

A mutation in the human heme A:farnesyltransferase gene (*COX10*) causes cytochrome c oxidase deficiency

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Cytochrome c oxidase (COX) defects are found in a clinically and genetically heterogeneous group of mitochondrial disorders. To date, mutations in only two nuclear genes causing COX deficiency have been described. We report here a genetic linkage study of a consanguineous family with an isolated COX defect and subsequent identification of a mutation in a third nuclear gene causing a deficiency of the enzyme. A genome-wide search for homozygosity allowed us to map the disease gene to chromosome 17p13.1–q11.1 ($Z_{\max} = 2.46$; $\theta = 0.00$ at the locus D17S799). This region encompasses two genes, *SCO1* and *COX10*, encoding proteins involved in COX assembly. Mutation analysis followed by a complementation study in yeast permitted us to ascribe the COX deficiency to a homozygous missense mutation in the *COX10* gene. This gene encodes heme A:farnesyltransferase, which catalyzes the first step in the conversion of protoheme to the heme A prosthetic groups of the enzyme. All three nuclear genes now linked to isolated COX deficiency are involved in the maturation and assembly of COX, emphasizing the major role of such genes in COX pathology.

INTRODUCTION

Cytochrome *c* oxidase (COX), the terminal complex of the mitochondrial respiratory chain, catalyzes the electron transfer from reduced cytochrome *c* to oxygen. Isolated COX deficiency is the most frequent cause of respiratory chain defects in humans and results in a variety of clinical manifestations including Leigh syndrome (1), hepatic failure (2) and encephalomyopathy (3). This clinical heterogeneity presumably stems from the large number of nuclear genes involved in the expression of the 13 COX subunit polypeptides and their subsequent maturation and assembly into the functional complex.

Mutations in the three mitochondrial genes encoding COX subunits, *COXI*, *COXII* and *COXIII*, have been reported in only a few patients (4–9). No mutations have been described in the

nuclear genes encoding COX subunits. This suggests that the prevalent non-maternally transmitted mutations causing COX deficiency are present in genes involved in COX assembly, as recently reported for the *SURF1* and *SCO2* genes (1,10,11). The high rate of recurrence risk and parental consanguinity in COX deficient families of Northern and Western African ancestry is highly suggestive of an autosomal recessive genotype (12). Therefore, in order to identify additional nuclear genes responsible for COX deficiency, we performed homozygosity mapping in a large African consanguineous family with COX deficiency. Here we report on the mapping of a new locus for isolated COX deficiency to chromosome 17p13.1–q11.1, which shows an autosomal recessive mode of inheritance. Further genetic examination allowed us to ascribe the disease to a homozygous missense mutation in the *COX10* gene.

RESULTS

Patients

A boy born to first cousin parents after a term pregnancy and normal delivery had an unremarkable development until the age of 18 months. He had an older sister who died at 5 years of age of a mitochondrial encephalopathy ascribed to cytochrome *c* oxidase deficiency (13). At 18 months, he had ataxia, and his neurological condition gradually worsened over the next 6 months. At 2 years of age, he presented with poor eye contact, severe muscle weakness, hypotonia, ataxia, ptosis, pyramidal syndrome and status epilepticus. Heart ultrasound was normal. Blood and CSF lactate were elevated (3.8 and 3.1 mmol/l, respectively), and GC/MS detected urinary lactate and Krebs' cycle intermediates. Increased urinary amino acids were suggestive of a proximal tubulopathy. He presented with isolated COX deficiency in muscle, lymphocytes and fibroblast cultures (13). He died at 2 years of age. His younger sister had progressive neurological deterioration with similar biochemical anomalies at the age of 2 years and died at 3 years of age (14). Neither of the patients presented large mitochondrial DNA (mtDNA) deletions or the MERRF, MELAS or NARP mtDNA mutations. Sequencing of the *COXI*, *COXII*

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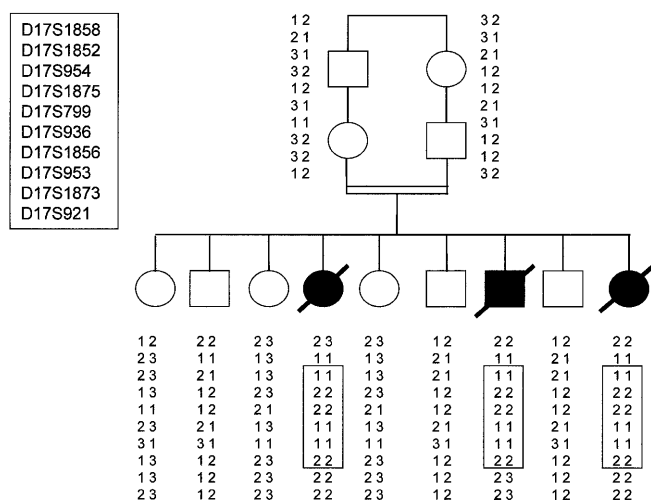


