

ORIGINAL ARTICLE

Mutations in *SDHD* lead to autosomal recessive encephalomyopathy and isolated mitochondrial complex II deficiency

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ABSTRACT

Background Defects of the mitochondrial respiratory chain complex II (succinate dehydrogenase (SDH) complex) are extremely rare. Of the four nuclear encoded proteins composing complex II, only mutations in the 70 kDa flavoprotein (SDHA) and the recently identified complex II assembly factor (SDHAF1) have been found to be causative for mitochondrial respiratory chain diseases. Mutations in the other three subunits (SDHB, SDHC, SDHD) and the second assembly factor (SDHAF2) have so far only been associated with hereditary paragangliomas and pheochromocytomas. Recessive germline mutations in *SDHB* have recently been associated with complex II deficiency and leukodystrophy in one patient.

Methods and results We present the clinical and molecular investigations of the first patient with biochemical evidence of a severe isolated complex II deficiency due to compound heterozygous *SDHD* gene mutations. The patient presented with early progressive encephalomyopathy due to compound heterozygous p. E69 K and p.*164Lext*3 *SDHD* mutations. Native polyacrylamide gel electrophoresis and western blotting demonstrated an impaired complex II assembly. Complementation of a patient cell line additionally supported the pathogenicity of the novel identified mutations in *SDHD*.

Conclusions This report describes the first case of isolated complex II deficiency due to recessive *SDHD* germline mutations. We therefore recommend screening for all SDH genes in isolated complex II deficiencies. It further emphasises the importance of appropriate genetic counselling to the family with regard to *SDHD* mutations and their role in tumorigenesis.

INTRODUCTION

The mitochondrial oxidative phosphorylation (OXPHOS) system is the final biochemical pathway in the production of adenosine triphosphate (ATP). Defects in the OXPHOS system result in devastating, mainly multisystem, diseases.¹ The system consists of five multiprotein complexes. The individual subunits are encoded by either the mitochondrial or the nuclear genome. Only the succinate dehydrogenase (SDH) complex (complex II, CII) is entirely encoded by the nuclear genome. It is composed of the four subunits SDHA, SDHB, SDHC, and SDHD. The catalytic core, which dehydrates

succinate to fumarate, is formed by the two larger hydrophilic subunits, SDHA and SDHB, which also harbour the redox cofactors that participate in electron transfer to ubiquinone. The cofactor FAD is covalently bound to SDHA which provides the succinate binding site, and SDHB possesses three Fe-S centres which mediate the electron transfer to ubiquinone. The smaller hydrophobic SDHC and SDHD subunits constitute the membrane anchor and ubiquinone binding sites of CII and localise CII to the inner mitochondrial membrane.^{2–4}

To date, two dedicated CII assembly factors, *SDHAF1*⁵ and *SDHAF2*,⁶ are known and required for stable assembly and full activity of CII. Complex II deficiency is a rare condition in humans and accounts for only 2–4% of OXPHOS defects.⁷ Its clinical presentation is highly variable, ranging from early onset encephalomyopathies to tumour susceptibility in adults. Recessive mutations in *SDHA* are predominantly associated with Leigh's syndrome in children or in a single case with late-onset neurodegenerative disease with progressive optic atrophy, ataxia, and myopathy.⁸ Recently, mutations in *SDHA* have been linked with dilated cardiomyopathy.⁹ In contrast, heterozygous mutations in the other three structural subunits, *SDHB*, *SDHC*, and *SDHD*, are responsible for dominantly inherited paragangliomas (PGL) and pheochromocytomas.^{10–12} However, recessive germline mutations in *SDHB* are also associated with a primary mitochondrial disease as shown recently in a patient with leukodystrophy.¹³ Mutations in *SDHAF1* have been associated with a highly progressive infantile leukoencephalopathy accompanied by an isolated complex II deficiency,⁵ whereas mutations in *SDHAF2* have been associated with hereditary paraganglioma.⁶

SUBJECT AND METHODS

Informed consent was obtained from all participating individuals and the study was approved by the local ethical committee.

