Additive effect of nuclear and mitochondrial mutations in a patient with mitochondrial encephalomyopathy

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Abstract

We describe the case of a woman in whom combination of a mitochondrial (MT-CYB) and a nuclear (SDHB) mutation was associated with clinical and metabolic features suggestive of a mitochondrial disorder. The mutations impaired overall energy metabolism in the patient’s muscle and fibroblasts and increased cellular susceptibility to oxidative stress. To clarify the contribution of each mutation to the phenotype, mutant yeast strains were generated. A significant defect in strains carrying the Sdh2 mutation, either alone or in combination with the cytb variant, was observed. Our data suggest that the SDHB mutation was causative of the mitochondrial disorder in our patient with a possible cumulative contribution of the MT-CYB variant. To our knowledge, this is the first association of bi-genomic variants in the mtDNA and in a nuclear gene encoding a subunit of complex II.

Introduction

The succinate dehydrogenase (SDH)–ubiquinone complex II (cII) is a mitochondrial enzyme complex that participates in both the citric acid cycle and the respiratory electron transfer chain (RC). Eukaryotic SDH is the only component of the RC totally encoded by the nuclear genome, and it is made up of four distinct subunits encoded by the SDHA, SDHB, SDHC and SDHD genes. SDHA and SDHB form the catalytic subunits on the matrix face of the inner mitochondrial membrane, whereas SDHC and SDHD anchor the catalytic dimer to the membrane. Two ancillary proteins (SDHAF1 and SDHAF2) regulate the correct assembly of the holocomplex. The amino acid sequences of the catalytic flavin and Fe–S-binding domains of cII, made up of subunits SDHA and SDHB, are evolutionarily conserved. The membrane domain is less conserved, although a four-helix bundle motif is ubiquitously present across species (1). Mutations in cII subunits are not particularly frequent in typical patients with defects of mitochondrial oxidative phosphorylation (OxPhos) (2, see also http://www.mitomap.org). The associated phenotypes include childhood-
onset mitochondrial encephalomyopathy (resembling a Leigh-like syndrome) and late-onset optic atrophy, ataxia and myopathy; each of these conditions has been observed in one case and both are linked to mutations in SDHA (3–5). An additional clinical presentation is infantile leukoencephalopathy associated with variants found in SDHAF1 or SDHB (2,6). Moreover, heterozygous variants in all the structural subunits of cIII as well as in the regulatory gene SDHAF2 determine or predispose to hereditary pheochromocytoma (PC)/paraganglioma (PGL) and gastrointestinal stromal tumors (reviewed in 7 and 8). Interestingly, mutations in SDHD and SDHAF2 cause highly penetrant multifocal tumor development only if paternally inherited, whereas maternal transmission rarely, if ever, promotes tumor development. This transmission pattern suggests genomic imprinting underlain by as yet unclear epigenetic mechanisms (9).

Ubiquinol-cytochrome c reductase (cIII) catalyzes the transfer of electrons from reduced coenzyme Q to cytochrome c. cIII works as a tightly associated dimer; in mammals, each monomer is made up of 11 subunits (10), with the single cytochrome c oxidase (cIII) catalyzes the transfer of electrons from reduced coenzyme Q to cytochrome c. cIII being encoded by the mitochondrial genome (mtDNA). Mutations in MT-CYB are usually somatic and in the vast majority of affected patients associated with exercise intolerance (11). Germline mutations are less common and usually present as multisystem phenotypes including mitochondrial encephalomyopathy with stroke-like episodes (MELAS)-type disorders (12–16).

Here, we report on a woman showing clinical and metabolic features suggestive of a mitochondrial disorder. Molecular investigations identified a heterozygous mutation in SDHD and a heteroplasmic mt.14841A>G variant in MT-CYB. The former change has already been described in association with malignant catecholamine-secreting PGL syndrome with an indolent course (17), whereas the latter, defined as a “helper” in extensive mtDNA-related databases, has been detected only once in a family segregating the syndrome of Leber hereditary optic neuropathy (LHON, 18). We combined morphological and biochemical investigations in muscle and cultured primary cells from the proposita—who did not have PGL—with studies in yeast mutant strains and on the basis of our findings propose that the two mutations act synergistically, eventually explaining the atypical clinical picture observed in this patient.