Figure 1. Pedigree and haplotype analysis of the COX deficient family. Haplotypes are given (top to bottom) for loci D17S1858, D17S1852, D17S954, D17S1875, D17S799, D17S936, D17S1856, D17S953, D17S1873 and D17S921.

and *COXIII* mitochondrial genes as well as the surrounding tRNA genes failed to reveal mutations (15).

Genetic linkage

A genome-wide search for homozygosity was undertaken using a total of 382 polymorphic markers. We obtained evidence for homozygosity in all affected individuals at loci D17S1852, D17S799 and D17S921. The shortest area of homozygosity in affected individuals was defined by the D17S1858–D17S1873 interval, but a recombination event in a healthy sib reduced the critical region to the 27.4 cM interval defined by loci D17S954 and D17S1873 on chromosome 17p13.1–q11.1 (Fig. 1). The maximum pair-wise LOD score was obtained at D17S799 ($Z_{\max} = 2.46$; $\theta = 0.00$). The lack of informativity accounts for the relatively low LOD score in this consanguineous family. No other homozygous region was found with other markers.

Candidate gene analysis

Based on homologies to COX-related genes in yeast, the 17p13–q11 region encompasses two candidate genes, *SCO1* and *COX10*. *SCO1* encodes a protein involved in mitochondrial copper transport and/or insertion in COX (16,17). *COX10* codes for the heme A:farnesyltransferase, which converts protoheme to heme O (18,19). The latter serves as the immediate precursor of heme A, the heme prosthetic group of the COXI subunit. Mutations in either of these two genes in yeast result in the absence of functional heme in the complex. A low temperature difference spectrum of the α region of cytochromes revealed the absence of aa3 heme in fibroblast cultures of the patient (Fig. 2), consistent with an abnormal function of either *COX10* or *SCO1*.

A search for mutations in the *COX10* gene was therefore undertaken in the family by direct sequencing of the seven exons of *COX10*. A homozygous C→A transversion was found in exon 4, corresponding to nucleotide 612 of the cDNA (Fig. 3a). This transversion results in the change of an

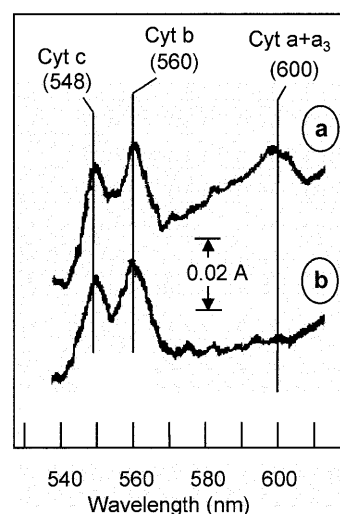


Figure 2. Low temperature (-196°C) difference spectra (α regions) of control (a) and patient (b) skin fibroblasts. Experimental conditions as described in Material and Methods.

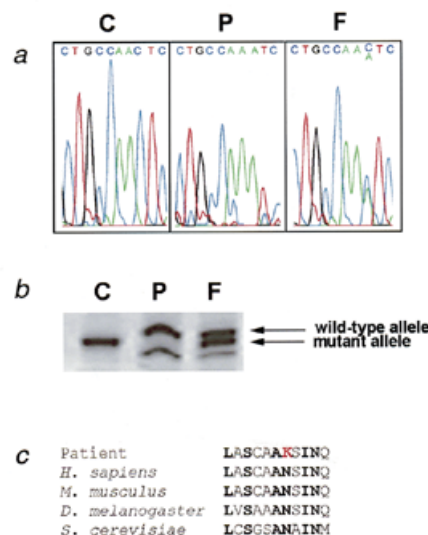


Figure 3. Molecular analysis of the *COX10* gene. (a) Sequence analysis of *COX10* gene; (b) SSCP screening of the C→A mutation at nt 612; P, patient; F, father; C, control. (c) Sequence alignment of the COX10 protein from a patient, controls and from non-human sources.

uncharged asparagine into a basic lysine residue (N204K) at a conserved position in the protein (Fig. 3c). Both parents were heterozygous for the 612 C→A transversion and unaffected children were either heterozygous or homozygous for the wild-type allele. The base change caused an abnormal SSCP migration pattern that segregated with the disease in the entire pedigree and was absent from 100 controls of the same ethnic origin (Fig. 3b).

