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Case Report

Pure myopathy with enlarged mitochondria associated to a new mutation in *MTND2* gene

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ABSTRACT

To date, only few mutations in the mitochondrial DNA (mtDNA)-encoded ND2 subunit of Complex I have been reported, usually presenting a severe phenotype characterized by early onset encephalomyopathy and early death. In this report, we describe a new mutation in the *MTND2* gene in a 21-year-old man with a mild myopathic phenotype characterized by exercise intolerance and increased plasma lactate at rest. Electromyography and brain NMR were normal, and no cardiac involvement was present. Muscle biopsy showed a massive presence of ragged red – COX-positive fibres, with enlarged mitochondria containing osmiophilic inclusions. Biochemical assays revealed a severe isolated complex I deficiency. We identified a novel, heteroplasmic mutation m.4831G>A in the *MTND2* gene, causing the p.Gly121Asp substitution in the ND2 protein. The mutation was present in the 95% of mitochondrial genomes from patient's muscle tissue, at a lower level in cells from the urinary tract and at a lowest level in lymphocytes from patient's blood; the base substitution was absent in fibroblasts and in the tissues from proband's healthy mother and brother. The specific skeletal muscle tissue involvement can explain the childhood-onset and the relatively benign, exclusively myopathic course of the disease.

1. Introduction

Respiratory chain defects (RCD) are usually phenotypically related to heterogeneous clinical features, ranging from fatal infantile multisystem syndromes to encephalomyopathies or isolated myopathies sometimes associated with cardiomyopathies. The age of onset varies from neonatal to childhood, up to adult life [1]. Hypotonia, lactic acidosis, cardiorespiratory failure and severe psychomotor delay are the most frequently reported features in paediatric patients [2], while myopathy, associated to central nervous system involvement (hearing loss, pigmentary retinopathy, seizures, ataxia, polyneuropathy, rarely movement disorders) is a main characteristic of the adult-onset pathologies. Respiratory chain defects are either related to mitochondrial DNA mutations, or to abnormalities in nuclear genes linked to mitochondrial function.

Complex I (CI, NADH dehydrogenase ubiquinone–ubiquinol reductase) is the largest complex of the respiratory chain. It catalyses the transfer of electrons from NADH to Coenzyme Q10, and consists of 45 subunits, seven of which (ND1–ND6, ND4L) encoded by the mitochondrial genome. In mitochondrial disorders, isolated CI deficiency is relatively frequent [3] , usually associated with severe, early-onset, multisystem phenotype. Due to the enzyme complexity, in almost half of the cases of CI defect, a genetic cause has not been identified yet. Only few mutations in the mitochondrial DNA (mtDNA)-encoded ND2 subunit (EC:1.6.5.3) have been reported, usually associated with Leigh syndrome [4], and Leber's hereditary optic neuropathy [5]; a single patient has been reported carrying a 2-bp deletion in *MTND2* gene and suffering from severe exercise intolerance [6].

Here we report a new mutation in the *MTND2* gene in a patient with a severe and isolated CI defect showing a relatively mild phenotype characterized by exercise intolerance and lactic acidosis.

2. Case report

The patient is a 21-year-old man, the first born after uncomplicated pregnancy and delivery from healthy unrelated parents. His only sibling – a younger brother aged 18 years old – has been healthy so far. Early

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psychomotor development was normal. First symptoms became evident at the age of 7 years, when he began to complain of overall fatigability presenting exclusively during physical activities, and worsened by exposure to cold temperatures. In the late five years , the exercise intolerance became so severe he could not keep up with his schoolmates when playing and running, requiring 30 to 60 min to regain the overall strength. The progressive fatigability eventually prevented him from riding a bike, then he became unable to carry ordinary burdens (i.e. backpack, books) while the tolerable walking distance gradually shortened to 150 m. Neither cognitive nor behavioural changes were described; he did not report any hearing, vision, speech impairment, he never lost consciousness, and he never experienced any selective muscle group weaknesses nor myoglobinuria. Starting from the age of 17 years, he began to exhibit frequent vomiting while exercising, symptoms that urged the patient's family to search for medical help.

