ONLINE MUTATION REPORT

Novel mutations in *COX15* in a long surviving Leigh syndrome patient with cytochrome c oxidase deficiency

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Background: Isolated cytochrome c oxidase (COX) deficiency is usually associated with mutations in several factors involved in the biogenesis of COX.

Methods: We describe a patient with atypical, long surviving Leigh syndrome carrying two novel mutations in the *COX15* gene, which encodes an enzyme involved in the biosynthesis of heme A.

Results: Only two *COX15* mutated patients, one with severe neonatal cardiomyopathy, the other with rapidly fatal Leigh syndrome, have been described to date. In contrast, our patient had a slowly progressive course with no heart involvement. COX deficiency was mild in muscle and a normal amount of fully assembled COX was present in cultured fibroblasts.

Conclusions: The clinical and biochemical phenotypes in *COX15* defects are more heterogeneous than in other conditions associated with COX deficiency, such as mutations in *SURF1*.

Cytochrome c oxidase (COX), the terminal component of the mitochondrial respiratory chain, catalyses the exergonic reduction of molecular oxygen to water. COX is a heteromeric complex consisting of three catalytic subunits, encoded by mitochondrial DNA (mtDNA), and ten "accessory" subunits, encoded by nuclear genes. The latter are presumably involved in the structural stabilisation and assembly of the complex, and in the modulation of its catalytic activity. Different clinical phenotypes associated with COX deficiency have been described, generally displaying an early onset of symptoms and a fatal outcome. Maternally inherited mutations in mtDNA encoded COX subunits are rare. Most of the syndromes are inherited as autosomal recessive traits. In several of these cases, the responsible gene remains unknown. No mutation in any of the nucleus encoded COX subunits has yet been identified. By contrast, all the genetically defined cases so far reported are due to mutations in genes encoding factors involved in the biogenesis and assembly of the complex, including *SURF1*, *COX10*, *SCO1*, *SCO2* and, more recently, *COX15*. Mutations in the *SURF1* gene, consistently associated with typical Leigh syndrome, are by far the most prevalent cause of infantile COX deficiency, while mutations in the other nuclear genes involved in COX biogenesis are much less common.^{1,2}

We report on a child with Leigh syndrome and isolated COX deficiency, in whom the biochemical defect is due to two different novel mutations in the *COX15* assembly gene. The clinical presentation is different from the rapidly progressive course of previous cases, as the patient is still surviving at 16 years of age.

CASE REPORT

The proband is the third born of unrelated Italian parents; two older sisters are healthy. From the first days of life he displayed poor sucking, feeding difficulties, and failure to thrive. A severe psychomotor delay was noted at 4 months of age, with diffuse hypotonia and muscle wasting and weakness. Subsequently he developed brisk deep tendon reflexes, cerebellar tremor, and eye movement incoordination. A first MRI at 18 months of age showed symmetric signal changes in the posterior part of the putamina and bilateral cerebellar white matter abnormalities. Lactate and pyruvate were markedly elevated in plasma (lactate 3.6 mM, normal values (nv) 0.5–1.8; pyruvate 0.19 mM, nv 0.06–0.13) and in CSF (lactate 3.8 mM, nv 0.8–2.0; pyruvate 0.17 mM, nv 0.045–0.125). Clinical and MRI features were consistent with the diagnosis of Leigh syndrome. Symptoms slowly worsened thereafter with virtual arrest of body growth, progressive loss of postural control, and onset of dystonic postures in the upper limbs, while the cognitive functions remained relatively better preserved. A follow up MRI study at 4 years of age disclosed a mild progression of the cerebellar white matter and putaminal lesions, and the appearance of bilateral signal changes in the head of the caudate nuclei (fig 1). His clinical features remained grossly unchanged thereafter, although plasma lactate and pyruvate progressively decreased to normal levels. Apart from the central nervous system and skeletal muscle, the patient showed no abnormality in other tissues or organs, including the heart, gastrointestinal tract, liver, kidneys, and haemopoietic system. The boy is now 16 years old.

METHODS

Morphological and biochemical analyses

Morphological analysis of skeletal muscle and biochemical assays of the individual respiratory chain complexes on muscle homogenate and cultured skin fibroblasts were carried out as described.³ Enzymatic activity of each complex was normalised to that of citrate synthase.

