

A Mutation in Mitochondrial DNA-Encoded Cytochrome c Oxidase II Gene in a Child With Alpers-Huttenlocher-Like Disease

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ABSTRACT. *Objective.* Cytochrome c oxidase (COX) deficiency has been demonstrated in some patients with Alpers-Huttenlocher disease, but no genetic background has been identified. Our objective was to determine the molecular defect underlying the mitochondrial respiratory chain deficiency in a child with Alpers-Huttenlocher-like progressive cerebrohepatic disease.

Methods. The entire coding region of mitochondrial DNA was analyzed by conformation-sensitive gel electrophoresis and sequencing. Biochemical and morphologic investigations were performed on tissue biopsy material, including oximetric and spectrophotometric analyses of oxidative phosphorylation, histochemistry, and electron microscopy.

Results. Postmortem histologic examination revealed a marked loss of neurons in the olivary nuclei and a spongy change in the calcarine cortex, fatty infiltration and micronodular cirrhosis of the liver, and atrophic ovaries. A novel heteroplasmic 7706G>A mutation was found in the COX II gene. The median degree of the mutant heteroplasmy was 90% in 5 tissues examined but was lower in the blood of asymptomatic maternal relatives. The distribution of the mutant heteroplasmy was skewed to the left in single muscle fibers of the proband and her mother. The 7706G>A mutation converts a hydrophobic alanine in a conserved transmembrane segment to hydrophilic threonine.

Conclusions. The 7706G>A mutation is pathogenic and may lead to impaired dioxygen transfer to the active site of COX. The clinical phenotype of this patient resembled that in Alpers-Huttenlocher disease, suggesting that analysis of mitochondrial DNA is worthwhile in patients with a progressive cerebrohepatic disease. *Pediatrics* 2003;111:e262–e268. URL: <http://www.pediatrics.org/cgi/content/full/111/3/e262>; *mitochondria, mitochondrial DNA, genetics, energy metabolism, pathology, cerebrohepatic disease.*

ABBREVIATIONS: COX, cytochrome c oxidase; mtDNA, mitochondrial DNA; CSGE, conformation-sensitive gel electrophoresis; PCR, polymerase chain reaction.

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Mitochondrial diseases are characterized by defects in the complexes of the mitochondrial respiratory chain, those in complex I or complex IV (cytochrome c oxidase [COX]) being the most common in childhood.¹ COX deficiency is associated with variable phenotypes such as subacute necrotizing encephalomyopathy (Leigh syndrome),² the Saguenay-Lac-Saint-Jean form of COX deficiency,³ fatal infantile myopathy with or without renal Fanconi syndrome,⁴ myopathy with cardiomyopathy,⁵ and benign reversible COX deficiency.⁶ In most families, COX deficiency seems to be inherited as an autosomal recessive trait, but no pathogenic mutations have been reported in genes encoding the COX subunits. Instead, several mutations have been described in nuclear genes coding for factors involved in the assembly of the COX enzyme complex.⁷ These genes include at least SURF1, COX10, SCO1, and SCO2.⁸

Twelve mutations (mtDNA) encoded subunits I to III, including missense mutations in the COX III gene in 2 patients with encephalomyopathy,^{9,10} microdeletion in the COX III gene in a patient with recurrent myoglobinuria,¹¹ an insertion in the COX III gene in a patient with a Leigh-like syndrome,¹² missense mutations in the COX I gene in 2 patients with acquired idiopathic sideroblastic anemia,¹³ a microdeletion in the COX I gene associated with motor neuron disease and a severe isolated muscle COX deficiency,¹⁴ and 2 stop-codon mutations in the COX I gene in patients with a multisystem mitochondrial disorder¹⁵ and recurrent myoglobinuria.¹⁶ 7587T>C, the first pathogenic mutation detected in the COX II gene,¹⁷ results in an inability to express this subunit, causing a specific decrease in COX activity. The 2 other mutations in this gene are 7671T>A¹⁸ and 7896G>A,¹⁹ which have been reported to cause defective assembly of COX.