Results
Clinical, biochemical and molecular studies in the patient

We studied a 34-year-old woman presenting with recurrent episodes of migraine, asthenia and fatigue, accompanied by increased urinary excretion of 3-methylglutaconic acid. The patient was adopted and she has one son, reportedly affected by migraine headache. Overall, the clinical and metabolic features of our patient, broadly falling in the spectrum observed in the MELAS-like syndrome, were compatible with a diagnosis of a mitochondrial disorder and suggested a possible alteration in the mtDNA. Whole-mtDNA sequencing, however, did not disclose variants commonly associated with MELAS (see www.mitomap.org) but identified the mt.14841A>G/p.N32S variant in MT-CYB, occurring on mitochondrial haplogroup K1a. This mutation, defined as “helper” in a single LHON pedigree (18), was het- eroplasmic in several tissues (ranging from 38% in blood to nearly homoplasmic levels in skin fibroblasts; see Fig. 1 for details). To further investigate how the MT-CYB change might be related to our patient’s clinical picture, we performed a muscle biopsy, which showed a slight histochemical reduction of SDH activity with normal cytochrome c oxidase (COX) reactivity and no evidence of ragged red fibers. Weak cytochemical staining for SDH was also seen in cultured skin fibroblasts (Supplementary

Figure 1. Genetic studies in the patient’s DNA. (A) PCR–RFLP analysis showing the mutation load of the mt.14841A>G mutation in different tissues. The restriction endonuclease HindIII cleaves mutated sequences into two fragments (sized 238 and 100 bp; arrows), whereas the wild-type samples remain uncut (338 bp). A Ctrl-PCR fragment of ∼1.4 kb was added to the reaction to check for HindIII complete digestion (uncut Ctrl-PCR = 1144 bp; digested Ctrl-PCR = 599 + 545 bp). Mutation load was 94.4% in skin fibroblasts (F), 43.1% in muscle (M), 38.0% in blood (B) and 39.3% in urine (U). L: DNA ladder; Un: uncut; C: Control. (B) Conservation of the MT-CYB mutated amino acid (N32S) in eukaryotes shown in Clustal Omega alignment. (C) Electropherogram of SDHb exon 7 region flanking the c.725G>A mutation identified in the patient. A wild-type sequence is also shown. An arrow indicates the mutation.
Material, Fig. S1). Accordingly, spectrophotometric determination of OxPhos activities showed a significant reduction of cII, cIII and cII + III (Fig. 2) in both skeletal muscle and fibroblasts, without any mitochondrial proliferation as determined by citrate synthase enzyme levels. The results of biochemical analyses prompted us to test genes encoding subunits of cII, known assembly factors of SDH and a regulatory gene (NFU1; 19,20). In SDHB, we detected the heterozygous c.725G>A/p.R242H mutation already described in familial PGL (17). cDNA studies did not show alterations in size or quantity of SDHB mRNA, and the c.725G>A/p.R242H mutation was found to be heterozygous at cDNA level too. The latter experiments suggested that the mutant allele was correctly expressed and that there were no additional mutations affecting splicing and generation of alternative transcripts. Consistent with these findings, 1D-PAGE in muscle and cultured primary cells showed a severe reduction (>50% compared with controls) in both SDHA and SDHB subunits as well as in subunits of cIII (using antibodies anti-Core2, RISP, TTC19; Fig. 3A), whereas subunits of the remaining RC complexes were normally expressed. BN-PAGE followed by western blotting in cultured cells revealed reduced levels of cII and cV (Fig. 3B). The latter finding appears in keeping with recent data associating cII and cV in enzymatically active, high-molecular-weight structures (21). Moreover, the observed reduced response of cII and cIII to their specific inhibitors may be ascribed to the mutation-related loss of binding of these inhibitors with their catalytic sites on the enzyme (Supplementary Material, Fig. S2;22). Confirming this hypothesis, analysis of the predicted structural model of the human SDHB subunit suggested that p.R242H induces alterations in the conformation of the protein (Supplementary Material, Fig. S3a and b), lowering the global stability of SDHB (ΔΔG: 1.50 ± 0.48 kcal/mol). Interestingly, the replacement of Arg 242 with His predicts a decrease in the positive surface extent of the mutant protein (Supplementary Material, Fig. S3c and d), which might alter the interaction of the SDHB with the SDHD subunit and impair cII assembly. In silico modeling of the mutant MT-CYB suggested that a Ser at residue 32 replacing Asn might impact on the nearby Trp 31
Supplementary Material, Fig. S4c and d), which is directly involved in quinone binding (Supplementary Material, Fig. S4e and f). This interaction might induce a change in the heme environment, which in turn would alter the catalytic activity of cII. Either a cII dysfunction or, to a lesser extent, a defective cIII would possibly lead to impaired total mitochondrial ATP output and overproduction of reactive oxygen species (ROS). Accordingly, we observed a significantly reduced amount of mitochondrial ATP in the presence of 2-deoxy-D-glucose (2-DG) in patient’s primary cells whereas glycolytic ATP was enhanced in the presence of oligomycin (Fig. 4A). Also, there was a statistically significant increase in ROS production in primary cells, both in basal condition and after a short-term H2O2 treatment (Fig. 4B), together with reduced levels in peripheral blood of total free glutathione concentration (159 µM versus a median value of 233 ± 17 µM in age-matched normal controls) and SOD activity (360 U/ml versus a median value of 421 ± 77 µM in age-matched normal controls) (Fig. 4C). Altogether, these findings suggested an increased susceptibility to oxidative stress in the patient’s tissues.