Genetic analysis

Molecular analysis was performed on genomic DNA extracted from cultured fibroblasts. Overlapping fragments of the *COX15* coding region were PCR amplified and products were assessed on agarose gel, purified, and sequenced according to Antonicka *et al*⁴ with slight modifications.

Western blot analysis of protein fraction resolved by 2D-BNE

For two dimensional Blue Native electrophoresis (2D-BNE), crude mitochondrial pellets, obtained as described,⁵ were resuspended in 100 µl of 1.5 M 6-aminohexanoic acid, 50 mM Bis-Tris, pH 7.0. Then 20 µl of 10% β-lauryl maltoside

Abbreviations: 2D-BNE, two dimensional Blue Native electrophoresis; COX, cytochrome c oxidase; mtDNA, mitochondrial DNA

Figure 1 Brain MRI. (A) Coronal proton density weighted image shows bilateral lesions in the putamina and in the head of caudate nuclei (arrows). (B) Axial T2 weighted image shows extensive cerebellar white matter involvement (arrows).

were added and the samples were incubated for 15 min on ice. Clearing of the samples was performed by centrifugation at 12 000×g for 20 min at 4°C. The supernatant was removed and 10 µl of 5% Serva Blue G in 1 M 6-aminohexanoic acid was added prior to loading. 2D-BNE and electroblotting procedures were performed as described.⁶ The blots were immunostained using mouse monoclonal antibodies raised against bovine COX subunit I (Molecular Probes, Eugene, OR), as previously described.³

RESULTS AND DISCUSSION

A muscle biopsy, performed at the age of 18 months, was morphologically normal. Biochemical assays (table 1) showed an isolated COX deficiency both in skeletal muscle and cultured fibroblasts (42% and 22% residual activity compared to the mean of controls, respectively). Accordingly, the cytochemical reaction to COX was moderately reduced in cultured fibroblasts as compared to normal control cells, but much stronger than that observed in fibroblasts from *SURF1* mutant Leigh syndrome patients (fig 2).

Mutation analysis revealed a compound heterozygous state for two novel mutations in the *COX15* gene. These mutations were absent in 40 alleles from ethnically matched controls, and are not listed in any SNP database. The first mutation, a 503C→G transversion, introduces a premature stop codon in exon 4 (H152X).⁷ The second mutation, a 1081T→C missense transition in exon 8, results in a serine to proline substitution at amino acid position 344, in the C-terminal end of the fifth predicted transmembrane domain of the protein. The S344 residue is conserved from vertebrates to *Neurospora crassa*, and is conservatively replaced by a threonine in a few species such as *Drosophila melanogaster*, an arthropod animal, and *Arabidopsis thaliana*, a vascular plant.

In humans, alternative splicing of the *COX15* gene generates two transcript variants (*COX15.1* and *COX15.2*; GenBank accession numbers NM_078470 and NM_004376) diverging in the 3'-UTR region and in the predicted C-terminal amino acid sequence. The functional significance of the two splice variants is still unknown.⁸ However, the mutations in our case affect the portion of the COX15 amino

acid sequence that is common to both variants. COX15 is a membrane bound mitochondrial protein involved in the last part of the biosynthetic pathway of heme A, an essential step in the biogenesis of COX. Studies on yeast mutants indicate that COX15 is part of a three component mono-oxygenase, catalysing the hydroxylation of the methyl group at position 8 of heme O, the precursor molecule of heme A. Heme O is the product of the farnesylation of pro-heme (heme B), a reaction catalysed by COX10, another enzyme involved in the early steps of COX formation. The alcohol group added to heme O is then oxidised into the corresponding aldehyde by an unknown enzyme, to eventually produce heme A. Accordingly, yeast *COX15* mutants have undetectable levels of heme A and detectable levels of heme O.^{9–11}

To study the assembly of COX in our *COX15* mutant patient, we performed Western blot analysis on mitochondrial proteins extracted from cultured fibroblasts and separated by 2D-BNE. In contrast with the accumulation of COX assembly intermediates, which is typical of *SURF1* defective mitochondria,⁵ the *COX15* mutant mitochondria contained a virtually normal amount of apparently fully assembled COX complex (fig 2).