We detected a complex IV defect together with a borderline decrease in complex I activity in the mitochondrial respiratory chain in a child whose clinical and morphologic features suggested a mitochondrial disorder. Therefore, we analyzed the entire coding region of the mtDNA of this patient by conformation-sensitive gel electrophoresis (CSGE) and sequencing. A novel heteroplasmic missense mutation was identified in the COX II gene leading to replacement of a conserved amino acid in the sub-

unit. Molecular modeling suggested a defect in the transfer of dioxygen into the catalytic center.

METHODS

Case Report

This girl was the third child of apparently healthy, nonconsanguineous parents. Her elder sister had Down syndrome, diabetes, and hypothyroidism, but her elder brother was healthy. The proband was born at full term after normal pregnancy and delivery but was small for gestational age. At 6 months of age, she was examined for feeding problems and poor weight gain (relative weight: -12% to -25% ; height: -2.5 to -5 standard deviations), and at 8 months she was admitted to hospital for failure to thrive. The heart was enlarged, and ultrasonographic examination suggested congestive cardiomyopathy. She also had developed hepatomegaly, and ultrasonographic examination revealed an enlarged, echogenic liver. A liver biopsy at 10 months showed a normal lobular structure but with considerable fatty infiltration and a moderate increase in collagen. Parenchymal cells were preserved only around the central vein (Fig 1A).

At the age of 10 months, histologic study of the skeletal muscle showed normal proportions and distributions of the fiber types. There was a slight increase of fat but no ragged red fibers. NICOTINAMIDE adenine dinucleotide-tetrazolium reductase and COX staining showed rough granular staining in type 1 fibers. Electron microscopy investigation revealed that the mitochondria were enlarged, especially inside myofibrils, and their size and shape varied. The activities of the respiratory chain enzymes were measured by spectrophotometric and oximetric methods in isolated muscle mitochondria.²⁰ Complex IV activity was decreased (68 nmol/min/mg; laboratory normal: 97–237 nmol/min/mg) and complex I activity slightly decreased (13 nmol/min/mg; laboratory normal: 14–72 nmol/min/mg), whereas the activities of complexes II + III were normal. In addition to this, mitochondrial respiration in the skeletal muscle with cytochrome c as the substrate of complex IV was 49 natom/min/mg (laboratory normal: 57–127 natom/min/mg) and slightly decreased with the substrate of complex I, ie, 41 natom/min/mg (laboratory normal: 42–120 natom/min/mg).

After an episode of deterioration precipitated by a viral infection, the girl was placed on a low-fat (20%–25%), high-carbohydrate diet with L-carnitine supplementation at a dose of 100 mg/kg/d. Serum free carnitine (17 $\mu\text{mol/L}$) was low on this occasion, whereas serum total carnitine was normal (52 $\mu\text{mol/L}$). L-Carnitine treatment was discontinued after 6 months, and the concentrations of total and free carnitine remained in the normal range thereafter. At the age of 5.5 years, her relative weight and height were within the normal limits.

Psychomotor development was slightly delayed, and she had ataxia and tremor. She sat independently at the age of 1 year 3 months and learned to walk at the age of 2 years 1 month but was never able to run. Her speech was delayed, and she had moderate mental retardation. She had muscle weakness and exercise intolerance, and at 6 years her condition began to deteriorate, so that she was exhausted after walking 50 to 100 m. At 6 years 8 months, she experienced an unexpected cardiac arrest. She was resuscitated, but brain death was diagnosed after 3 days.

At the age of 8 months, abnormal results were obtained for serum aspartate aminotransferase (242 U/L; laboratory normal: <50 U/L), alanine aminotransferase (105 U/L; laboratory normal: <35 U/L), lactate (2.70–4.36 mmol/L; laboratory normal: 1.00–1.80 mmol/L), pyruvate (120–226 $\mu\text{mol/L}$; laboratory normal: 45–85 $\mu\text{mol/L}$), and the lactate/pyruvate ratio (22–27; laboratory normal: <20). Blood gas analysis showed slight metabolic alkalosis during the first 2 years but was within the normal range thereafter. Normal values were found for serum creatinine kinase, lactate dehydrogenase, creatinine, serum very long-chain fatty acids, plasma lysosomal enzymes, plasma and urinary levels of amino acids, and urinary excretion of organic acids and oligosaccharides. Electroencephalography showed slightly attenuated and disorganized background activity. Magnetic resonance imaging of the brain revealed a supracerebellar arachnoidal cyst, but the brain parenchyma was normal, including the basal ganglia.

