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RAPID COMMUNICATION

Mutant NDUFV2 Subunit of Mitochondrial Complex I Causes Early Onset Hypertrophic Cardiomyopathy and Encephalopathy

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Respiratory chain complex I deficiencies represent a genetically heterogeneous group of diseases resulting from mutations in either mitochondrial or nuclear DNA. Combination of denaturing high performance liquid chromatography and sequence analysis allowed us to show that a 4-bp deletion in intron 2 (IVS2+5_+8delGTAA) of the NDUFV2 gene (encoding NADH dehydrogenase ubiquinone flavoprotein 2) causes complex I deficiency and early onset hypertrophic cardiomyopathy with trunk hypotonia in three affected sibs of a consanguineous family. The homozygous mutation altering the consensus splice–donor site of exon 2 resulted in 70% decreased NDUFV2 protein and complex I deficiency. While mutation in a number of genes encoding complex I subunits essentially result in neurological symptoms, this first mutation in NDUFV2 is strikingly associated with cardiomyopathy, as previously observed in the unique case of NDFUS2 mutations. Hum Mutat 21:582–586, 2003. © 2003 Wiley-Liss, Inc.

KEY WORDS: cardiomyopathy; encephalopathy; mutation analysis; NADH: ubiquinone oxidoreductase; NDUFV2

DATABASES:
NDUFV2 – OMIM: 600532; GenBank: NM_021074

INTRODUCTION

Reduced nicotinamide adenine dinucleotide (NADH): ubiquinone oxidoreductase (mitochondrial respiratory chain complex I) catalyzes electron transfer from NADH to the ubiquinone pool with simultaneous vectorial protons pumping across the inner mitochondrial membrane. This enzymatic complex contains more than 40 subunits encoded by either the mitochondrial DNA or the nuclear genome [Fearnley and Walker, 1992]. Using chaotropic agents, the complex has been separated into three different fractions [Galante and Hatefi, 1979; Loeffen et al., 2000]. The largest, hydrophobic, membrane fraction (HP) contains all of the mtDNA encoded subunits plus 25 nuclear encoded subunits, while the matrix protruding fractions harboring the flavin and the iron-sulfur cluster moieties (FP and IP fraction, respectively) are exclusively encoded by nuclear genes [Triepels et al., 2001].

Mutations have been identified in all the seven subunits encoded by the mtDNA, resulting in a variety of clinical symptoms, ranging from organ-specific involvement, e.g., Leber hereditary optic neuropathy (LHON), to multivisceral disease, e.g., mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) [DiMauro and Schon, 2001]. In addition, mutations have been found in six of the nuclear genes, encoding proteins distributed in all the three HP, FP, and IP fractions. Mutation in five of these genes (NDUFS1, NDUFS4, NDUFS7, NDUFS8, NDUFV1) [Loeffen et al., 1998; Van den Heuvel et al., 1998; Budde et al., 2000;

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Petruzzella et al., 2001; Triepels et al., 1999; Schuelke et al., 1999; Bénit et al., 2001] results in neurological disease, mostly Leigh or Leigh-like syndromes, while mutation of the NDUFS2 gene has been shown to be associated with hypertrophic cardiomyopathy and encephalomyopathy [Loeffen et al., 2001].

Here, we show that the skipping of exon 2 of the NDUFV2 gene (MIM# 600532) transcript encoding a complex I catalytic subunit markedly decreases the NDUFV2 protein and complex I activity, and is associated with early onset hypertrophic cardiomyopathy and encephalopathy.

MATERIALS AND METHODS

Patient 1, the second child of first cousin African parents, was born after an uncomplicated full-term pregnancy (birth weight: 2,750 g, height: 45 cm). At 5 days of life, he presented hypertrophic cardiomyopathy and trunkal hypotonia. Feeding difficulties and growth retardation were noted at day 8 (weight: –2.5 SD, height: –3 SD, head circumference: –2 SD). Persistent hyperlactatemia was observed (above 5 mmol/l, normal <2) and he died at 3 months of age. The first child, a girl, is healthy. Two younger brothers (Patients 2 and 3) subsequently died of hypertrophic cardiomyopathy in their first year of life.