Besides our patient, *COX15* mutations have previously been reported in only two other cases (table 2). Albeit rare, defects in this gene appear to be associated with considerable clinical and biochemical heterogeneity. The first child presented soon after birth with muscle hypotonia, epilepsy, and lactic acidosis, but the clinical course was dominated by the development of a rapidly fatal hypertrophic cardiomyopathy.^{4 12} The COX residual activity was markedly reduced in heart tissue (6%) and was low (25–30%) in liver, kidney, and cultured fibroblasts but normal in skeletal muscle. The child was a compound heterozygote with a missense mutation (700C→T, R217W) in exon 5 on one allele and a splice site mutation (C447-3G) in intron 3 on the other allele, resulting in the deletion of exon 4. Interestingly, the BNE pattern obtained from heart mitochondria was similar to that of our proband, that is, fully assembled COX, with no assembly intermediates,⁴ although the amount of cross-reacting material was much lower, in agreement with the

Table 1 Biochemical activities*				
	Complex I/CS†	Complex II+III/CS	Complex IV/CS	Citrate synthase
Muscle	10.4 (nv 15 ± 4)	9.5 (nv 12 ± 3)	46 (nv 106 ± 28)	82 (nv 133 ± 27)
Fibroblasts	–	–	31 (nv 140 ± 27)	140 (nv 150 ± 25)

*nM/min/mg protein; nv, normal values; †citrate synthase.

Table 2 Clinical features of COX15 mutated patients			
	Kennaway <i>et al</i> ¹² ; Antonicka <i>et al</i> ⁴	Oquendo <i>et al</i> ¹³	Present case
Gender	Female	Male	Male
Family history	–	–	–
Age at onset/at death	Birth/24 days	7 months/3 years 11 months	4 months/still alive (16 years)
Neurological signs	Hypotonia Epileptic seizures	Axial hypotonia Limb spasticity Bulbar signs Nystagmus, retinopathy microcephaly	Axial hypotonia Limb spasticity Cerebellar tremor Eye movement incoordination Dystonia
Hypertrophic cardiomyopathy	+	–	–
Other clinical features	Midfacial hypoplasia	Gastrointestinal dysfunction	Somatic growth below 3rd centile
Plasma lactate (mM)	Increased	5.2	3.6
CSF lactate (mM)	NS	6.2	3.8
Brain MRI	NS	Basal nuclei Brainstem tegmentum Cerebral peduncles	Basal ganglia (putamina, caudate) Cerebellar white matter
Mutations	700C→T, C447-3G	700C→T, 700C→T	503C→G, 1081T→C

NS, not specified in the report.

extreme reduction of COX activity in cardiac mitochondria. Similar to yeast *COX15* mutants, the cytochrome spectrum showed low levels of heme A and accumulation of heme O.

More recently, the same 700C→T missense mutation (R217W) has been reported in a homozygous child with rapidly progressive Leigh syndrome and chronic gastrointestinal dysfunction, who died at 4 years of age. The R217 is a highly conserved residue located in the region between the third and the fourth transmembrane domain of the protein. Residual COX activity in cultured fibroblasts was undetectable by both spectrophotometric and cytochemical assays.¹³

By contrast, the clinical course in our patient was slow, with partial preservation of the cognitive functions and survival beyond adolescence. The residual COX activity in muscle and fibroblasts was only moderately reduced; this could in turn account for the slower clinical progression.

However, wide tissue variability of the biochemical defect was observed also in the previous two *COX15* mutant cases; therefore, a mild reduction of COX activity in muscle and fibroblasts does not exclude a more severe biochemical defect in other tissues, including the brain. An interesting possibility is that the S344P change has a hypomorphic, rather than null, effect, determining a partial reduction, rather than abolition, of the *COX15* catalytic activity on heme A biosynthesis. This hypothesis could also explain the presence of a normal amount of fully assembled COX found in our patient, in contrast with the lower than normal amount found in the first *COX15* mutant case, and also in other defects affecting the heme A biosynthesis, notably mutations in the *COX10* gene.¹⁴

Figure 2 Analysis of COX. Left panel: Immunoblot staining of 2D-BNE using an anti-COX I specific monoclonal antibody. The analysis was performed on cultured skin fibroblasts from a control, from our *COX15* patient, and from a *SURF1*^{−/−} patient. S1 and S2 are assembly intermediates of the COX holoenzyme, corresponding to S4. The arrows indicate the direction of the electrophoretic run in the first and second dimensions, respectively. Right panel: COX cytochemical staining of the cultured fibroblast lines used for the 2D-BNE immunoblot analysis.

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