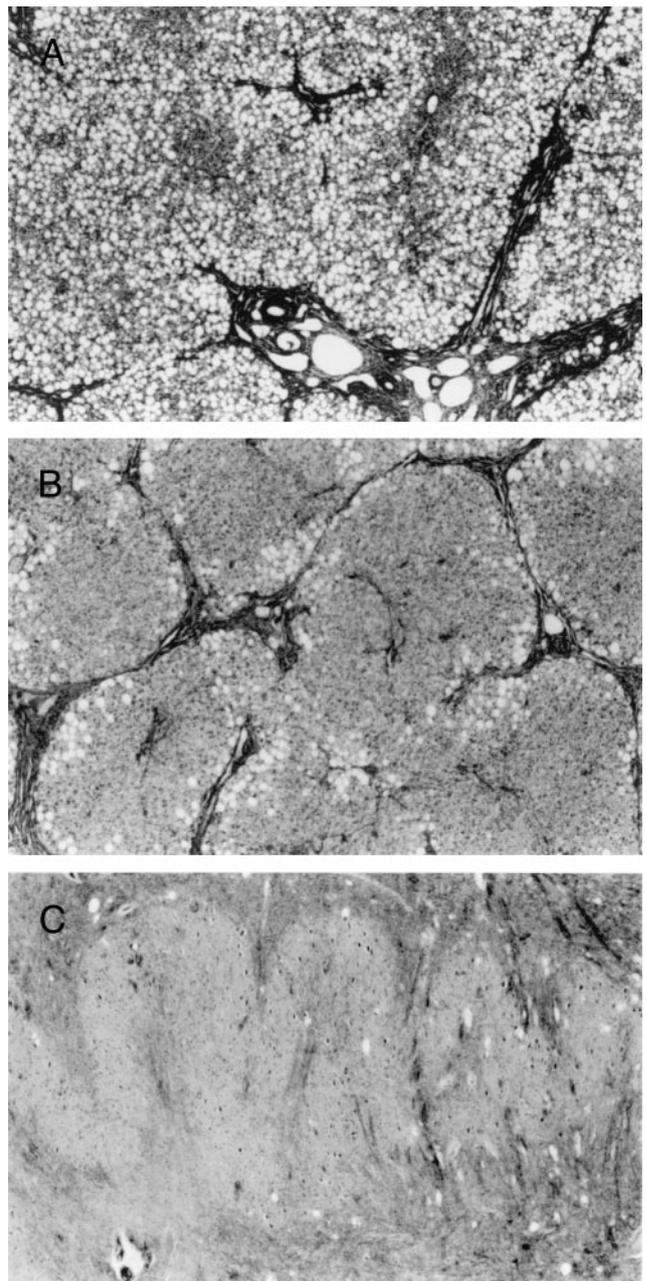


Fig 1. Histopathologic findings. A, The liver at the age of 10 months, showing a severe increase in fat and moderate fibrosis (Herovici staining $\times 20$). B, Postmortem liver, showing micronodular cirrhosis (Herovici staining $\times 20$). C, Dorsal part of the olivary nucleus, with severe depletion of neurons (Luxol fast blue/cresyl violet staining $\times 20$).

Histologic Examination

An autopsy was performed 2 hours after death. The left frontal lobe was frozen, and the rest of the brain was fixed in 4% phosphate-buffered formaldehyde and dissected. Tissue samples obtained at autopsy were embedded in paraffin, and the sections were stained with hematoxylin-eosin or Luxol fast blue/cresyl violet. Specimens from the muscle and liver biopsies and autopsy samples from the heart and liver were fixed in glutaraldehyde, embedded in Epon, and processed for electron microscopy.

For enzyme histochemical staining, frozen muscle biopsy sections (10- μm thick) were mounted on polylysine-coated slides. The specimens were double-stained for COX and succinate dehydrogenase. Blue fibers were considered COX negative, and fibers with brown color were defined to be COX positive.

