

Paule Bénit · Julie Steffann · Sophie Lebon · Dominique Chretien · Noman Kadhom · Pascale de Lonlay
Alice Goldenberg · Yves Dumez · Marc Dommergues · Pierre Rustin · Arnold Munnich · Agnès Rötig

Genotyping microsatellite DNA markers at putative disease loci in inbred/multiplex families with respiratory chain complex I deficiency allows rapid identification of a novel nonsense mutation (IVS1nt –1) in the *NDUFS4* gene in Leigh syndrome

Received: 17 September 2002 / Accepted: 11 November 2002 / Published online: 4 March 2003

© Springer-Verlag 2003

Abstract Complex I deficiency, the most common cause of mitochondrial disorders, accounts for a variety of clinical symptoms and its genetic heterogeneity makes identification of the disease genes particularly tedious. Indeed, most of the 43 complex I subunits are encoded by nuclear genes, only seven of them being mitochondrially encoded. In order to offer urgent prenatal diagnosis, we have studied an inbred/multiplex family with complex I deficiency by using microsatellite DNA markers flanking the putative disease loci. Microsatellite DNA markers have allowed us to exclude the *NDUFS7*, *NDUFS8*, *NDUFV1* and *NDUFS1* genes and to find homozygosity at the *NDUFS4* locus. Direct sequencing has led to identification of a homozygous splice acceptor site mutation in intron 1 of the *NDUFS4* gene (IVS1nt –1, G→A); this was not found in chorion villi of the ongoing pregnancy. We suggest that genotyping microsatellite DNA markers at putative disease loci in inbred/multiplex families helps to identify the disease-causing mutation. More generally, we suggest giving consideration to a more systematic microsatellite analysis of putative disease loci for identification of disease genes in inbred/multiplex families affected with genetically heterogeneous conditions.

Introduction

Reduced nicotinamide adenine dinucleotide (NADH): ubiquinone oxidoreductase (complex I) catalyzes electron transfer from NADH to ubiquinone. This enzyme, the largest complex of the mitochondrial respiratory chain, contains more than 40 subunits (Fearnley and Walker 1992). Complex I deficiency, the most common cause of mitochondrial disorders, represents one third of all cases of

respiratory chain deficiency (von Kleist-Retzow et al. 1998; Kirby et al. 1999). This disease accounts for a variety of clinical symptoms, ranging from neurological disorders to cardiomyopathy, liver failure and myopathy (von Kleist-Retzow et al. 1998; Loeffen et al. 2000; Smeitink et al. 2001). Most of the 43 complex I subunits are encoded by nuclear genes; only seven of them are mitochondrially encoded. This genetic heterogeneity makes diagnosis of the disease genes in affected families particularly tedious. Indeed, mutations in a number of nuclear complex I genes have been identified in patients with Leigh syndrome (*NDUFS8*, *NDUFS4*, *NDUFS7*, *NDUFV1*, *NDUFS1*; Loeffen et al. 1998; van den Heuvel et al. 1998; Budde et al. 2000; Petruzzella et al. 2001; Triepels et al. 1999; Schuelke et al. 1999; Bénit et al. 2001) and cardiomyopathy/encephalomyopathy (*NDUFS2*; Loeffen et al. 2001).

Here, we show that genotyping microsatellite DNA markers at putative disease loci in inbred/multiplex families helps to solve this genetic complexity and has allowed us to identify rapidly a novel homozygous nonsense mutation of the *NDUFS4* gene in a sibship with Leigh disease and severe complex I deficiency. More generally, we suggest giving consideration to a microsatellite-based exclusion of putative disease loci for prenatal diagnosis of genetically heterogeneous conditions.

Materials and methods

Nomenclature

Gene mutation nomenclature used in this article follows the recommendations of den Dunnen and Antonarakis (2001). Gene symbols used in this article follow the recommendations of the HUGO Gene Nomenclature Committee (Povey et al. 2001).