Patient

The patient was the second female child of non-consanguineous Swiss parents, with normal pregnancy and birth. Clinical examination was normal. There was no relevant family history.

After respiratory syncytial virus (RSV) bronchiolitis at 3 months of age, the parents observed a

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developmental regression with reduced motor activity, followed by a slower pace of psychomotor development. At 10 months the patient had pendular nystagmus in all directions, muscle hypotonia, and severely retarded development (Griffith Developmental Quotient (DQ) 53). Biochemical analyses were unremarkable, including normal lactate, glucose, electrolytes, liver enzymes, blood gases, amino acids and congenital disorders of glycosylation (CDG) screening and organic acids. Brain MRI, EEG, and screening for hearing were normal. The ocular fundi were normal apart from bilateral pale papillae.

By 2 years, the patient had developed secondary microcephaly, ataxia and dystonia, and her vision had deteriorated. Routine laboratory results were always normal outside severe catabolic episodes. A further extended metabolic work-up was normal. A second brain MRI and spectroscopy were equally normal apart from a relatively low NAA peak. No abnormal peak could be detected, including for lactate or succinate. The parents later refused further follow-up imaging, because they had observed developmental regression after each MRI in the context of prolonged fasting.

Subsequently, several episodes of developmental regression were observed. They occurred after periods of prolonged fasting or one of her frequent respiratory or gastrointestinal infections. Her maximum developmental age was 9–11 months at 3–4 years of age.

After 4.5 years of age, she developed polymorphic epileptic seizures, and later also continuous intractable myoclonic movements. At 7 years of age, in the context of aspiration pneumonia, she had lactic acidosis (lactate 10.2 mmol/L), with an increased anion gap; creatine kinase (CK) was 2496 U/L (reference range (NR) <154 U/L), aspartate aminotransferase (ASAT) 177 U/L (NR 11–47 U/L), and alanine aminotransferase (ALAT) 32 U/L (NR 7–24 U/L). Urinary organic acid analysis showed pronounced lactic aciduria and ketonuria, related metabolites, as well as Krebs cycle intermediates, including borderline elevation of succinate (0.07 mmol/mol creatinine, reference range <0.06 mmol/mol). This prompted muscle and skin biopsies for mitochondrial work-up. A gastrostomy was placed and a fat-reduced and carbohydrate-rich diet introduced, but this had no apparent clinical effect. Only during glucose infusion (4–7 mg/kg/min) did myoclonic movements improve drastically. The patient died at the age of 10 years.

Biochemical assays

Isolation of mitochondria from skin fibroblasts and preparation of skeletal muscle homogenates (600×g supernatants) were performed as described.¹⁴ The activities of the individual respiratory chain (RC) complexes and the mitochondrial matrix marker enzyme citrate synthase (CS) were measured spectrophotometrically with a UV-1601 spectrophotometer (Shimadzu) using 1 mL sample cuvettes thermostatically maintained at 30°C.^{14 15} Complex II activity was measured as thenoyltrifluoroacetone (TTFA)-sensitive succinate ubiquinone reductase activity, as published.¹⁶ All RC activities are expressed as ratios to the mitochondrial marker enzyme CS (mU/mU CS). Fibroblast control values were established from patients with circumcisions or auriculoplasty and muscle controls were obtained from biopsies of patients without clinical and biochemical suspicion of a mitochondrial disorder.

Molecular genetics

Genomic DNA was extracted from EDTA-stabilised venous blood samples applying the QIAamp DNA kit according to the manufacturer's instructions. All coding regions including intron–exon boundaries of *SDHA*, *SDHB*, *SDHC*, *SDHD*,

SDHAF1, and *SDHAF2* were amplified from genomic DNA by means of PCR using primers listed in the online supplementary data. Mutation analysis of the amplified exons was performed by single strand conformation polymorphism (SSCP) as described previously.¹⁷ PCR products were sequenced using BigDye Terminator Chemistry (Applied Biosystems) and separated on an ABI 3100 DNA Sequencer. Data were analysed with SeqScape V.2.1.1 software (Applied Biosystems).

