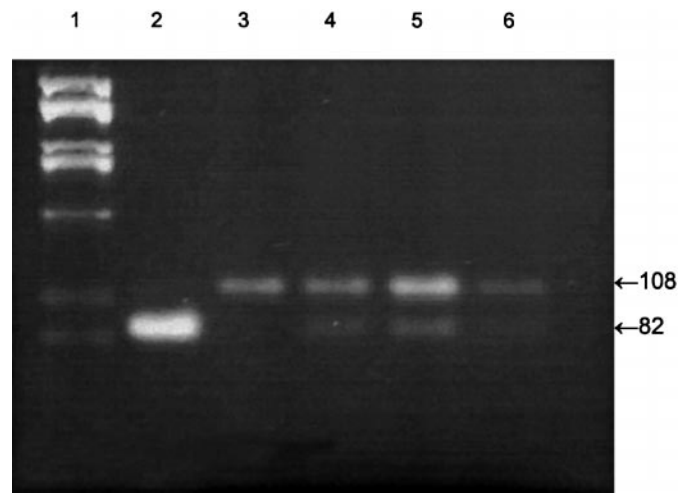

FIG. 2. Restriction enzyme analysis for the C316T mutation within family 2. In lane 1 is a pUC18 HaeIII marker, the smallest fragments correspond with 102 and 80 bp. Lane 2 is the control, homozygous wildtype. Lane 3 is the patient, with the homozygous mutation. Heterozygosity of the mutation of the father and mother are shown in lane 4 and 5, respectively. Lane 6 is the brother of the patient, heterozygous for the mutation.

in patient 1. The mutation causes a change of amino acid tryptophan to a stop in the mature protein. The protein is therefore truncated at amino acid 96. The mutation was confirmed by PCR-PIRA. Both parents and two other sibs are all heterozygous for the mutation. In patient 2 a mutation was found at cDNA position 316, a transition of a CGA (arg) to TGA(stop), resulting in a truncated NDUFS4 protein at amino acid position 106. Again the mutation was confirmed by PCR-PIRA. The consanguineous parents and a brother are heterozygous for the mutation. The observed frequency of mutations in this subunit makes it a mutational hot-spot.

All known mutations in the NDUFS4 subunit of complex I result in truncated proteins and cause the conserved phosphorylation site to disappear. The bovine equivalent of the NDUFS4 subunit, the 18-kDa subunit, is phosphorylated by a mitochondrial cAMP dependent protein kinase. Studies with 3T3 Balb/c mouse fibroblasts show that an increase in cAMP, induced by cholera toxin, promotes the phosphorylation of the 18-kDa subunit. This phosphorylation results in a marked enhancement of the complex I activity. Phosphorylation of the bovine 18-kDa subunit positively regulates complex I, causing a strong respiratory and complex I stimulation associated with the elevation of cellular cAMP *in vivo* (15, 16).

It is remarkable that not only the complex I activity is lowered, but also the complex III activity is decreased in NDUFS4 mutated patients. The complex III activity of patient one in this report could only be measured in fibroblasts. Patient 2 showed a decreased complex III activity in both fibroblasts and muscle tissue.

Although further studies are warranted a recent report of Schägger and Pfeiffer (17), who present evidence that the classical description of the OXPHOS system composition of mammalian cells is incorrect, may give an explanation for the observed findings. By blue native gel electrophoresis they showed that, an observation also made in lower species, complex I and III together with complex IV occur in supercomplexes instead of isolated complexes as was until recently the common view. Alternations in the NDUFS4 protein, as a consequence of the observed mutations in our patients, may interfere in the formation of such supercomplexes leading to a combined complex I and III deficiency. Accordingly to this hypothesis our findings support a physical interaction between complex I and III. Of course other explanations to clarify our findings like enhanced radical production caused by the complex I deficiency secondary influencing complex III activity may be present. Further studies in order to try to obtain a definite explanation will be planned.

Our results show that a mutation in a structural complex I gene can cause a combined complex I and III deficiency. It is worthwhile to investigate the NDUFS4 subunit for mutations in patients with a combined complex I and III deficiency.

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