The first clinical evaluation performed at 20 years of age showed asthenic habitus, no dysmorphic facial features but he showed high-arched palate and malocclusion of teeth, normal head circumference, low body mass index (BMI, 18 kg/m^2) with diffuse muscle hypotrophy. The overall neurological examination was normal; neither pyramidal nor cerebellar signs were observed. Muscle strength and tone were unremarkable, with mildly decreased deep tendon reflexes. Clinical tests for myasthenia, prostigmine test, anti-AchR and anti-MuSK antibodies were negative. However, he referred general weakness during arm uplifting >60 s. and five squats. The psychological testing and psychiatric status were normal (full-scale IQ = 97, WAIS-IV). Neurophthalmologic findings, electroretinogram and audiometry were normal. Electrophysiological assessment (electroencephalogram, multimodal evoked potentials, ENG, EMG with repetitive stimulation) and Brain MRI with spectroscopy were normal. Thyroid status and routine blood tests for renal and liver function were within normal ranges, so as CK values (190 U/L). Lactate at rest were increased in the blood (9.9 mmol/L, n.v. <2 mmol/L) increasing at 14,4 mmol/L after exercise , while normal in cerebrospinal fluid. Electrocardiogram and echocardiogram were normal, while 24 h-Holter ECG detected a supraventricular tachycardia (190/min) during minor physical activity (e.g. slow walking), with normal heart rate at rest (80/min) and ergometry test. Respiratory assessment (clinical examination, spirometry, arterial blood gas analysis) was normal. Since a mitochondrial disorder was suspected, the patient underwent a muscle biopsy and was afterwards put on oral antioxidant therapy (Coenzyme Q10 180 mg per day and vitamin B complex). After the starting of antioxidant therapy, he showed an improvement of his condition, with a better tolerance of physical activity better and a decrease of his overall fatigability with shortening of the post-exercise recovery time; no effects were reported on exercise tolerance. The patient is still on therapy, and it is clinically stable.

3. Materials and methods

Histological and ultrastructural assessment of the muscle biopsy were performed using standard histological, histochemical and electron microscopy techniques [7].

Mitochondrial respiratory chain (MRC) enzymes activity was assayed by standard spectrophotometric techniques in muscle homogenate and in digitonin-treated cultured fibroblasts obtained from skin biopsy [8]. Each MRC enzyme specific activity was normalized to that of citrate synthase (CS), a standard marker of cellular mitochondrial content. Normal activity range is expressed as mean value ± standard deviation; residual activity was expressed in percentage.

The entire mtDNA was PCR-amplified and sequenced as described in Bugiani et al. [8].

Restriction fragment length polymorphism (RFLP) analysis was used to confirm and quantify the mutation using the *Nla*IV enzyme (NEB).

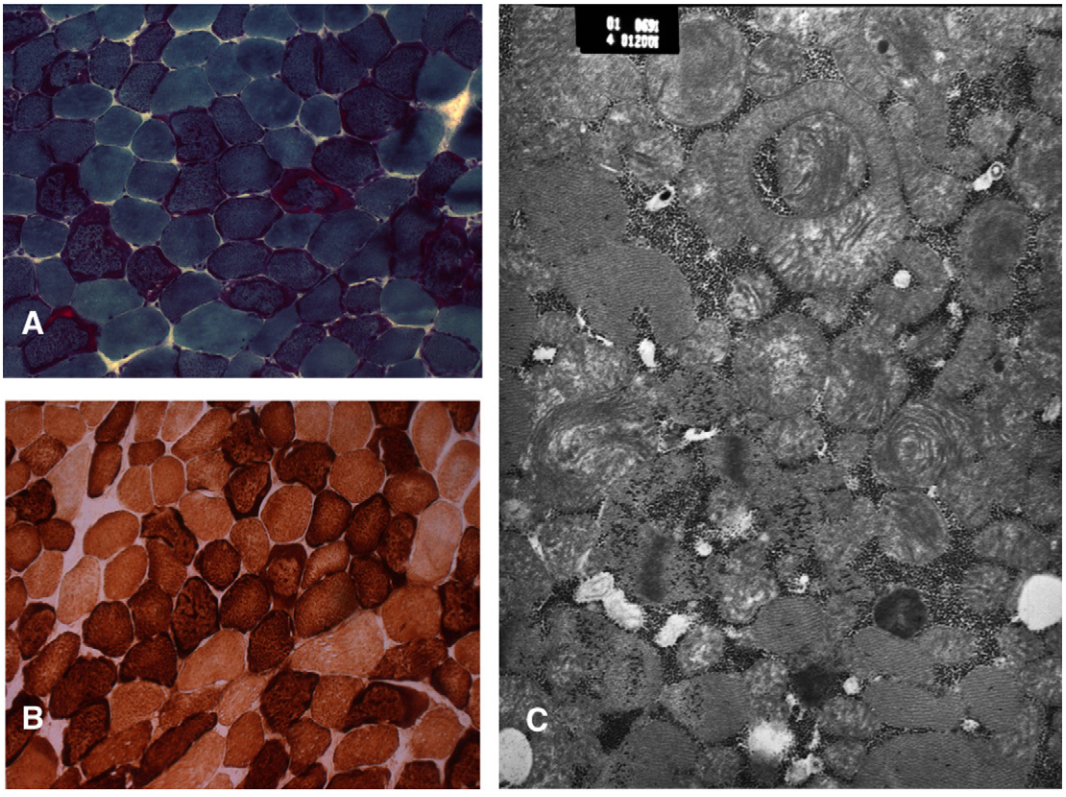

Fig. 1. Muscle biopsy. A: Gomori trichrome stain showing numerous ragged red fibres. B: Cox staining showing that the ragged red fibres are COX-positive. C: Electron microscopy showing enlarged mitochondria with osmiophilic inclusions.