SDS and native-PAGE and western blotting

SDS-PAGE

For western blotting 7 µg of isolated mitochondria of fibroblasts and 10 µg of muscle homogenate were separated by 15% SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis). The membrane was cut into two pieces between 37 kDa and 50 kDa. Proteins were visualised using antibodies against complex II 70 kDa (SDHA, upper part) and 30 kDa (SDHB, lower part) subunit. The membrane hybridised with the antibody against SDHB was stripped and probed again with a porin antibody. All primary antibodies were purchased from Mitoscience/Abcam. For detection, blots were treated with appropriate horseradish peroxidase (HRP)-conjugated immunoglobulins, stained with ECL (GE Healthcare) and exposed to film.

Native-PAGE

For western blotting 15 µg of isolated fibroblast mitochondria and 25 µg of 600×g supernatants of muscle homogenates were centrifuged (30 min, 4°C, 13 000 rpm) and the oxidative phosphorylation complexes were solubilised by 5 mg digitonin per mg protein before separation by native 4.5–13% PAGE as recommended.¹⁸ After blotting, the membrane was cut below 440 kDa into two pieces. The upper part was hybridised with a monoclonal antibody against the core II subunit of complex III and the lower part using monoclonal antibody raised against complex II (SDHA, 70 kDa subunit (Mitoscience)).

Lentiviral based complementation assay

For wild-type and mutant *SDHD* cDNA synthesis total RNA from control and patient fibroblasts was isolated using the QIAgen RNeasy Kit according to the manufacturer's instructions. Random oligohexamer primed RNA was reverse transcribed using the SuperScript II First-Strand cDNA Synthesis System (Invitrogen) according to the manufacturer's recommendations. One-twenty-fifth of single stranded cDNA was used as a template to amplify *SDHD* using the following two oligonucleotides located in the 5'- and 3'- UTRs, respectively: *SDHD*-5'-UTR: 5'-GAGCCCTCAGGAACGAGA-3', and *SDHD*-3'-UTR: 5'-CAGAGGCAAAGAGGCATACAT-3'. Cycling conditions using HotStar Taq DNA Polymerase (Qiagen) were 95°C for 15 min, 32 cycles of 15 s at 95°C, 15 s at 58°C and 1 min at 72°C, and a final extension step of 5 min at 72°C. RT-PCR products were subsequently subcloned into pCR2.1-TOPO plasmid using the TOPO-TA Cloning Kit (Invitrogen). Single colonies were picked and inserts were directly amplified using M13 forward and reverse primers and subjected to DNA sequencing as described above to obtain the three plasmids p.*SDHD*-wt-cDNA, p.*SDHD*-E69K-cDNA, and *SDHD*-*164Lext*3-cDNA. The obtained inserts were further subcloned into an FIV (feline immunodeficiency virus) based lentiviral system and used for transduction of patient cell line as described previously.^{19 20}

Oxygen consumption

The OROBOROS oxygraph was used to measure oxygen consumption in permeabilised cells using a substrate-uncoupler-inhibitor