Western blot analysis revealed a mild reduction of COX subunit steady-state levels (Fig. 4) with more marked reduction of subunits III and VIc (~50% of normal levels) and barely detectable levels of subunit II (<3%). We subsequently screened the *COX10* gene in seven additional patients with isolated COX deficiency and roughly similar COX subunit

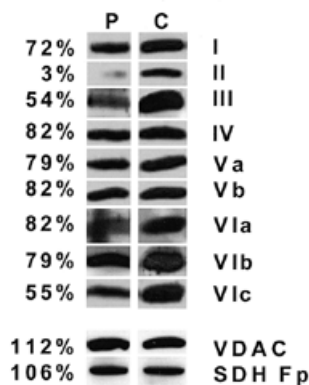


Figure 4. Immunoblots of mitochondrial proteins isolated from patient (P) and control (C) skin fibroblasts. The upper blots were developed with specific antibodies against the various COX subunits, the last but one blot was developed with a monoclonal antibody against VDAC and the last blot was developed with monoclonal antibody against flavoprotein (Fp) of succinate dehydrogenase. Numbers indicate the percentage of each subunit in patient compared with control.

profile. No mutations could be identified in any of these patients (data not shown). In an additional screening of four unrelated patients with similar clinical presentations we also failed to find any mutations in the *COX10* gene.

Functional complementation

To confirm the deleterious nature of the N204K *COX10* genotype, the human wild-type and mutant genes were tested for their ability to complement the respiratory defect of a yeast *cox10* null mutant. Indeed, due to the high residual activity present in the fibroblast of the patient, transfection of these cells may result in not absolutely clear-cut results. The mutation was introduced into a wild-type *COX10* cDNA clone by PCR. The wild-type and mutant *COX10* genes were cloned into three types of plasmid: (i) a high-copy plasmid containing the yeast 2μ origin of replication; (ii) a low-copy CEN plasmid; and (iii) an integrative plasmid linearized at *LEU2* to direct integration in the homologous locus in chromosomal DNA (20). The six different plasmids were used to transform a yeast *cox10* null strain previously shown to be readily complemented by the wild-type human gene (21). Growth of the mutant on glycerol-rich medium was restored by the wild-type but not the N204K gene when present in low copy (Fig. 5). Similar results were obtained when the two genes were integrated at the *leu2* locus of the host (not shown). However, a small percentage (5–10%) of cells carrying the integrated mutant gene showed some growth on the non-fermentable carbon source. This is probably due to multiple integrations of the gene (20). Both the wild-type and the mutant N204K genes complemented the *COX10* deficient strain when introduced on the high copy plasmid (Fig. 5), indicating retention of some residual function by the mutant protein. In keeping with this, it is worth remembering that a residual COX activity was retained in the patient's lymphocytes (patient: 47 nmol/min/mg protein; normal range: 72–203, $n = 85$). The inability of the mutant gene to complement the yeast *cox10* null strain, when expressed in low copy strongly supports the view that COX deficiency is a consequence of the N204K *COX10* mutation in our patients.

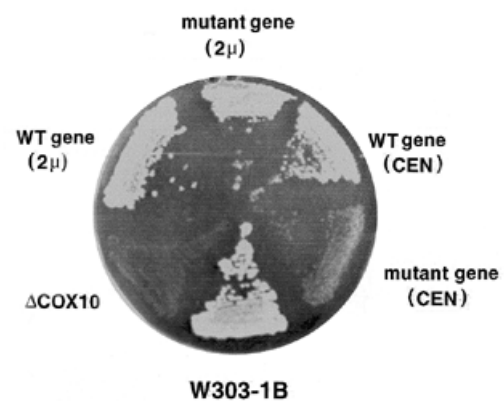


Figure 5. Growth of wild-type yeast, a *cox10* null mutant and transformants on rich medium (yeast extract and peptone) containing glycerol as the carbon source. The following strains were spread on glucose-rich medium: the wild-type haploid strain W303-1B; a yeast *cox10* null mutant (Δ COX10); the yeast null mutant transformed with the wild-type gene on the multicopy plasmid pG19/HST5 (WT gene, 2μ); the null mutant transformed with the mutant gene on the multicopy plasmid pG19/HST6 (mutant gene, 2μ); the null mutant transformed with the wild-type gene on the low copy plasmid pG19/HST9 (WT gene, CEN); and the null mutant transformed with the mutant gene on the low copy plasmid pG19/st10 (mutant gene, CEN). The plate was replicated on glycerol-rich medium and the photograph was taken 24 h after incubation at 30°C.