Controls

Blood samples were obtained from 480 healthy, anonymous people. The mtDNA haplogroups were determined by analysis of restriction fragment length polymorphisms.²¹ The research protocol was approved by the ethics committees of the Medical Faculty, University of Oulu, and of the Finnish Red Cross.

Molecular Methods

DNA Extraction

Total DNA was isolated from the blood cells using a QIAamp Blood Kit (Qiagen, Hilden, Germany) and from skeletal muscle, cardiac muscle, kidney, liver, and brain by using standard extraction with phenol and chloroform.

Sequence Analysis

Screening for mtDNA polymorphisms and mutations was conducted by CSGE and subsequent sequencing.^{22,23} The heteroduplexes that differed in their mobility in electrophoresis from wild-type homoduplexes were analyzed by automated sequencing (ABI Prism 377 Sequencer using Dye Terminator Cycle Sequencing Ready Kit; Perkin Elmer, Foster City, CA) after treatment with exonuclease I and shrimp alkaline phosphatase.²⁴ The primers used for sequencing were the same as those used in the amplification reactions for CSGE.

COX VIc gene was sequenced in the proband and her mother and grandmother. A fragment spanning exon 1 (NCBI accession number AF067636) was amplified using a primer pair GCAGCTC-CAATCAATGCTTCCA and GCTATAGTTCCTGTCACACTGAG, and a fragment spanning exon 2 (NCBI accession number AF067637) was amplified using a primer pair TAAACTCTAATGGATGGTTGCTT and CATTGTGAGGGACAGTCACCTG. The fragments were sequenced in both strands using the same primers as those used in the amplification reactions.

Detection of Heteroplasmy at Nucleotide 7706

Restriction fragment analysis was used to determine the degree of mutant heteroplasmy in tissues from the proband (blood, skeletal muscle, brain, cardiac muscle, kidney, and liver) and in blood from her mother and grandmother and an anonymous control. The mtDNA region was amplified in polymerase chain reaction (PCR) in the presence of ³⁵S-dATP using a forward primer 7533–7557 and a reverse primer 7893–7915. The wild-type genome harbors a cleavage site at nt 7610, and the amplified fragment (383 bp) thus will be digested into fragments of 306 bp and 77 bp. The 7706G>A mutation leads to the gain of an *Acc I* cleavage site at nt 7704, and the amplified DNA fragment harboring this mutation will be digested into fragments of 212 bp, 94 bp, and 77 bp. After overnight digestion at 37°C with 10 U of *Acc I*, the DNA fragments were separated in a 10% polyacrylamide gel. The intensities of the bands on autoradiography film were quantified using a GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA).

Single-Fiber mtDNA Analysis

Muscle fibers were dissected with a sharp tungsten needle.²⁵ The proportion of mutant mtDNAs was quantified in 65 fibers from the proband and 53 fibers from the mother. For this purpose, each isolated muscle fiber was incubated for 10 minutes at 94°C in 5 µL of a solution containing 200 mM KOH and 50 mM dithiothreitol. This solution was then neutralized by adding 5 µL of 900 mM Tris-HCl (pH 8.3) with 300 mM KCl and 200 mM HCl. DNA was amplified in PCR using a standard protocol with the exception that 10 pmol of fluorescein-labeled (FAM) forward primer was added for the last amplification cycle. The fragments obtained by *Acc I* digestion were electrophoresed in a 4% polyacrylamide gel using model 377 DNA sequencer (Applied Biosystems) and analyzed by the Genotyper version 2.0 software. The relative percentages of mutated and wild-type mtDNAs were calculated from the peak area of cleaved and uncleaved mtDNAs.

Cloning

The heteroplasmy of the 7706G>A mutation was verified by cloning an amplified fragment spanning between nts 7533 and 7915 of the mtDNA into a pCR 2.1-TOPO vector (TOPO TA

Cloning Kit; Invitrogen, Leek, The Netherlands). Positive clones were cultured overnight in 2 mL of LB medium containing 50 µg/mL ampicillin, and portions of 1 µL from the cultures were then incubated for 10 minutes at 94°C to lyse the cells and inactivate the nucleases and amplified in the presence of a vector-specific pair of primers. Nested PCR was conducted using the same pair of primers as in the restriction fragment analysis of 7706G>A (see above). For detecting 7706G>A, the amplified DNA fragments were digested with *Acc I* and cleavage was verified on 3% MetaPhor agarose (FMC BioProducts, Rockland, ME). Sequencing was conducted using plasmid-specific and insert-specific primers.