Genotype-phenotype correlations

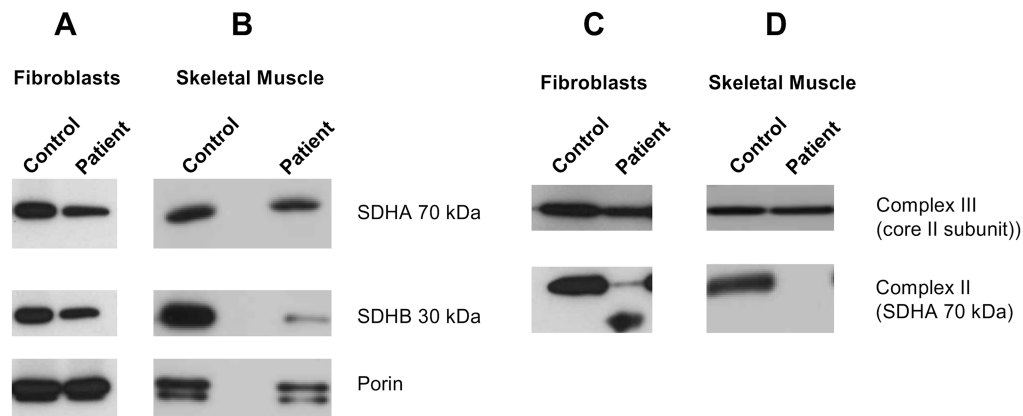


Figure 1 Steady state levels of complex II proteins and respiratory chain complexes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot (WB) analysis of (A) mitochondria from fibroblasts and (B) muscle homogenates using antibodies against SDHA 70 kDa subunit and SDHB 30 kDa subunit. Porin is shown as loading control. Whereas there is only a mild decrease of SDHA and SDHB subunits in fibroblasts, the decrease is much more prominent in muscle native-PAGE and WB analysis of (C) fibroblast mitochondria and (D) muscle homogenates using an antibody against the 70 kDa subunit (SDHA) of complex II. As a loading control complex III was visualised using an antibody against the core II subunit of complex III. In muscle there is no complex II visible whereas in fibroblasts there is a faint band showing fully assembled complex II and a prominent signal below, representing presumably the SDHA/SDHB dimer.

titration regimen as previously described.²¹ The oxygen consumption rates were calculated and expressed as specific oxygen consumption rates ($\text{pmol O}_2/\text{s}/10^6$ cells). The succinate related pyruvate respiration (SRPR) is defined as the ratio of the pyruvate (CI dependent) respiration over succinate (CII dependent) respiration. The pyruvate respiration was determined with malate (2 mM) and pyruvate (5 mM) after addition of adenosine diphosphate (ADP) (2 mM). The succinate respiration was determined after addition of succinate (10 mM), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and rotenone (0.5 μM). All measurements were performed in triplicate.

RESULTS

RC analysis

Biochemical measurements of the RC enzymes in skeletal muscle revealed a significantly decreased complex II activity with 5% of residual activity (table 1), whereas the other RC enzymes were normal. In fibroblasts the activity of complex II was not deficient, when expressed as a ratio to CS, but was decreased when expressed relative to protein, and activity ratios of CI/CII (patient 2.36; normal range 0.51–1.78) and CIV/CII (patient 5.15; normal range 0.92–4.75) also suggested a complex II defect. The complex II defect was, however, clearly detected in oxygen consumption studies of fibroblasts revealing a pathological SRPR of 1.37 (control range 0.91–1.24) (figure 3B).

Immunoblot analysis

Fibroblast mitochondria and skeletal muscle homogenates from patient and control were compared on immunoblots after separation by SDS-PAGE and native-PAGE.

Immunoblot analysis after one dimensional SDS gel electrophoresis showed slightly diminished amounts of SDHA and SDHB in mitochondria from the patient's fibroblasts (figure 1A). In the patient's muscle homogenate SDHA was present in comparable amounts, whereas SDHB was considerably decreased, most probably reflecting different tissue stability (figure 1B).

Protein blot analysis of native-PAGE using anti-SDHA antibody points to very low amounts of fully assembled complex II in fibroblasts and large amounts of SDH (SDHB+SDHA) (figure 1C). In muscle homogenate there was a complete lack of assembled complex II and no subcomplex visible (figure 1D).