Patients

Patients II-2 and II-4 were born to first cousin Moroccan parents after a term pregnancy and normal delivery. Three brothers are healthy (II-1, II-3, II-5; Fig. 1). The first girl (patient II-2) did well in her first 2 months of life (birth weight: 3 kg, occipito-frontal circumference: 34 cm). Poor sucking, drowsiness and floppiness were

P. Bénit · J. Steffann · S. Lebon · D. Chretien · N. Kadhom
P. de Lonlay · A. Goldenberg · Y. Dumez · M. Dommergues
P. Rustin · A. Munnich (✉) · A. Rötig
Département de Génétique, Maternité and INSERM U393,
Hôpital Necker-Enfants Malades,
149 Rue de Sèvres, 75015 Paris, France
Tel.: +33-144381584, Fax: +33-147348514,
e-mail: munnich@necker.fr

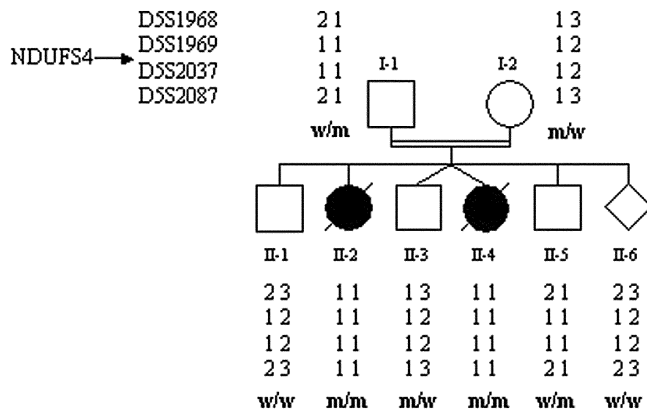


Fig. 1 Pedigree and haplotype analysis of the complex I deficient family (*w* wild-type, *m* mutant). Haplotypes are given for loci D5S1968, D5S1969, D5S2037 and D5S2087 (*top to bottom*)

first noted at 10 weeks of age. She had trunk hypotonia, poor spontaneous movements, poor reactivity, a squint and absent deep tendon reflexes but no other organ involvement was noted. Elevated plasma lactates (6–7 mmol/l, normal: less than 2 mmol/l) and bilateral hypodensity of the periventricular white matter on brain computer tomography (CT) were suggestive of Leigh syndrome of metabolic origin. She died at 4 months of age of major swallowing difficulties, hypoventilation and severe brainstem involvement. Her sister (patient II-4) was small for gestational age (birth weight: 1700 g) but she did well until the age of 3 months. At 3.5 months, psychomotor regression with drowsiness and floppiness were noted. She could not smile or follow objects with her eyes and presented recurrent attacks of bradycardia and bradypnea, suggestive of severe brainstem involvement. Brain CT scan showed cortical atrophy and bilateral hypodensity of the peduncles and striatum, suggestive of Leigh syndrome (plasma lactate 2.5–3.3 mmol/l; cerebrospinal fluid lactate 3.9 mmol/l, normal: less than 2.4 mmol/l). The pregnant mother requested prenatal diagnosis at 10 weeks of gestation.

Methods

Spectrophotometric assays of respiratory chain enzymes were performed on muscle and liver homogenates and on cultured skin fibroblasts as described (Rustin et al. 1994). For haplotyping, the mi-

Table 1 Respiratory chain enzyme activities in skeletal muscle and liver homogenate of patient II-4 and in controls. Abnormal values are in *bold*. In the liver, the activity of oligomycin-sensitive ATPase (complex V) was found to be low compared with that of cytochrome *c* oxidase, a frequent feature in frozen samples