**Structural and functional modeling in *Saccharomyces cerevisiae***

Yeast cII and cIII are very similar to their human counterparts; therefore, the effects of human gene mutations can be analyzed in yeast strains (23,24). We observed that strains harboring either the homoplasmic N31S variant—equivalent to the N32S variant in MT-CYB—or the homozygous R235H variant—corresponding to the R242H mutation found in SDHB, seen in our patient—or even an additional mutant strain carrying both mutations affected cellular bioenergy. In particular, cII activity was severely decreased (by 60–70%) in the Sdh2-R235H strain and in the double mutant (Fig. 5A). In contrast, cIII activity was only slightly decreased (by 15–20%) in cytb-N31S and in the double mutants (Fig. 5B). Interestingly, the activity of succinate-cytochrome c reductase (cII + III, Fig. 5C), which was reduced by 45% in Sdh2-R235H, was lowered by 60% in the double mutants, possibly indicating the cumulative effects of the two mutations. As expected, cytb-N31S had no effects on cII activity and Sdh2-R235H did not impair cIII activity. The reduction in cII activity was confirmed by monitoring the activity of the whole respiratory chain. A 25–30% decrease in O2 consumption was observed with Sdh2-R235H and in the double mutants when succinate was used as the electron donor (Supplementary Material, Fig. S5). While the mutations affected the activity of the complexes, they did not seem to impair their assembly in yeast strains as judged by the optical spectra (Supplementary Material, Fig. S6).

The mitochondrial membrane extracted from control and mutant strains exhibited similar yeast cIII (Supplementary Material, Fig. S6a) and yeast cII (Supplementary Material, Fig. S6b)
cytochrome b contents, indicating that yeast cII and cIII are not disassembled by their respective mutation but are impaired in their catalytic function.

Notwithstanding a decrease in activity, we observed no drastic effect of single or double mutants on growth or respiratory substrates (results not shown) probably because in yeast the main electron flux pathway in the respiratory chain involves the type II NADH dehydrogenases leading to higher NADH quinone reductase activity compared with cII activity (25).

**Discussion**

This report presents the peculiar case of a woman with clinical features of mitochondrial encephalomyopathy resembling MELAS and harboring a heterozygous mutation in SDHB in combination with the heteroplasmic p.N32S variant in MT-CYB. While mutations in SDHB have only recently been associated with more canonical mitochondrial clinical phenotypes, changes in MT-CYB have already been related to a MELAS-like phenotype in a handful of patients (23). Overall, our findings, reinforced by results in yeast models, seem to indicate a significant role of the genetic variants, either alone or in combination and propose the existence of intergenicomic interactions in a case where a heteroplasmic mtDNA variant modulates the effects or the penetrance of an established pathogenic mutation in nuclear DNA (nDNA).