Molecular genetic analysis

The molecular genetic analysis of *SDHA* revealed no pathogenic mutation, and deep intronic mutations resulting in an aberrant *SDHA*-mRNA splicing were excluded by *SDHA* cDNA analysis using RNA isolated from fibroblasts (results not shown). Due to the complete loss of assembled complex II in patient muscle, an assembly defect was suspected making *SDHAF1* a good candidate for further molecular genetic analysis. Again, in *SDHAF1* no pathogenic mutations could be identified. This prompted us to analyse the remaining genes known to be required for proper SDH function (*SDHB*, *SDHC*, *SDHD*, and *SDHAF2*).

Table 1 Respiratory chain complex activities in skeletal muscle and isolated mitochondria from skin fibroblasts of the index patient*

	CI/CS	CII/CS	CIII/CS	CIV/CS	CV/CS	CS†
Muscle						
Patient	0.12 (63%)	0.01(5%)	0.55 (71%)	0.76 (66%)	0.56 (143%)	134 (126%)
Mean±SD (n=31)	0.19±0.04	0.21±0.05	0.78±0.15	1.16±0.28	0.39±0.13	105±25
fibroblasts						
Patient	0.50 (172%)	0.21 (63%)	0.97 (161%)	1.22 (162%)	0.22 (110%)	125 (69%)
Mean±SD (n=22)	0.29±0.06	0.33±0.09	0.60±0.15	0.75±0.17	0.20±0.07	181±30

Values in parentheses present activities as a percentage of the control mean.

*Values are given as mU/mU citrate synthase.

†CS, citrate synthase activity is expressed as mU/mg protein.

Molecular genetic analysis of the coding sequence and the adjacent exon-intron boundaries of *SDHD* revealed two heterozygous missense mutations, which have not been described so far: c.205G>A in exon 3, predicting the p.E69K missense variant, and c.479G>T in exon 4, predicting a change of the stop codon into a codon for leucine, followed by proline, phenylalanine and a stop codon: p.*164Lext*3 (figure 2A). The mutations are localised in highly conserved regions of *SDHD* (figure 2B). Both mutations were absent in 200 control individuals. Further analysis identified the mother of the patient as a heterozygous carrier of the c.479G>T variant and the father as a heterozygous carrier of the c.205G>A mutation (figure 2A).

Complementation studies of *SDHD* wild-type and mutant alleles

To test whether the newly identified missense mutations in *SDHD* are indeed causing complex II deficiency we expressed individually the wild-type allele, E69K and *164Lext*3 variants of *SDHD* cDNA in the patient fibroblast cell line using a lentiviral based transduction system (figure 3). Only wild-type *SDHD* was able to restore the amount of assembled complex II as demonstrated in native-PAGE (figure 3A). The visible sub-complex presumably presenting the *SDHA/SDHB* dimer

vanished completely in favour of fully assembled complex II. Functional restoration of complex II is further shown with the normalisation of the SRPR. Upon transviral transduction only patient fibroblasts transduced with wild-type *SDHD* showed a normal SRPR (figure 3B).

DISCUSSION

Complex II (succinate:ubiquinone oxidoreductase) is a tetrameric protein that localises to the inner mitochondrial membrane and is bifunctional.² It oxidises succinate to fumarate in the citric acid cycle and transfers electrons to ubiquinone in the mitochondrial electron transport chain.⁵ Deficiency of complex II is a rare subgroup of mitochondrial RC defects. The prevalence is quoted differently in various studies depending on their patient cohorts. Vladutiu and Heffner²² found 23% of all muscle biopsies with RC defects to have complex II deficiency, while other groups reported only 2%.⁷ Ghezzi *et al*⁵ and Scaglia *et al*²³ quoted a slightly higher prevalence of about 8% in their patient cohorts. The phenotype of complex II deficiency is not well defined and varies from multisystem failure and death in infancy to adult onset myopathy without cognitive impairment.²⁴

Here, we report a child who presented with progressive psychomotor retardation, pendular nystagmus, and seizures during infancy, in whom biochemical analyses of RC activities in