Enzyme	Muscle		Liver	
	Patient II-4	Controls (<i>n</i> =51)	Patient II-4	Controls (<i>n</i> =51)
	Activities (nmol/min per mg protein)			
NADH quinone reductase	8	10–23	11	15–28
Succinate quinone dichlorophenol indophenol reductase	64	21–44	168	111–167
Decylubiquinone cytochrome <i>c</i> reductase	690	248–453	437	289–453
Cytochrome <i>c</i> oxidase	303	85–214	340	125–231
ATPase	–	–	72	61–105
	Activity ratios			
Cytochrome <i>c</i> oxidase/NADH quinone reductase	48.2	9.2±1.2	31.5	8.6±3.1
Cytochrome <i>c</i> oxidase/succinate quinone dichlorophenol indophenol reductase	4.7	5.3±0.2	2	1.5±.4
Cytochrome <i>c</i> oxidase/decylubiquinone cytochrome <i>c</i> reductase	0.4	0.5±0.1	0.8	0.6±.1
Cytochrome <i>c</i> oxidase/ATPase	–	–	4.7	2.2±.4

rosatellite DNA markers of the Genethon map flanking the putative disease loci were tested in the parents, the affected and the unaffected sibs. The most informative flanking microsatellites were used from chromosome 2 (D2S155-*NDUFS1*-D2S369-D2S2358), chromosome 5 (D5S1968–0.3cM-D5S1969–0.4cM-*NDUFS4*-0.3cM-D5S2037–8.2cM-D5S2087), chromosome 11 (D11S4191-D11S4113-*NDUFS8-NDUFV1*-D11S4139-D11S4136) and chromosome 19 (D19S886-*NDUFS7*-D19S883-D19S878).

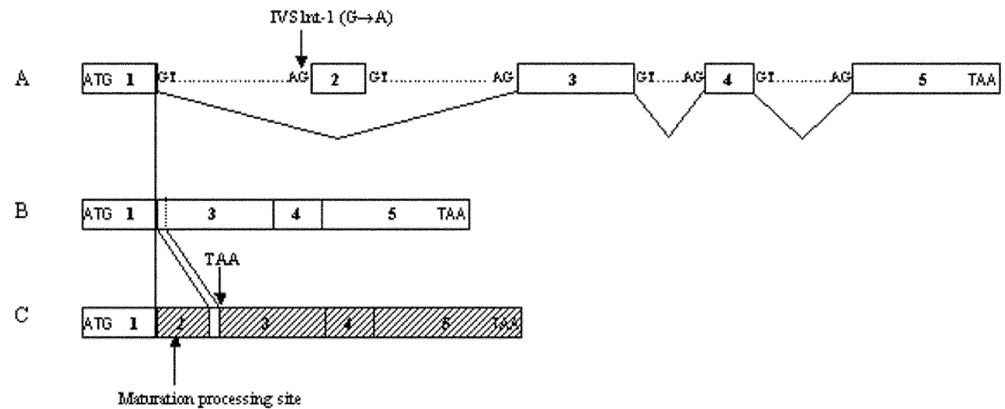
Linkage analysis was performed using version 5.1 of the Linkage program (Lathrop et al. 1985). Pairwise linkage was performed between the disease locus and marker loci. Haplotype studies were performed at each possible disease locus and the most likely haplotype was inferred by minimizing the number of crossover events in the sibship. Homozygosity mapping based on genetic analysis of the inbred family relies on the finding that, in an affected child born to consanguineous parents, the region spanning the disease locus is homozygote by descent (Lander and Botstein 1987). Homozygosity depends on (1) the distance between the marker and the disease locus, (2) the degree of inbreeding, (3) the mutant gene frequency and (4) the allele frequency for each locus tested.

When the two affected children were homozygous and haploidentical at polymorphic loci flanking a putative disease gene, the corresponding genomic DNA was sequenced in the proband by using the Big Dye terminator cycle sequencing kit (ABI Prism). Total RNAs were extracted from cultured skin fibroblasts by using the Rnasin Kit (Quiagen) and reverse-transcribed with random hexamers (GenAmp RNA PCR core kit, Perkin-Elmer). The reverse transcription/polymerase chain reaction (RT-PCR) amplification of the *NDUFS4* cDNA was carried out with forward (5'-AGTG-TTTGCCTGCAGCAAG-3') and reverse (5'-CATCAAAGGAT-TTCCCATCGC-3') primers.