DISCUSSION

We report a third nuclear gene responsible for complex IV deficiency, COX10. This gene has been identified by a combination of conventional linkage analysis in a large consanguineous family of African ancestry and a candidate gene screening. This allowed us to find a homozygous substitution in the *COX10* gene resulting in an asparagine to lysine substitution (N204K) at a conserved position in the protein. The inability of the mutant gene to complement the yeast *cox10* null strain, when expressed in low copy, confirms that this substitution is the disease-causing mutation.

The *COX10* gene encodes a heme A:farnesyltransferase. The gene was originally isolated in yeast and shown to be required for the biogenesis of functional cytochrome *c* oxidase in yeast (22). The human cDNA has been isolated by functional complementation of a yeast *cox10* mutant. It encodes a protein predicted to contain seven to nine transmembrane domains localized in the mitochondrial inner membrane (21). The protein structure is conserved from *Escherichia coli* to human, with the amino acid sequence of transmembrane segments II to V being highly conserved. A study of *E. coli* mutants has shown that the II/III loop, which is on the mitochondrial matrix side of the enzyme in eukaryotes, is essential for catalytic function (19). This loop contains a highly conserved motif involved in the transfer of a farnesyl moiety to the pyrrol ring A of ferrous protoheme IX. This farnesyl chain of the heme would then be used as a lipophilic anchor holding the heme at the proper position within the oxidase complex (19). The substitution of a lysine for an asparagine (N204K), identified in our patients, is in the second transmembrane segment of the heme A:farnesyltransferase at the II/III loop junction. A substitution in this region is likely to elicit a conformational change in the loop with adverse effects on heme A synthesis.

Isolated COX deficiency is a frequent cause of respiratory chain dysfunction. Sequencing of the mitochondrial genes that encode the three catalytic core subunits has allowed the identification of mutations in the *COXI*, *COXII* and *COXIII* genes, in a limited number of patients (4–9). In addition to the *COX10* mutation reported in this study, mutations in two other nuclear genes involved in COX assembly have been described but no mutations have been reported in any of the structural nuclear genes coding for COX subunits. Functional complementation studies, using microcell-mediated chromosome transfer have led to the discovery of mutations in the *SURF1* gene as a frequent cause of a COX defect in patients with Leigh's syndrome (1,10). Systematic sequencing of candidate genes led to the identification of mutations in the *SCO2* gene underlying COX deficiency in fatal infantile cardioencephalomyopathy (11). We used homozygosity mapping to identify a disease-causing mutation in the *COX10* gene. The yeast homologues of these three genes are involved in COX assembly (16,18,23). Yeast strains with mutations in the *SURF1* homologue display inadequate electron transfer between COX and other components of respiratory chain (23). The *SCO2* gene product plays a role in the transport of the copper to COX (16), whereas *COX10* gene codes for heme A:farnesyltransferase which functions in the maturation of the heme A prosthetic group of COX (18).

Patients with mutations in any one of these three genes present with a severe and isolated COX deficiency associated with an abnormal expression pattern of COX subunits; however, the COX subunit anomalies resulting from *SURF1*, *SCO2* and *COX10* mutations differ noticeably. Western blot analysis has indicated that in *SURF1* patients, COX subunit levels are very much reduced with the exception of subunits Va and Vb (24). Immunohistochemistry revealed severe reductions of subunits I and II in *SCO2* patients, while subunits IV and Va levels are less affected (11). We show here on Western blots that a mutation in the *COX10* gene has a major effect on the level of subunit II. The steady-state levels of other subunits, including subunit I, are relatively normal. This is remarkable because subunit I in the *COX10* patients can not be associated with heme aa3 prosthetic groups. This suggests that the heme groups are not necessary to stabilize subunit I and subassemblies of the enzyme complex may be present which protect the subunits from proteolytic degradation by mitochondrial proteases (25). The clinical phenotypes caused by mutations in these three genes differ as well. *SURF1* mutations are associated with subacute necrotizing encephalomyopathy, also known as 'Leigh disease'. Patients with *SCO2* mutations presented with encephalocardiomyopathy, while patients with the *COX10* mutation reported here, presented with tubulopathy and leukodystrophy.

At present, only a limited number of genes involved in the biogenesis of COX are known. However, this is likely to change as the search for the molecular bases of human mitochondrial diseases takes advantage of the ever increasing functional data available for yeast. At least a dozen nuclear yeast gene products have been described as acting in the late stages of the COX assembly pathway (26). Even though the functions of many of these mitochondrial proteins still need to be clarified, they are good candidates for future studies in human COX deficiencies.