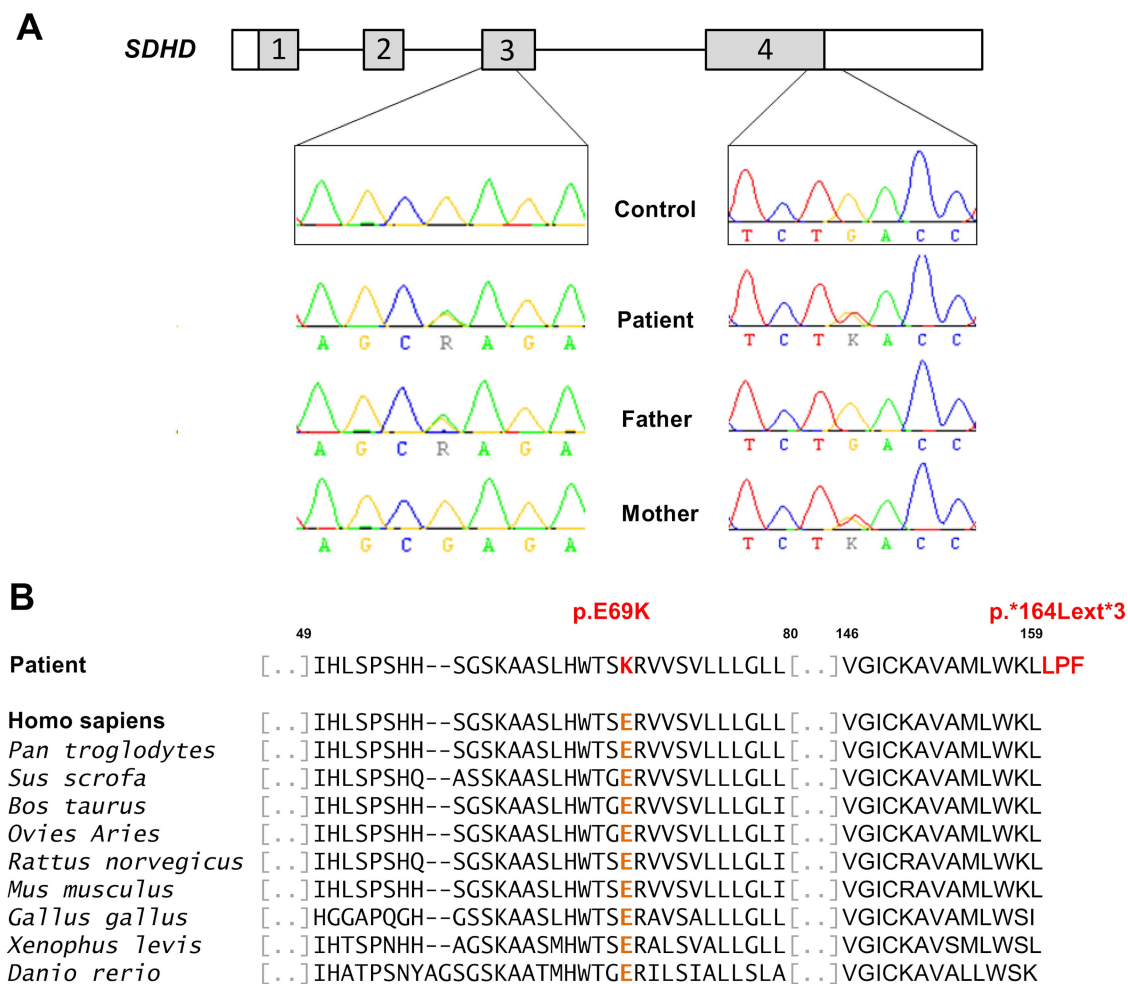


Figure 2 Molecular genetic analysis of the *SDHD* gene in the family. (A) Electropherograms surrounding the mutations in *SDHD*. Heterozygous c.205G>A (p.E69K) and c.479G>T (p.*164Lext3) *SDHD* mutations were identified in the patient and parental DNA analysis, supporting recessive inheritance. (B) Phylogenetic alignment of the *SDHD* gene. The p.E69K mutation affects a strictly conserved residue in the *SDHD*-encoded subunit. The p.*164Lext3 mutation extends the *SDHD* C-terminus by the three additional amino acids leucine, proline, and phenylalanine.