Table 1
Respiratory chain complexes activities in muscle and fibroblasts from the patient.

	CI	CII	CIII	CIV	CS
Muscle	2,0	29,0	94	155	466
Control values	19,5 ± 3,4	19,9 ± 4,4	100 ± 15	189 ± 33	133 ± 39
Fibroblasts	25,0	20,4	103	206	124
Control values	23,7 ± 4,6	19,2 ± 3,4	108 ± 20	150 ± 30	113 ± 18

Activities of complexes are expressed as nmol/min mg of protein and are normalized to the citrate synthase activity. Control values are shown as means ± s.d.

4. Results

Quadriceps muscle biopsy disclosed the presence of numerous ragged red - COX-positive fibres, with accumulations enlarged mitochondria and mitochondrial proliferations (Fig. 1A, B). The electron microscopy confirmed the presence of numerous and enlarged mitochondria, often with abnormal proliferating cristae and rounded osmiophilic inclusions (Fig. 1C).

Biochemical assays of MRC complexes performed on muscle homogenate showed a severe isolated CI deficiency equal to 10% of control mean with very high CS activity equal to 345% of control mean; all MRC activities was normal on patient's fibroblasts (Table 1). We performed the sequence analysis of entire mtDNA, classified into haplogroup J1c3 by HaploGrep2 inherited from the mother ,as confirmed by the same analysis performed on her DNA, and identified a new mutation in *MTND2* gene (m.4831G>A) that causes the substitution of the Glycine in position 121 with an Aspartic acid (p.Gly121Asp) in the protein, in a site between two intermembrane domains. The mutation has never been reported, the p.Gly121Asp change scored very highly for likelihood to be deleterious according to ad-hoc softwares for pathogenicity prediction (damaging for Polyphen2: p = 1.000; Panther: 0.96; MutPred: 0.969; SIFT and MutationTaster) and the amino-acid involved is highly conserved in the phylogenies (Fig. 2A).

PCR-RFLP analysis performed to quantify the mutation in different tissues using the *Nla*IV enzyme (Fig. 2B) showed the presence of the m.4831G>A change in the 95% of mitochondrial genomes from patient's muscle, in the 40% of genomes from urinary tract cells and only in <5% of genomes from patient's peripheral blood lymphocytes. The mutation was absent in fibroblasts obtained from skin biopsy as in the tissues (blood and urine) from the healthy mother and younger brother (Fig. 2C).

5. Discussion

Isolated CI deficiency is a frequent cause of mitochondrial dysfunction, usually related to severe and early-onset multisystem phenotype