Results

Enzyme investigations on skeletal muscle and liver homogenate revealed a severe complex I deficiency in patient II-4 accompanied by increased activity of most respiratory chain complexes, especially in skeletal muscle, suggestive of a mitochondrial accumulation (Table 1). Microsatellite DNA markers flanking *NDUFS7* (pairwise lod-score $Z=-1.24$ at a recombination fraction $\theta=0.05$ at the D19S886 locus), *NDUFS8-NDUFV1* ($Z=-1.7$ at $\theta=0.05$ at the D11S4139 locus) and *NDUFS1* ($Z=-\infty$ at $\theta=0$ at the

Fig. 2A–C Schematic representation of the *NDUFS4* splice mutation IVS1nt –1 (G→A). **A** Genomic DNA. Exon numbers are shown. **B** Patient's cDNA. **C** Mutant *NDUFS4* protein. *Open bars* Mutant protein, *hatched bars* absent domains of the mutant protein



D2S2358 locus) genes allowed us to exclude these genes, based on heterozygosity of II-2 and II-4 at these loci (not shown). By contrast, the two patients were homozygous for polymorphic markers flanking the *NDUFS4* gene on chromosome 5 ($Z_{\max}=2.1$ at $\theta=0$ at the D5S2087 locus), whereas their parents and their unaffected sibs were heterozygous at these loci (Fig. 1). Sequencing genomic DNA of the affected sibs revealed a homozygous splice acceptor site mutation of intron 1 of the *NDUFS4* gene (IVS1nt –1, G→A), whereas the carrier parents were heterozygous for this mutation. RT-PCR analysis of cultured skin fibroblasts of patient II-2 detected a shorter *NDUFS4* cDNA (279 bp) compared with that of the control (358 bp) and sequencing this amplification product disclosed a 76-bp deletion corresponding to the complete skipping of exon 2. This splic-

ing mutation resulted in a shortened RNA transcript encoding a predicted truncated protein of only 39 amino acids (controls: 175 amino acids), containing an altered mitochondrial targeting sequence and missing the cleavage site required for the proper maturation of the preprotein (Fig. 2). This mutation was not found in chorion villi of the ongoing pregnancy as the fetus II-6 was found to have inherited the two wild-type alleles at the *NDUFS4* locus.

Discussion

Taking advantage of consanguinity, we first excluded the *NDUFS1*, *NDUFV1*, *NDUFS7* and *NDUFS8* genes but not the *NDUFS4* gene in an inbred family requesting pre-

Table 2 Mutations in nuclear DNA associated with complex I deficiencies

Gene	Mutations	Clinical presentation	Chromosome localisation	References
<i>NDUFV1</i>	R59X/T423 M	Encephalomyopathy	11q13	Schuelke et al. (1999)
	A341V/A341V	Leukodystrophy and myoclonic epilepsy	–	Schuelke et al. (1999)
	E214 K/IVS8nt+4	Leigh syndrome	–	Bénit et al. (2001)
	A432P/del nt 989–990	Leigh syndrome	–	Bénit et al. (2001)
<i>NDUFS1</i>	Y204C/C206G	Leigh syndrome	–	Bénit et al. (2001)
	D252G/del codon 222	Leukodystrophy	2q33–34	Bénit et al. (2001)
	R241 W/R557X	Leigh syndrome	–	Bénit et al. (2001)
<i>NDUFS2</i>	M707 V/large scale deletion	Leigh syndrome	–	Bénit et al. (2001)
	R228Q/228	Hypertrophic cardiomyopathy and encephalomyopathy	1q23	Loeffen et al. (2001)
	P229Q/P229Q	Hypertrophic cardiomyopathy and encephalomyopathy	–	Loeffen et al. (2001)
<i>NDUFS4</i>	S413P/S413P	Hypertrophic cardiomyopathy and encephalomyopathy	–	Loeffen et al. (2001)
	Homozygous 5 bp duplication	Leigh syndrome	5q11	van den Heuvel et al. (1998)
	W96X/W96X	Leigh-like syndrome	–	Petruzzella et al. (2001)
<i>NDUFS7</i>	R316X/R316X	Leigh-like syndrome	–	Budde et al. (2000)
	V122 M/V122 M	Leigh syndrome	19p13	Tripels et al. (1999)
<i>NDUFS8</i>	P79L/R102H	Leigh syndrome	11q13	Loeffen et al. (1998)