Polarographic tests and/or spectrophotometric assays were carried out on skeletal muscle mitochondria, heart homogenate, and cultured skin fibroblasts as previously described [Rustin et al., 1994]. Total RNAs were extracted from cultured skin fibroblasts using the Rnasin kit (Qiagen, Cortabeuf, France) and reverse transcribed using random hexamer primers (GenAmp RNA PCR core Kit, PE Applied Biosystems, Perkin Elmer, France). The RT-PCR amplification of specific RNA was performed in two overlapping fragments spanning the entire coding region of the *NDUFV2* gene. Fragment 1 was amplified using oligonucleotides 5′-GGAAGGTGAACAGTGTGGCC-3′ (forward) and 5′-GCAGGGTGTAGTAGTGCAGAC-3′ (reverse), fragment 2 was amplified using oligonucleotides 5′-ATACAATGTATAATCGAAAGCCAG-3′ (forward) and 5′-CATATTTTATTTCTCTAGTGACAT-3′ (reverse). Sequencing was directly performed using the Big dye terminator cycle sequencing kit (ABI Prism).

Mitochondria-enriched preparations were obtained from exponentially growing cultured skin fibroblasts as previously described [Bourgeron et al., 1992]. Protein concentrations were determined using the Bradford method. For immunodetection, mitochondrial proteins (5 μg) were separated on 12% polyacrylamide gels, electroblotted onto PVDF membranes, and incubated with antibodies as described [Chevallet et al., 1997]. Antibodies were raised in rabbit against peptides corresponding to the major complex I subunit and cytochrome c oxidase subunit II [Procaccio et al., 1999].

RESULTS

Polarographic assays showed a low malate oxidation by mitochondria isolated from Patient 1 skeletal muscle as compared to controls (Table 1). The analysis of activity ratios [Chretien et al., 1997] indicates that those involving complex I dependent activities (e.g., malate oxidation, NADH cytochrome *c* reductase activity) were significantly abnormal, corresponding to a 50–60% complex I deficiency in the skeletal muscle of Patient 1. Consistently, the endomyocardial biopsy of the patient revealed a 50% decrease of complex I activity. A similar figure was obtained in cultured skin fibroblasts of the patient (Table 1) and his affected brother (Patient 2, not shown). Noticeably, similar partial defect of complex I has been shown to possibly result in a full blockade of complex I dependent substrate oxidation in vivo [Geromel et al., 1997].

The systematic DHPLC analysis of nuclearly encoded complex I subunit genes ruled out *NDUFV1*, *NDUFS8*, *NDUFS7*, *NDUFB6*, *NDUFA8*, *NDUFS1*,

TABLE 1. Respiratory Enzyme Activities in Skeletal Muscle Mitochondria, Heart Homogenate, and Cultured Skin Fibroblasts of Patient 1 and Control

	Patient 1	Control (n=80)
Muscle mitochondria	Activities (nmol/min/mg prot)	
Malate oxidase	**14**	(20–44)
Succinate oxidase	51	(34–92)
Duroquinol oxidase	99	(54–152)
NCCR	196	(120–523)
SCCR	582	(150–803)
QCCR	784	(334–2037)
COX	1635	(575–2419)
	Activity ratios	
Succ ox/mal ox	**3.6**	(1.9 ± 0.5)
DQ ox /mal ox	**7.0**	(2.9 ± 0.4)
DQ ox /succ ox	1.9	(2.0 ± 0.4)
COX/NCCR	**8.4**	(3.4 ± 0.8)
COX/SCCR	2.8	(3.2 ± 0.3)
COX/QCCR	2.1	(1.5 ± 0.2)
Heart homogenates	Activities (nmol/min/mg prot)	
NCCR	172.3	(100–243)
SCCR	315.4	(92–206)
QCCR	482.6	(191–789)
COX	1172	(359–1315)
	Activity ratios	
COX/NCCR	**6.8**	(3.4 ± 0.5)
COX/SCCR	3.71	(3.4 ± 0.3)
SCCR/NCCR	**1.83**	(1.0 ± 0.2)
Fibroblasts	Activities (nmol/min/mg prot)	
Malate oxidase	**4.1**	(5.0–11.7)
Succinate oxidase	20.5	(9.4–20)
NQR	**17**	(22–31)
COX	101	(61–140)
	Activity ratios	
Succ ox/mal ox	**5.0**	(1.7 ± 0.3)
COX/NQR	**5.9**	(4.3 ± 0.5)

Substrate oxidation was polarographically measured as described in Materials and Methods section. Abnormal values are in bold. CI-IV, the various complexes of the respiratory chain; NCCR, NADH cytochrome c reductase (CI+CIII); NQR, NADH quinone reductase (CI); SCCR, succinate cytochrome c reductase (CII+III); QCCR, decylubiquinone cytochrome c reductase (CIII); COX, cytochrome coxidase (CIV).