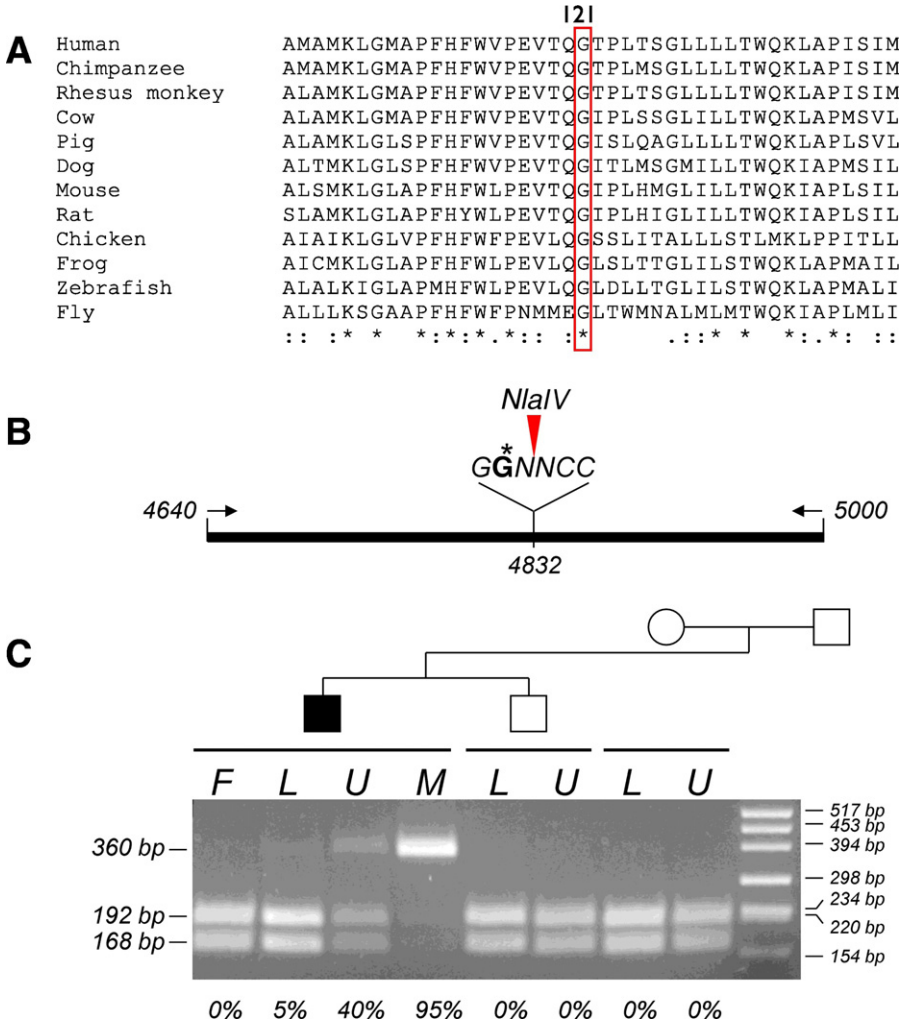

Fig. 2. A: Multi-alignment analysis. The comparison of the aminoacid sequences around Gly-121 in ND2 protein using clustalW between different species shows the high conservation of residue. B: Strategy of restriction fragment length polymorphism (RFLP) analysis with *Nla*IV enzyme. In the wt mtDNA molecules the *Nla*IV enzyme cut the 360 bp amplified fragment in two smaller fragment (192 bp and 168 bp), the mutation m.4831G>A abolishes the restriction site resulting the uncutted band. C: Result of RFLP analysis in patient and his family. The mutation is present in 95% of mtDNA from patient's muscle tissue (M), in 40% of genomes from urinary sediment (U), in <5% from peripheral blood lymphocytes (L) and is absent in fibroblasts (F) as well as in tissues from the healthy mother and younger brother (L and U).

and associated with mutations in nuclear genes coding for CI structural proteins. The less frequent mutations described in *MTND* genes usually cause Leigh syndromes or encephalomyopathies. We report a novel mutation in mitochondrial *MTND2* gene coding for a CI subunit, characterized by a mild phenotype with exclusive muscular involvement presented as exercise intolerance and high blood lactate at rest. Vomiting while exercising might be due to frequent physiological causes or rare somatic causes, and it is often a hallmark of exercise intolerance. In our patient Coenzyme Q10 therapy could ameliorate the capability to endure physical activity, but was unable to treat the overall fatigability.

The severe CI deficiency observed in patient's muscle homogenate is related to the almost homoplasmic mutation in this tissue, while in fibroblasts, where the mutation was absent, the activity was normal. This evidence confirms the pathogenicity of the mutation which, by changing an amino-acid highly preserved between species that links two intermembrane domains, probably affects their stability and function. Furthermore, the high mutation load on muscle tissue can explain the onset in childhood and the relatively benign course of the disease involving only skeletal muscle, but raises the question of how and when the specific tissue segregation ensued in this patient. The absence of the mutation in the healthy mother and brother suggests the exclusive presence of a lower mutation load in the ovarian cells or a de novo genesis in our patient. Extreme exercise intolerance and isolated mitochondrial myopathy has been reported in mitochondrial DNA mutations in at least 5 genes: *MTCYTB MTND2*, *MTND4*, *MTND5*, and *tRNAs*.

Our case resembles that of several mutations in *MTCYTB*, another mtDNA gene that encodes for the cytochrome *b*, a subunit of Complex III, which are often sporadic, in which a pure myopathy with exercise intolerance has been described [9,10,11]. Since now only few patients showing an exclusively mild muscular phenotype have been described carrying mutations in *MTND4* and *MTND5* genes coding for CI [12,13] and only one patient has been reported with a phenotype similar to our patient, characterized by severe exercise intolerance and lactic acidosis, due to a deletion of 2 bp in *MTND2* gene [6]. In all this patients the muscle biopsy showed ragged-red and COX-positive fibres; the mutations were present at high level only in muscle tissue, as seen in our patient. Our case however presents peculiar histopathological characteristics. At Gomori Trichrome stain, the accumulation of mitochondria is present in a large number of muscle fibres in particular in type 2 fibres, which show a peculiar large accumulation of mitochondria positive for COX and SDH staining that correlate with the biochemical data of a significative increment of citrate synthetase activity and a normal COX activity. Nevertheless the presence of mitochondrial proliferation with enlarged organelles containing osmiophilic inclusions and abnormal cristae confirm the severe involvement of mitochondria in muscle

In conclusion, in the presence of isolated exercise intolerance with high blood lactate, histopathological signs of mitochondrial myopathy and defects of CI activity, the sequence analysis of mitochondrial DNA should be performed.

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