Other Methods

Interatomic contacts of structural units as given in the crystallographic data file 2OCC.PDB²⁶ deposited at the Research Collaboratory for Structural Bioinformatics Protein Data Bank and available through www.pdb-browsers.ebi.ac.uk were analyzed with the CSU software,²⁷ and the coordinates were used for drawing the model.

RESULTS

Postmortem Findings

Histologic investigation of the liver showed moderate fatty infiltration and micronodular cirrhosis (Fig 1B). Neuropathologic examination showed that the number of dorsal neurons in the olives was severely diminished and that there was gliosis in the olivary nuclei (Fig 1C). The ventral regions showed similar but milder changes, whereas the calcarine cortex showed spongy changes, and there were severe global ischemic-anoxic changes. The heart was enlarged (weight: 141 g; expected: 107 g), and electron microscopy of the cardiac muscle revealed an increased number of structurally normal mitochondria, interstitial edema, and increased lipofuscin. The uterus was rudimentary (7 mm), and the ovaries were extremely atrophic, with only a few primary follicles and 1 oocyte.

Analysis of the mtDNA of the Proband

The coding region of the mtDNA of the proband was found to differ from the revised Cambridge reference sequence in 24 nucleotide positions.²⁸ Twenty-two of the variants were common polymorphisms that characterize mtDNA haplogroup K.²⁹ The remaining 2 substitutions included a synonymous mutation 9093A>G in the ATPase6 gene and a missense mutation 7706G>A in the COX II gene.

Heteroplasmic 7706G>A Mutation in the COX II Gene

The 7706G>A mutation in the COX II gene was found in the proband and in her mother and maternal grandmother, and both digested and undigested fragments were detected in each subject, suggesting heteroplasmy (Fig 2). The degree of mutant heteroplasmy was 87% in the blood of the proband, 87% in the heart, 90% in the skeletal muscle, 90% in the kidney, 91% in the liver, and 91% in the brain. The mutant heteroplasmy was 72% in the blood of the proband's asymptomatic mother and 66% in the blood of her asymptomatic grandmother. The proportion of mutant mtDNA in the skeletal muscle of the proband, as determined by subcloning, was 88% (61 of 69 clones harbored the mutation).

The distribution of mtDNA with 7706G>A in muscle of the proband and her mother was studied

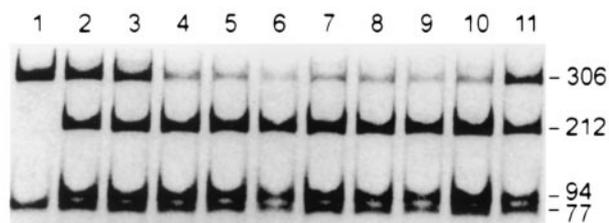


Fig 2. Restriction fragment analysis of the 7706G>A mutation. The heteroplasmy of the 7706G>A mutation was detected by restriction fragment analysis using *Acc I*. Lane 1, blood mtDNA from a healthy control; lane 2, blood mtDNA from the proband's grandmother; lane 3, blood mtDNA from the proband's mother; lanes 4 to 10, mtDNAs from different tissues of the proband: lane 4, blood; lane 5, kidney; lane 6, liver; lane 7, brain; lane 8, cardiac muscle; lane 9, skeletal muscle at the age of 1 year 8 months; lane 10, skeletal muscle at the age of 10 months; lane 11, blood mtDNA from an anonymous control belonging to haplogroup W.

by single-fiber PCR analysis. The proportion of mutant mtDNA was $80 \pm 7\%$ in the muscle of the proband and $54 \pm 16\%$ in that of the mother (Fig 3). In both cases, the distribution was found to be skewed to the left (Kolmogorov-Smirnov test, $P < .001$). We found only a few COX-deficient fibers, but clearly COX-negative fibers or ragged red fibers were not present.