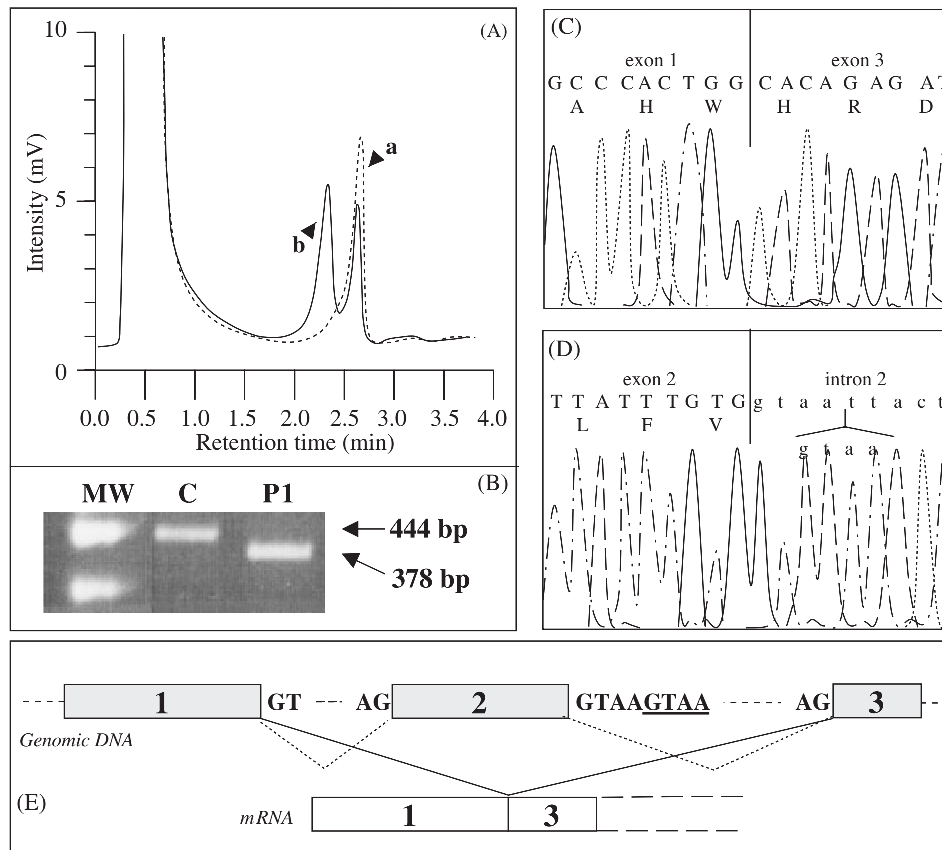

FIGURE 1. **Molecular analysis of the *NDUFV2* gene. A: DHPLC pattern of NDUFV2 RT-PCR of control (a) and combined patient plus control fragments 1 (b). Notice the double peak in patient (b), indicative of an abnormal F1 fragment eluding with a shorter retention time. Fragment 1 was amplified using oligonucleotides 5′- GGAAGGTGAACAGTGTGGCC-3′ (forward) and 5′-GCAGGGTGTAGTAGTGCAGAC-3′ (reverse). B: RT-PCR amplification of *NDUFV2* fragment 1. C,D: Sequence analysis was performed on cDNA (C) and on genomic DNA (D) for Patient 1 (C: control, P1: Patient 1). E: Genomic structure of NDUFV2 gene (exon 1 to 3). Underlined nucleotides in the genomic DNA indicate the 4-bp deletion IVS2+5_+8delGTTA. Dotted line indicates splicing as predicted for the wild-type sequence, continuous line represents the abnormal splicing resulting from the 4-bp deletion and the subsequent skipping of exon 2.**

and *NDUFS2* as disease-causing genes in this consanguineous family. Yet, an abnormal DHPLC pattern in the NDUFV2 gene (i.e., an additional peak caused by the reduced retention time of heteroduplex DNAs) was observed (Fig. 1A). RT-PCR amplification of cultured skin fibroblast mRNAs of Patients 1 and 2 in two overlapping fragments revealed a unique PCR product for fragment 1 that was shorter for Patient 1 (378 bp) as compared to control (444 bp; Fig. 1B). The unique corresponding RNA species will only allow the synthesis of a mutant protein. Direct sequencing of the abnormal amplification product revealed a homozygous 66-bp deletion consistent with the skipping of exon 2 (Fig. 1C) and sequencing genomic DNA in the three affected sibs revealed a homozygous 4-bp deletion of intron 2 (nt + 5 to + 8; IVS2+5_+8delGTTA; wild type: gtaagtaattac, mutant: gtaattac) altering the consensus splice-donor site of intron 2 (Fig. 1D). Although most known mutations (deletions) in donor splice sites affect one of the first two GT nucleotides of the consensus slice sequence, mutations affecting nucleotides located as far as +5, +8, or even +36 nucleotides from the donor site have been previously reported [Hadjisavvas et al., 2003; Buzin et al., 2003]. Analysis of the cDNA indicates that the intron 1 donor site was used instead of the nonfunctional intron 2 donor site causing the observed exon 2 skipping (Fig. 1E).