There are three major considerations emerging from this study. First, our patient showed a milder clinical phenotype than the severe encephalopathy recently described in SDHB (6), possibly with a dominant transmission. The occurrence of dominant and recessive inheritance of mutations in the same gene has already been described in relation to other mitochondrial related genes, including POLG (26). The clinical manifestations in our case probably reflect a milder effect of the mutation in her brain, and it is possible that the transient nature of her symptoms (migraine, easy fatigue) indicate a general genetic susceptibility possibly influenced by environmental factors or other epigenetic mechanisms such as those invoked for example in cases harboring SDHD mutations (8, 9). The different mutation load of the MT-CYB variant or the specific mitochondrial haplogroup (K1a in the proposita) or both might represent additional factors altering penetrance and clinical expressivity, as already reported in a single LHON pedigree (18). Our in silico modeling indicated that the structural changes resulting from the MT-CYB mutation alter the bheme environment through a conformational change of the highly conserved Trp 31 residue; moreover, a MT-CYB Trp 31C mutation affecting the same residue in yeast (27) has been associated with a lowering of the redox potential of bheme. The combined effects of the two mutations, or their synergy suggested by yeast analyses, together with the marked decrease in cII and cIII activities in the patient’s cells, could have impacted on her neurological disease. Second, despite her predisposing genotype, our patient did not present clinically evident manifestations of PGL. However, and in keeping with data published elsewhere (8), the evidence of a metabolic switch toward tumorigenesis is likely because of an increased glycolytic ATP production in her primary cells at the expense of OxPhos activities, the accumulation of succinate and elevated ROS levels. Moreover, PGL shows reduced penetrance (40%) up to the age of 40 years, and we cannot exclude that our patient will develop overt manifestations in later life. Also, we were unable to establish the genotype of the patient’s parents (she was adopted) or of her child (consent was not given). Hence, we cannot draw conclusions on whether the mutation was inherited and transmitted, as already seen in unrelated kindred (28, 29). A contingent protective role of the p.N32S variant in MT-CYB might also be hypothesized if one recalls that other mtDNA changes, namely mt9055G>A in MT-ATP6 in women with Parkinson’s disease (30).
and m.3745G>A in MT-ND1 underlying adaptation to high altitudes and extreme climates (31), have been proposed as factors modulating the clinical manifestations. In clinical practice, according to her genotype, the patient has modified her physical lifestyle and will undergo clinical monitoring to allow early detection and treatment of any PGL-related dysfunction. A third and final comment is prompted by the slightly more severe functional effect measured in the yeast double-mutant strains. Translating this finding to human tissues, it would appear that defective OXPHOS complexes in the patient had a detrimental effect on total mitochondrial ATP output and favored ROS production, given that both cII (32) and, to a lesser extent, cII (33,34) play a role in enhanced oxidative stress, as shown herein. Whether this bioenergetic failure might be responsible for damaged nociceptive system and trigeminal cells in our patient (35), as it is suggested by the efficacy of antioxidant cocktail therapy, is a possibility that will require further follow-up.

Taken together, the human and yeast results in the present case suggested that the SDHb mutation has a direct role in the patient’s disease, whereas the significance of the MT-CYB mutation is less obvious, although it seemed to worsen all the bioenergetic endpoints analyzed, at cellular, protein and biochemical levels. An additive contribution of an mtDNA variant to an RC change associated with an nDNA gene mutation seems an intriguing possibility. In the future, with the ever-expanding clinical use of new sequencing technology in mitochondrial disorders, we might expect to see further combinations of several mutations in functionally connected genes and also in OXPHOS-related proteins. To borrow a term used in relation to other inherited clinical syndromes, we might hypothesize a “mito-interactome” as a novel face of human mitochondrial pathologies.