7706G>A was not found among controls belonging to haplogroup K ($n = 12$), but surprisingly, it was found in 1 of the 468 controls belonging to other haplogroups. MtDNA from this control belonged to

haplogroup W and the 7706G>A mutation was heteroplasmic, the proportion of the mutant genome being 68% in blood (Fig 2).

Topology of COX Subunit II

Analysis of the atomic coordinates of crystalline bovine COX²⁶ showed that COX subunit II and subunit VIc are in close apposition in the holoenzyme. Residue 41 (isoleucine in the bovine enzyme) was found to form a stabilizing hydrophobic-hydrophobic contact with Ile-21, Ala-24, and Phe-25 (homologous to Met-23, Ala-26, and Phe-27, respectively, in humans) of subunit VIc. There was a cluster of hydrophobic residues between the tail of the hydroxylfarnesylethyl side chain of haeme a_3 and residue 41 in both transmembrane helices of subunit II (Fig 4).

To exclude compensatory replacements in the amino acid positions 23, 26, or 27 of COX subunit VIc, we sequenced the 2 exons of the COX VIc gene. Only 1 synonymous substitution was found at codon 56 in exon 2 in the proband and in her mother and grandmother.

DISCUSSION

We report here on a novel heteroplasmic 7706G>A mutation in COX II gene in a patient with encephalomyopathy, ataxia, cardiomyopathy, fatty liver, gonadal dysgenesis, and lactic acidosis, features similar to those associated with isolated

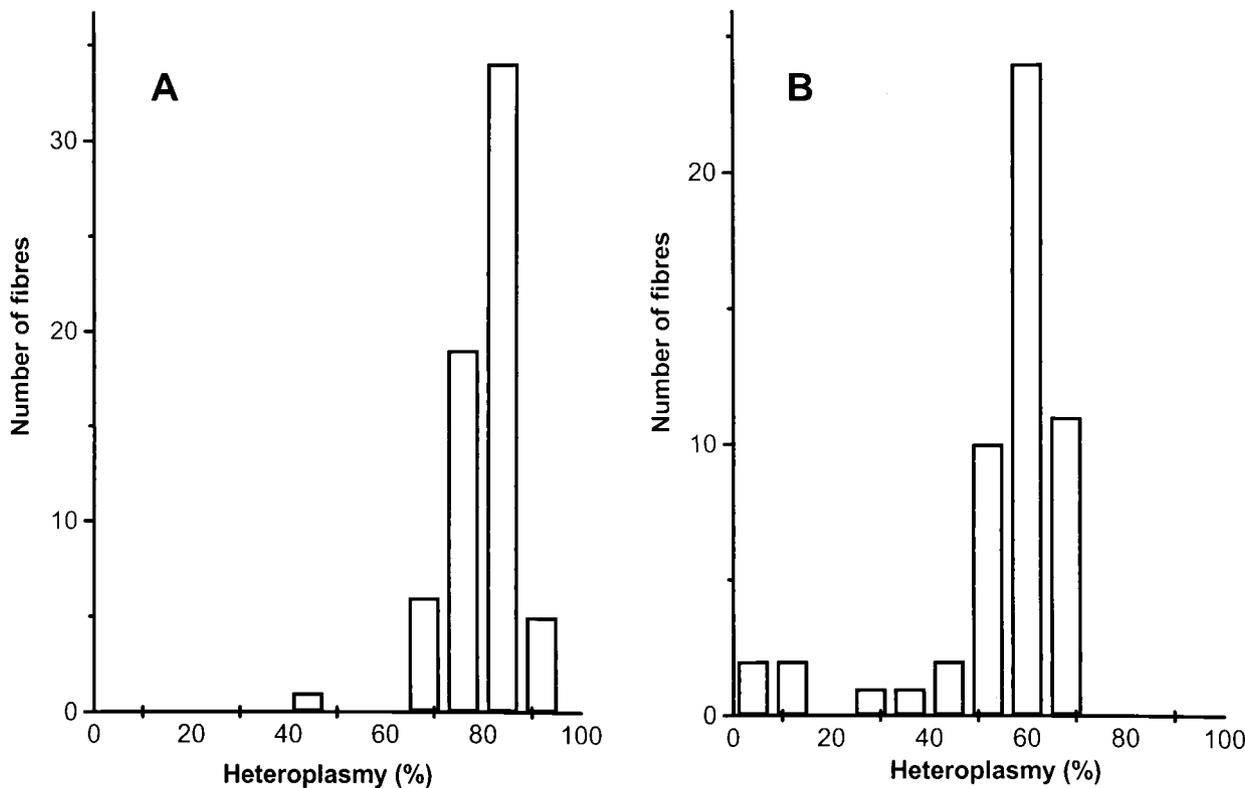
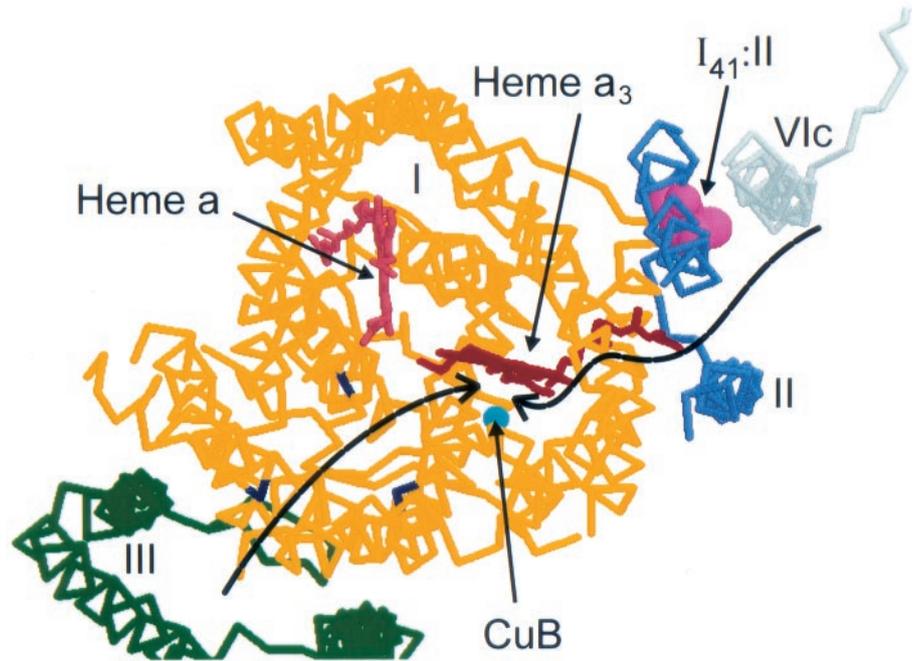