natal diagnosis for Leigh disease and complex I deficiency. We found an hitherto unreported *NDUFS4* splicing mutation of intron 1 (*IVS1nt-1*) causing the complete skipping of exon 2 in the *NDUFS4* mRNA. This splicing mutation resulted in a shortened RNA transcript encoding a predicted truncated protein of only 39 amino acids, containing an altered mitochondrial targeting sequence and missing the cleavage site required for the proper maturation of the preprotein (Fig. 2). Based on this finding, we were able to carry out a rapid prenatal diagnosis for the next pregnancy.

Over the years, the genetic heterogeneity of clinically homogeneous conditions has become a major issue of medical genetics, particularly for prenatal diagnosis and genetic counselling of inherited diseases. On the other hand, most of the disease genes have been (or are being) mapped and identified. For this reason, the systematic sequencing of all putative disease genes in inbred families may not be mandatory, as studying the segregation of informative microsatellite DNA markers flanking the disease loci should help to exclude several genes, i.e. by focusing mutation search on loci of haploidentity and/or shared homozygosity in affected individuals. Similarly, haploidentity of healthy and affected individuals and/or divergences between affected individuals at a given locus allow(s) a particular locus to be rapidly excluded. Based on this approach, the number of disease genes to be sequenced should be markedly reduced. Because of the small size of each single inbred family, it should be borne in mind, however, that one expects exclusions and indications of consistence rather than significant linkage at putative disease loci.

Finally, this approach may prove particularly rewarding in cases of major genetic heterogeneity, such as in Leigh disease where at least six complex I nuclear genes and two mitochondrially encoded complex I subunits may be involved. The first mutation identified in a nuclear encoded complex I gene was a 5-bp *NDUFS4* duplication found in a patient with Leigh-like syndrome (van den Heuvel et al. 1998). Other *NDUFS4* mutations have been subsequently reported in patients with Leigh disease or Leigh-like presentation (Table 2). The systematic study of nuclear encoded subunits has detected disease-causing mutations in several other genes in cases of complex I deficient Leigh syndrome (*NDUFS1*, *NDUFV1*, *NDUFS7*, *NDUFS8*) and in cardiomyopathy (*NDUFS2*; Table 2).

In conclusion, because of the increasing genetic complexity of inherited diseases and the cost of screening procedures requested for genetic counselling and prenatal diagnoses, we suggest giving consideration to a more systematic genetic analyses of putative disease loci in inbred families with genetically heterogeneous conditions.

Acknowledgements We thank Josseline Kaplan for her help in lodscore calculations. This work was supported in part by the Association Française contre les Myopathies.