Material and Methods

Clinical features

The patient is a 34-year-old woman who reported slow weight gain in early childhood and normal motor and language development. She had been adopted and her family history is unknown but her pre- and perinatal history is thought to have been unremarkable. Since her teens, the patient has suffered from recurrent attacks of migraine with aura (lasting 1–3 days) associated with vertigo, and throat and chest tightness. She displays no motor symptoms during the auras but always experiences fatigue upon resolution of the attacks. A progressive worsening of this fatigue over time prompted her to seek expert clinical advice. MRI scan of the head and neck and an X ray of the cervical spine were normal. A neurological examination at age 32 showed mild bilateral ptosis without ophthalmoparesis, and moderate hypotonia in the upper and lower limbs without signs of muscle wasting. Deep tendon reflexes were enhanced in the lower limbs, but there was no Babinski sign. Metabolic work-up repeatedly showed preprandial (417 µmol/l; normal 158–337) and postprandial hyperalaninemia (538 µmol/l; normal 206–498), hyperglycemia (409 µmol/l; normal 158–337), and hyperlactinemia (259 µmol/l; normal 105–236). The patient also presented increased urinary excretion of 3-methylglutaconic acid (30 µmol/l; normal <9) in association with elevated urinary lactic acid (61 micromol/l; normal <25) and methymalonic aciduria (14 µmol/l; normal <2) on several occasions. When we last saw her, her neuromuscular examination was consistent with easy fatigue, delayed recovery from exertion and myalgia after physical activity. She showed mild bilateral ptosis, muscle weakness mainly distally, brisk deep tendon reflexes in the lower limbs and bilateral pes cavus. She also reported migraine attacks on a weekly/monthly basis. Treatment with a cocktail of carnitine, ubiquinone, vitamin B1 and B2 has significantly improved her muscle power and reduced the frequency (but not the severity) of her migraine attacks during the past eight months. Her 14-year-old son also experiences fatigue and sporadic migraine attacks but was not examined by us.

Molecular and biochemical analyses

Total DNA purification from tissues (skeletal muscle, cultured skin fibroblasts, urine sediment and peripheral blood) was performed according to standard procedures. Complete mtDNA sequencing as well as analyses of the coding region and flanking intron–exon boundaries of the known SDHx genes and assembly factors and the NFU1 gene used traditional Sanger methodologies. An ad hoc designed PCR–RFLP strategy was used to quantify the abundance of the mt.14841A–G mutation in tissues (see legend to Fig. 1). Total RNA was isolated from cultured fibroblasts (RNeasy Mini Kit, Qiagen, Switzerland) and reversely transcribed to cDNA with the High Capacity cDNA Reverse Transcription Kit (Life Technology). Overlapping primers were designed to amplify and sequence the entire SDHB cDNA (primers available upon request). A diagnostic biopsy of the deltoid muscle was processed for routine morphology and histochemistry using standard methods. A skin punch biopsy was also performed, and fibroblasts were grown in DMEM medium supplemented with 10% fetal bovine serum, 4.5 g/l glucose and 50 µg/ml uridine.

Spectrophotometric determination of RC enzyme activities in muscle homogenate and skin fibroblasts was performed as reported elsewhere (36) and expressed as nmol/min/mg of fresh tissue for muscle, or mg of protein for fibroblasts. The specificity of cI and cII activities was measured running two parallel reactions with and without the specific inhibitors, malonate and antimycin A, respectively. The degree of inhibition indicates a high specificity of the reaction. The specific activities were calculated as inhibitor-sensitive activity (36).

Western blotting of OXPHOS subunits and BNPAGE analyses followed methodologies already reported elsewhere (37,38). We used monoclonal antibodies against subunits NDUF53 (cI), SDHA (cI, 70 kDa), SDHB (cII, 30 kDa), Core protein 2 and RISP (cII), COXI and COXII (cIV), Cytos (all antibodies were from Mitosciences, Eugene, OR, USA) and a polyclonal antibody, anti-TTC19 (Sigma–Aldrich, St. Louis, MO, USA). Porin/VDAC (Mitosciences) was used as control for loading. Quantitative blot analysis was performed using ImageJ software (http://rsbweb.nih.gov/ij/).

Cellular analyses

ATP levels were assayed using the Luminescence ATP Detection Assay System (ATP-lite one step, Perkin Elmer, Boston, MA, USA) as reported previously by us (38). For the evaluation of intracellular ROS production, we used the CM-H2DCFDA probe (Molecular Probes, Life Technology) as described elsewhere (38).