Fig 3. Distribution of the proportion of mutant mtDNA in single muscle fibers. The proportion of mutant mtDNAs was quantified in 65 fibers from the proband (A) and 53 fibers from the mother (B). Amplification was performed as described in Fig 2 with the exception that 10 pmol of fluorescein-labeled (FAM) forward primer was added for the last amplification cycle. The fragments obtained by *Acc I* digestion were electrophoresed in a 4% polyacrylamide gel using model 377 DNA sequencer and analyzed by the Genotyper version 2.0 software. The relative percentages of mutated and wild-type mtDNAs were calculated from the peak area of cleaved and uncleaved mtDNAs. The bin size is 8. The scale on the x axis is shown numerically with 1 minor tick.

Fig 4. Topology of human amino acid substitutions in subunit II of COX in the context of putative dioxygen channels. The model is drawn on the basis of the coordinates published for the oxidized bovine enzyme.²⁶ Only selected subunits and functional groups of the enzyme are shown. Yellow, subunit I; blue, subunit II; gray, subunit VIc. Position of residue 41 in subunit II in relation to 2 putative dioxygen channels and subunit VIc. View perpendicular to the mitochondrial membrane plane. This graphic was produced with the Rasmol program.



COX deficiency.^{2-5,8,30} The most conspicuous neuropathological finding was degeneration of the olivary nuclei, which is similar to that described in some patients with the mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes syndrome³¹ or the myoclonic epilepsy and ragged red fibers syndrome,³² and in a patient with the Leigh syndrome caused by the 8344A>G mutation.³³ Loss of neurons in the olivary nuclei thus is not an uncommon finding in patients with mtDNA mutations.