The truncated RNA transcript is predicted to encode a mutated protein lacking part of the mitochondrial targeting sequence and missing the cleavage-processing site of the protein [Toda et al., 1989]. The mutant protein is expected to be 22 amino acids shorter than the wild-type protein. Yet, because it lacks the targeting sequence cleavage site, its length is indistinguishable from the wild-type NDUFV2 protein. The mutation was absent in 150 alleles from healthy controls and 59 expressed sequence Tags from GenBank. The DNA of the parents and other siblings were not available.

Western blot analysis of mitochondria isolated from cultured skin fibroblasts revealed a significant

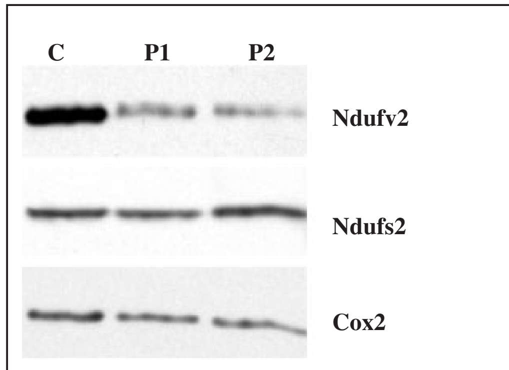

FIGURE 2. **Immunoblots with mitochondrial proteins isolated from Patient 1 (P1), Patient 2 (P2), and control skin fibroblasts (C). The blot was developed with monoclonal antibodies against NDUFV2, NDUFS2, and Cox2.**

reduction in the amount of the NDUFV2 protein in the two patients (residual protein amount of 33% and 25% of controls from Patients 1 and 2, respectively). The amount of another nuclearly encoded complex I subunit, NDUFS2, was similar to controls in both patients when expressed in absolute values or relative to a mitochondrially encoded complex IV subunit, COXII (Fig. 2). Noticeably, no detectable NDUFV2 protein could be detected in the cytosolic fraction (not shown). Fibroblasts from Patient 3 were not available.

DISCUSSION

In the family of hypertrophic cardiomyopathy and complex I deficiency reported here, the skipping of NDUFV2 exon 2 results in a 66-bp truncated RNA transcript predicted to encode a protein with an altered mitochondrial targeting sequence and a missing cleavage site, hampering the normal processing of the protein. The resulting decrease in the NDUFV2 protein content was paralleled by a decrease in complex I activity, in accordance with the catalytic function previously ascribed to this subunit.

Mutations in both NDUFS2 [Loeffen et al., 2001] and NDUFV2 (this work) appear to result in roughly similar clinical presentation associating a hypertrophic cardiomyopathy with an encephalopathy. In contrast, no heart involvement has been noticed in patients harboring mutations in five on six of the other nuclear genes encoding complex I subunits [Loeffen et al., 1998; Van den Heuvel et al., 1998; Schuelke et al., 1999; Triepels et al., 1999; Budde et al., 2000; Petruzzella et al., 2001; Bénit et al., 2001]. To date, no simple explanation explains this striking clinical variability. Noticeably, similar variability has been reported for mutations affecting other respiratory chain complexes [Munnich and Rustin, 2001]. Complex I genes are typical house keeping genes, without known isoforms. They all encode subunits presumably implicated in the catalysis of a similar process, i.e., electron transfer from NADH to ubiquinone. Most of the reported mutations severely hamper complex I activity. Cardiomyopathy has been only reported in patients with either NDUFS2 or NDUFV2 mutation. Both NDUS2 and NDUFV2 proteins are part of the matrix protruding part of complex I being not directly involved in proton pumping. But this stands true for NDUFV1, NDUFS1, and NDUFS4 as well. The possibility that only part of the reported mutations causes superoxide overproduction in the heart might however be hypothesized, as on one hand superoxide overproduction is known to readily trigger cardiomyocyte hypertrophy [Siwik et al., 1999], and on the other hand only a subset of complex I deficiencies actually results is superoxide overproduction [Pitkänen and Robinson, 1996; Geromel et al., 2001].

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