MATERIALS AND METHODS

DNA analyses

DNA was extracted from peripheral blood. A genome-wide search for homozygosity was undertaken with 382 pairs of fluorescent oligonucleotides from the Genescan Linkage Mapping Set, version II (Perkin-Elmer Applied Biosystems, Foster City, CA) under conditions recommended by the manufacturer. Amplified fragments were electrophoresed and analyzed with an automatic sequencer (ABI 377). The polymorphic markers have an average spacing of 10 cM throughout the genome. Linkage analysis was performed using M-LINK and LINKMAP, version 5.1.

Mutation screening

The seven exons of the *COX10* gene were amplified by PCR using the following *COX10*-specific intronic primers (forward/reverse, 5'–3'):

exon 1: TTCGGAGCCCGCCCGCCGGAAGT, CGGCCTCGCAC-GTGGTAATA,
 exon 2: TAGTCATCATTTGATAAGAAG, ACTTTAATTAAT-AGGAATTTCTG,
 exon 3: GTTGCTAAATAACCATTGAGAG, CACTAAAGATA-GAGTTTCAAATAAG,
 exon 4: TGGCCTTTACAGTTGGGACTCCT, AGCCATCTAGGA-AAAAGTGACAA,
 exon 5: TCGAAATTAATAATGAAGTTTA, TAGTGGGAAATGATTACAGATGAACAAG,
 exon 6: GATTTTAGGTAGTGTGATTGA, AGGAAATGCCTCCTTACCCGAGTGT,
 exon 7 (1): ATTCTTTGGAAATCTCTGGTGATGAC, TCCAGAA-TTACCACAACATGCTCG,
 exon 7 (2): TTCTTCTGCAGCCTGTGGGA, CTTTGAGGGACCT-GAGCTCA,
 exon 7 (3): TTTTGGTTCCATCCTTACCAC, TATTTGGTAACC-GAGCCACA,
 exon 7 (4): TTAGCCTCCACATGTGCAATG, ACTCCTGTTCTT-GTATAACCATT.

Amplification products were electrophoresed through a 2% low melting point agarose gel, purified and directly sequenced using the PRISM™ Ready Reaction Sequencing Kit (Perkin-Elmer) on an automatic sequencer (Applied Biosystems). SSCP analysis was performed using the GeneGel Excel 12.5/24 Kit from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Complementation of a yeast *cox10* null mutant

The N204K mutation was introduced into the wild-type human cDNA sequence by amplification of the 114 nucleotide region spanning the *NheI* and *StuI* sites with primers 5'-GGCGAG-GCCTTGATCCTGTGCTGCCAA(A)TCCATCAATCAG (the C→A mutation is indicated in parentheses) and 5'-GGACACAGCTAGCAATGG. The PCR-generated fragment with the mutation was digested with *NheI* and *StuI* and substituted for the native fragment in pG19/HST5, a plasmid identical to pG19/HST2 (21) except that the 1.5 kb *HindIII*-*EcoRI* fragment containing human *COX10* is in the 2 μ plasmid Yep351 (27). The resultant plasmid (pG19/HST6) was confirmed by sequencing to have only the C→A transversion in the *NheI*-*StuI* region. Both wild-type and mutant genes were transferred to the integrative plasmid YEp351 (27) yielding pG19/HST7 (wild-type) and pG19/HST8 (mutant). These plasmids were linearized at the *ClaI* site of *LEU2*. The two alleles

were also cloned in the CEN plasmid pRS316 (28); these plasmids were designated as pG19/HST9 (wild-type) and pG19/HST10 (mutant). All six constructs were used to transform the haploid strain of *Saccharomyces cerevisiae* aW303Δ COX10 bearing the *cox10* null allele (22).

Immunoblot analysis

Western blot analysis was performed as described (24). Quantification of COX subunit was performed using Sigma Gel software.

Low temperature difference spectra

Low temperature difference spectra performed at liquid nitrogen temperature (−196°C) were recorded in 80 μl cells with a computerized UV-3000 Shimadzu spectrophotometer. Baseline (oxidized minus oxidized) was subtracted from difference spectra (reduced minus oxidized). Quantitative measurements of cytochromes were performed according to Chance (29). The study was carried out in 300 mM mannitol, 10 mM KH₂PO₄ pH 7.2, 10 mM KCl, 5 mM MgCl₂ and 1 g/l of bovine serum albumin (30).

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