The prominent histologic features included lipid accumulation and micronodular cirrhosis of the liver, which also characterize Alpers-Huttenlocher disease (MIM 203700).³⁴ In this disease, the hepatocytes typically show marked accumulation of small lipid droplets, alternating with densely packed mitochondria, a pale matrix, and loss of granules.³⁵ Furthermore, spongiosis of the cerebral cortex, particularly of the occipital lobes, is a feature of Alpers-Huttenlocher disease, and quite remarkably, a marked spongy change in the calcarine cortex was also found in our patient. COX deficiency has been demonstrated in at least some patients with Alpers-Huttenlocher disease,^{36,37} but no genetic background has been identified. Our findings suggest that a missense mutation in the COX II gene leads to a cerebrohepatic disease that has clinical features in common with Alpers-Huttenlocher disease.

The 7706G>A mutation was heteroplasmic, and the degree of mutant heteroplasmy was high in affected tissues, suggesting a pathogenic role. Furthermore, the proportion of the mutant mtDNA in single muscle fibers of the proband and her mother showed skewed distribution to the left, suggesting that mtDNAs harboring 7706G>A are subject to negative selection. That 7706G>A was absent in unrelated haplogroup-matched controls suggests that it does not belong to a specific subcluster within haplogroup

K, and it has not been found among 560 complete mtDNAs.³⁸ Surprisingly, an anonymous control belonging to haplogroup W harbored 7706G>A, indicating that the mutation had arisen at least twice in the population. The degree of the 7706G>A mutation heteroplasmy in the control was similar to that in the asymptomatic relatives of the proband. This finding suggests that the control with 7706G>A may be an asymptomatic carrier of a pathogenic mutation in a similar manner to asymptomatic people who harbor 3243A>G in many mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes pedigrees.³⁹ In addition to COX deficiency, our patient had a borderline decrease in the complex I activity, but this finding could not be verified in the lack of fresh tissue from the patient.

The 7706G>A mutation converts a hydrophobic alanine to a hydrophilic threonine in position 41 (Ala41Thr) in a moderately conserved transmembrane segment of COX subunit II. The topology of this amino acid can be visualized with the aid of the atomic coordinates of the bovine enzyme derived from radiograph crystallographic data.²⁶ A mechanism involving impaired access of oxygen to the active site may be proposed for the pathogenicity of 7706G>A. The oxygen-reducing site, comprising the binuclear haeme a₃-Cu_B center, is located on subunit I and is covered by subunits II and VIc. Two routes for oxygen have been proposed (Fig 4). Mutagenesis in *Paracoccus denitrificans* has suggested a role for a route that uses a series of hydrophobic amino acids from the direction of subunit III.⁴⁰ Alternatively, dioxygen transfer may use the hydrophobic hydroxylfarnesylethyl side chain of haeme a₃.²⁶ Its tip forms a hydrophobic-hydrophobic contact with Ile-72 in a transmembrane helix of subunit II. A chain of hydrophobic amino acids extends from Ile-72 of subunit II to Ile-21 of subunit VIc, and Ala-41 of subunit II

(Ile-41 in the bovine enzyme) is located in this path (Fig 4). Sequencing of the COX VIc gene of the proband did not reveal any nonsynonymous changes.

Decreased COX activity is the most common deficiency that affects the mitochondrial respiratory chain, but mutations in the genes encoding the subunits are rare. Indeed, the ratio of replacement to silent substitution is approximately 3 times greater for the nicotinamide adenine dinucleotide dehydrogenase genes than for the COX genes both within and between species, consistent with a greater degree of selective constraint for the COX genes.⁴¹ The 7706G>A mutation leads to amino acid replacement in a moderately conserved segment in COX subunit II, which we interpret as disturbing the transfer of dioxygen to the active site of the COX holoenzyme. However, a biochemical verification of the pathogenic nature of the mutation is lacking. Therefore, we cannot rule out other possibilities in pathogenesis, such as an interaction between Ala41Thr and a substitution elsewhere in COX subunits. The novel mutation in the COX II gene reported here was associated with a multisystem disorder resembling Alpers-Huttenlocher disease, suggesting that analysis of mtDNA may be worthwhile in patients with a progressive cerebrohepatic disease.

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