Genotype-phenotype correlations

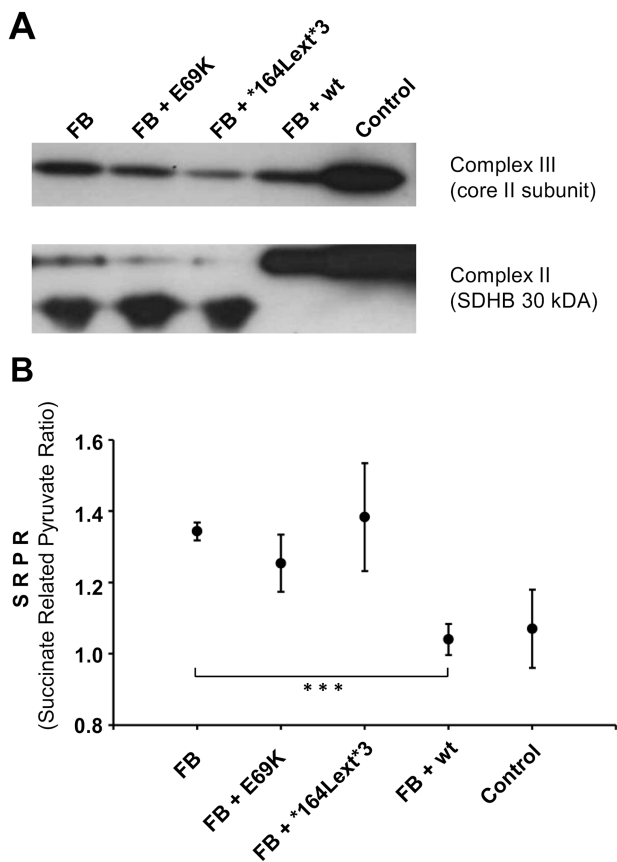


Figure 3 Functional complementation of patient's fibroblasts (FB). (A) Total amount of mature complex II is restored in fibroblasts of the patient transfected with wild-type *SDHD* cDNA (FB+wt) as determined by native-PAGE followed by western blotting using the anti-SDHB antibody. Both mutant *SDHD* (FB+E69K and FB+*164Lext*3) showed no effect on *SDHD* restoration. Control: wild-type fibroblasts. Equal loading was confirmed using anti-core 2 of complex III antibody. (B) Complementation of the patient's fibroblasts with wild-type *SDHD* cDNA (FB+wt) normalises the decreased succinate-related pyruvate respiration. Both mutant *SDHD* (FB+E69K and FB+*164Lext*3) showed no effect on complex II restoration. Controls: wild-type fibroblasts (n=27). Statistical differences between mean values were evaluated using the unpaired t test. Data represent the mean of three independent experiments; error bars indicate $\pm 1SD$. ***p \leq 0.001.

skeletal muscle demonstrated a severe, isolated complex II deficiency. Mitochondrial complex II deficiency is known to be caused by mutations in *SDHA* and *SDHAF1* genes.^{5 25 26} Very recently, recessive mutations in the *SDHB* gene were found to be associated with leukodystrophy in one patient.¹³ Thus, sequencing of these genes was prioritised, which, however, did not reveal any mutations. The extension of the mutation screen to *SDHC*, *SDHD*, and *SDHAF2* genes led to the discovery of two novel heterozygous *SDHD* variants (c.205G>A and c.479G>T). Recessive inheritance of the complex II defect was confirmed by parental DNA analyses showing that both parents are heterozygous carriers. *SDHD* together with *SDHC* constitute the membrane domain of SDH and anchor it to the inner mitochondrial membrane.²⁻⁴ *SDHD* has four α -helices with the N terminus at the matrix side and the C terminus located in the intermembrane space,² whereas all α -helices are localised in the inner mitochondrial membrane.