References

- Bénit P, Chretien D, Kadhon N, Lonlay-Debeney P de, Cormier-Daire V, Cabral A, Peudenier S, Rustin P, Munnich A, Rötig A (2001) Large-scale deletion and point mutations of the nuclear *NDUFV1* and *NDUFS1* genes in mitochondrial complex I deficiency. *Am J Hum Genet* 68:1344–1352
- Budde SM, Heuvel LP van den, Janssen AJ, Smeets RJ, Buskens CA, DeMeirleir L, Coster R van, Baethmann M, Voit T, Trijbels JM, Smeitink JA (2000) Combined enzymatic complex I and III deficiency associated with mutations in the nuclear encoded *NDUFS4* gene. *Biochem Biophys Res Commun* 275:63–68
- Dunnen JT den, Antonarakis SE (2001) Nomenclature for the description of human sequence variations. *Hum Genet* 109:121–124
- Fearnley IM, Walker JE (1992) Conservation of sequences of subunits of mitochondrial complex I and their relationships with other proteins. *Biochim Biophys Acta* 1140:105–134
- Heuvel L van den, Ruitenbeek W, Smeets R, Gelman-Kohan Z, Elpeleg O, Loeffen J, Trijbels F, Mariman E, Bruijn D de, Smeitink J (1998) Demonstration of a new pathogenic mutation in human complex I deficiency: a 5-bp duplication in the nuclear gene encoding the 18-kD (AQDQ) subunit. *Am J Hum Genet* 62:262–268
- Kirby DM, Crawford M, Cleary MA, Dahl HH, Dennett X, Thorburn DR (1999) Respiratory chain complex I deficiency: an underdiagnosed energy generation disorder. *Neurology* 52:1255–1264
- Kleist-Retzow JC von, Cormier-Daire V, Lonlay P de, Parfait B, Chretien D, Rustin P, Feingold J, Rötig A, Munnich A (1998) A high rate (20%–30%) of parental consanguinity in cytochrome-oxidase deficiency. *Am J Hum Genet* 63:428–435
- Lander ES, Botstein D (1987) Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children. *Science* 236:1567–1570
- Lathrop GM, Lalouel JM, Julier C, Ott J (1985) Multilocus linkage analysis in humans: detection of linkage and estimation of recombination. *Am J Hum Genet* 37:482–498
- Loeffen J, Smeitink J, Triepels R, Smeets R, Schuelke M, Sengers R, Trijbels F, Hamel B, Mullaart R, Heuvel L van den (1998) The first nuclear-encoded complex I mutation in a patient with Leigh syndrome. *Am J Hum Genet* 63:1598–1608
- Loeffen JL, Smeitink JA, Trijbels JM, Janssen AJ, Triepels RH, Sengers RC, Heuvel LP van den (2000) Isolated complex I deficiency in children: clinical, biochemical and genetic aspects. *Hum Mutat* 15:123–134
- Loeffen J, Elpeleg O, Smeitink J, Smeets R, Stockler-Ipsiroglu S, Mandel H, Sengers R, Trijbels F, Heuvel L van den (2001) Mutations in the complex I *NDUFS2* gene of patients with cardiomyopathy and encephalomyopathy. *Ann Neurol* 49:195–201
- Petruzzella V, Vergari R, Puzifferri I, Boffoli D, Lamantea E, Zeviani M, Papa S (2001) A nonsense mutation in the *NDUFS4* gene encoding the 18 kDa (AQDQ) subunit of complex I abolishes assembly and activity of the complex in a patient with Leigh-like syndrome. *Hum Mol Genet* 10:529–535
- Povey S, Lovering R, Bruford E, Wright M, Lush M, Wain H (2001) The HUGO Gene Nomenclature Committee (HGNC). *Hum Genet* 109:678–680
- Rustin P, Chretien D, Bourgeron T, Gérard B, Rötig A, Saudubray JM, Munnich A (1994) Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta* 228:35–51
- Schuelke M, Smeitink J, Mariman E, Loeffen J, Plecko B, Trijbels F, Stockler-Ipsiroglu S, Heuvel L van den (1999) Mutant *NDUFV1* subunit of mitochondrial complex I causes leukodystrophy and myoclonic epilepsy. *Nat Genet* 21:260–261
- Smeitink J, Sengers R, Trijbels F, Heuvel L van den (2001) Human NADH:ubiquinone oxidoreductase. *J Bioenerg Biomembr* 33:259–266
- Triepels RH, Heuvel LP van den, Loeffen JL, Buskens CA, Smeets RJ, Rubio Gozalbo ME, Budde SM, Mariman EC, Wijburg FA, Barth PG, Trijbels JM, Smeitink JA (1999) Leigh syndrome associated with a mutation in the *NDUFS7* (PSST) nuclear encoded subunit of complex I. *Ann Neurol* 45:787–790