Redox markers

Blood samples were collected into Vacutainer tubes (Becton Dickinson, Rutherford, NY, USA) containing EDTA and immediately centrifuged (450 x g, for 3 min) to obtain erythrocytes. GSH levels and SOD (EC 1.15.1.1) activity were assayed spectrophotometrically as previously reported (39,40).
Generation of the yeast mutant strains

The introduction of the mutation into the mitochondrially encoded cytochrome b was performed using the technique of mitochondrial transformation by microprojectile bombardment as previously described (41). In order to produce the sdh2 mutant, the yeast gene (corresponding to human SDHB) with its own promoter and terminator was PCR amplified and cloned in a centromeric plasmid. Mutagenesis was then performed using the Quickchange Site-Directed Mutagenesis Kit (Stratagene). The genomic SDH2 gene was deleted by homologous recombination with a Sdh2::URA3 PCR product. Then, the asdh2 mutant was transformed with the plasmid carrying the cloned wild-type or mutated SDH2 gene. The mutant strain combining the mutated cytochrome b and the mutated SDH2 was constructed by cytoduction. All the strains analyzed were constructed from W303-1B [Mat alpha, ade2, his3, leu2, trp1, ura3 (intronless mtDNA)].

Determination of the activities of cII and cIII in yeast

Determination of biochemical activities in yeast strains was carried out on isolated mitochondria at 25°C in phosphate buffer (50 mM potassium phosphate, 50 mM EDTA, pH 7.4) using the dual wavelength mode with an Aminco DW2A spectrophotometer (42). cII activity, or succinate–quinone reductase activity, was measured by following the disappearance of oxidized quinone DB (40 μM) at 275/325 nm with succinate (10 mM) as the electron donor in the presence of 2 mM KCN. cII, or DBH–cytochrome c reductase activity, was assayed by measuring the reduction of cytochrome c (40 μM) at 550/540 nm in the presence of 2 mM KCN with decylubiquinol (50 μM DBH) as the electron donor. Extinction coefficients of 16, 18 and 24 mm−1 cm−1 were used for calculation of DB, cyt c + c1 and cyt b, respectively.

Protein modeling

The three-dimensional model of the human MT-CYB was obtained by homology using the program Modeller (version 9.9; 43) and the crystal structures of bovine (PDB ID: 1BE3; chain C; sequence identity: 75.5%) and chicken (PDB ID: 1BEH; chain C; sequence identity: 72.4%) as templates. In total, 100 unique models were generated, and the PROCHECK program (44) was used to assess the stereochemical quality of the predicted models and select the best structure (Ramachandran plot: residues in favored regions, 95.5%; residues in allowed regions, 3.9%; residues in generously allowed regions, 0.3%; residues in disallowed regions, 0.3%). The three-dimensional structure of the human SDHb (residues 37–275) was modeled using the program MODELLER and the crystal structures of porcine (PDB ID: 1Z0Y, 1ZP0, 3SF6, 3SF; chain B; sequence identity: 97.5%) and chicken SDHb (PDB ID: 1YQ3, 1YQ4, 2FBW, 2H88, 2H89; chain B; sequence identity: 91.2–90.8%) as a template. The quality of the resulting models was assessed using the QMEAN server (45); QMEAN score for the best structure: 0.719). The effects of mutations on MT-CYB (N32S) and SDHb (R242H) structure and stability were predicted using the Rosetta Backrub server (46) and FoldX program (47). Binding regions for prosthetic/metal groups were identified using the 3DLigandSite server (48). Molecular interactions between heme prosthetic group and wild-type or the N32S mutation in MT-CYB were analyzed using LigPilot (v.1.4.4; 49). Energy minimization of protein models was carried out using the KoBoMIn web server (50). The electrostatic surface potentials for the predicted human SDHb and porcine SDHD (PDB codes: 1Z0Y) were computed using the Adaptive Poisson–Boltzmann Solver software (51). UCSF Chimera software (version 1.6; 52) was used for inspection of models and for molecular graphics.

Supplementary Material

Supplementary Material is available at HMG online.

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