The c.205G>A mutation is predicted to result in an amino acid substitution at the strictly conserved position 69 from glutamic acid to lysine in the first hydrophobic α -helix. The c.479G>T

mutation affects the stop codon; as a consequence the peptide is predicted to be extended by the three amino acids leucine, proline, and phenylalanine in the fourth hydrophobic α -helix, suggesting an impaired integration of *SDHD* into the inner mitochondrial membrane—thus mimicking a complex II assembly defect as shown using native-PAGE. Complex II assembly and normal function was restored in patient fibroblasts by expression of wild-type but not with mutant *SDHD* cDNAs. Together these data confirmed the pathogenicity of the *SDHD* mutations. Interestingly, SDH act as classical tumour suppressor genes.²⁷ Germline mutations in *SDHD*, *SDHB*, and *SDHC* genes were observed in patients with hereditary PGL and pheochromocytomas.¹⁰⁻¹² Recently, mutations in genes encoding *SDHA* and the SDH assembly factor 2 (*SDHAF2*) were also found to be associated with PGL and pheochromocytoma syndrome (HPGL/PCC).^{6 28} The genetic lesions in the SDH genes predisposing to HPGL/PCC syndrome are heterozygous germline mutations, which are supposed to inactivate protein function. If there is a loss of the remaining wild-type allele in somatic cells—that is, loss of heterozygosity, resulting in a complete loss of the enzyme function—the neoplastic transformation occurs.

While the identified recessive mutations caused severe neurological symptoms and a profound complex II deficiency in the proband, the parents were identified as heterozygous carriers of a germline mutation and, hence, suggests that they have an increased risk for tumourigenesis. Although these two mutations p.E69 K and p.*160Lext*3 are not among the >130 mutations that have been identified in the *SDHD* gene in subjects with HPGL/PCC reported in the Leiden open Variation Database (<http://chromium.liacs.nl/LOVD2/SDH/home.php>), and there is also no indication of cancer susceptibility in the family, the parents have been referred for surveillance. Screening with 24 h measurements of blood pressure and urinary catecholamines of both parents was normal.

Alston and colleagues¹³ recommend screening of the *SDHA*, *SDHB*, and *SDHAF1* genes for patients with a biochemically and histochemically characterised isolated complex II deficiency. Based on our findings that mutations in *SDHD* genes cause isolated complex II deficiency, we recommend screening of the entire known SDH subunits and assembly factors (*SDHB*, *SDHC*, *SDHD*, and *SDHAF2* genes) if the initial screening of the *SDHA* and *SDHAF1* genes for patients with a biochemically characterised isolated complex II deficiency revealed wild-type sequences. Identification of the underlying genetic defect of isolated complex II deficiency allows appropriate genetic counselling to be given to the family. In view of the increased cancer susceptibility, particularly in relation to *SDHD* defects, routine surveillance would allow early detection of tumours and appropriate intervention in heterozygous carriers.

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Contributors Subject ascertainment and recruitment were carried out by JM and MG. Sequencing, genotyping and cDNA subcloning were carried out and interpreted by CBJ, SG and AS. Cell transductions were performed by HP and BH. Western blots and OXPHOS measurements were carried out and interpreted by JM, DH and AH. The study was conceived, designed and drafted by AS. All authors critically revised the manuscript and gave final approval.

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Competing interests None.

Patient consent Obtained.

Ethics approval Local ethic committee.

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Mutations in *SDHD* lead to autosomal recessive encephalomyopathy and isolated mitochondrial complex II deficiency

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