Exercise Intolerance and Myoglobinuria Associated with a Novel Maternally Inherited *MT-ND1* Mutation

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Abstract The most common clinical phenotype caused by a mtDNA mutation in complex I of the mitochondrial respiratory chain is Leber hereditary optic neuropathy. We report a family with a novel maternally inherited homoplasmic mtDNA m.4087A>G mutation in the ND1 gene (*MT-ND1*) associated with isolated myopathy, recurrent episodes of myoglobinuria, and rhabdomyolysis. DNA from blood in seven family members and muscle from four family members were PCR amplified and sequenced directly and assessed for the m.4087A>G variation in *MT-ND1*. Mitochondrial enzyme activity in all muscle biopsies was measured. PCR and direct sequencing of the *MT-ND1* genes from blood showed that all seven family members were homoplasmic for the m.4087A>G mutation (NC_012920.1:c.781A>G). The mutation predicts a threonine to alanine substitution at position 261 (p.T261A). The same mutation was found in muscle of all four family members available for muscle biopsy, and biochemical analyses revealed an isolated complex I defect in muscle of all family members (range 22–52% of normal). Muscle morphology showed severe myopathic changes with internal nuclei in multiple fibers of all family members. Monosymptomatic myopathy with recurrent myoglobinuria is a rare phenotype of mitochondrial myopathies. We report this phenotype in a family affected by a novel homoplasmic

mutation in *MT-ND1*. It is the first time such a phenotype has been associated with complex I gene mutations and a homoplasmic mutation of mtDNA.

Introduction

Complex I, the largest of the mitochondrial respiratory chain (RC) complexes, consists of at least 46 subunits of which seven are encoded by mitochondrial DNA (mtDNA). The remaining 39 subunits are encoded by nuclear DNA (Wong 2007). The most common clinical phenotype caused by a mtDNA mutation in complex I is Leber hereditary optic neuropathy (LHON), a condition associated with subacute visual failure, which is often caused by a mutation in the mitochondrial ND1 subunit gene (Schapira 2006). Mutations in mtDNA can be homoplasmic (all mtDNA copies carry the mutation) or heteroplasmic (mutated mtDNA coexists with wild-type mtDNA) (Taylor and Turnbull 2005). Most patients with LHON are homoplasmic for mtDNA mutations, and the penetrance of the mutations is variable among family members (Schapira 2006; Taylor and Turnbull 2005). We report a family with a novel maternally inherited homoplasmic mtDNA m.4087A>G mutation in the ND1 gene (*MT-ND1*) associated with a new phenotype.

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Patients and Methods

Case Report

The proband is a 41-year-old man with an isolated myopathy. He had congenital ptosis, which is unusual for patients with mitochondrial disease, but not without precedence. He had rigid spine, increased CK and lactate

levels, and episodic myoglobinuria. Throughout childhood he felt physically weaker than his peers. At age 17, he was hospitalized due to a physical collapse. His CK values were highly elevated, and a muscle biopsy showed nonspecific myopathic changes, but no specific diagnosis was reached. At age 38, he developed rhabdomyolysis and muscle stiffness following injection of suxamethonium during surgery for appendicitis. On physical examination, he had decreased eye movements in all directions and he had previously undergone bilateral surgery for ptosis. He had pronounced rigid spine, scapula alata, and reduced proximal strength of the upper extremities (strength 4; MRC scale) but normal strength in the lower extremities.

Other Affected Family Members

The sister of the proband had an episode of rhabdomyolysis in her thigh muscles, at age 20, during biking while working as a mail delivery person. At the age of 43, she was hospitalized with severe pain and rhabdomyolysis. CK was elevated to 35,000 U/l (normal values: <210 U/l). On physical examination, she did not have ptosis or rigid spine like her brother. Her 16-year-old daughter frequently experienced exercise-induced muscle cramps and pain. The sister's 11-year-old seemingly unaffected son was clinically examined, and a blood sample was secured from him. A 71-year-old maternal aunt had hearing impairment since age 50 years. In her youth, she experienced muscle cramps and physical collapse after strenuous work but did not seek medical advice. The mother of the proband aged 74, who is in a nursing home because of brain damage following Herpes encephalitis, and the grandmother, who recently passed away at age 101, were both unavailable for clinical examination, but blood samples were secured from both. The grandmother and aunt wore hearing aids.

Muscle Biopsies

Percutaneous needle muscle biopsies were performed in the vastus lateralis muscle of the proband, his sister, niece, and the maternal aunt. The biopsies were frozen immediately in isopentane cooled by liquid nitrogen and kept in a freezer at -80°C until histochemical and biochemical analyses. Serial frozen 10 μm sections of muscle tissue were stained with hematoxylin and eosin (H&E), Gömöri trichrome, COX/SDH, and ATPase. Mitochondrial enzyme activity in all muscle biopsies was measured at 37°C as previously described (Larsen et al. 2012).

Western Blotting

Western blot analysis was performed essentially as previously reported (Ostergaard et al. 2007). The membrane was

incubated with an antibody against ND1 (1:1000, H00004535-A01, Abnova.) and an antibody against Porin (Proteintech, Chicago, IL, USA), which was used as a loading control. The secondary antibodies were goat anti-mouse/goat anti-rabbit (Dako, Glostrup, Denmark). The ND1 antibody was raised against amino acids 21–71 (YP_003024026).

DNA Analysis and HUMARA Assay

Total DNA was isolated from blood and muscle samples by standard methods. The mtDNA-encoded complex I genes were PCR amplified and sequenced directly using template DNA derived from muscle of the proband. Whole blood-derived DNA from the proband, his maternal grandmother, his mother, his maternal aunt, his sister, and his niece and nephew were subsequently assessed for the m.4087A>G variation in *MT-ND1*. The remaining complex I mtDNA genes were PCR amplified and sequenced directly on DNA derived from muscle of the proband to exclude other potential pathogenic variations.

To test for a potential X-linked mode of inheritance, we carried out a HUMARA assay (Allen et al. 1992) to assess potential biased X-chromosome inactivation in the sister of the proband.

Bicycle Ergometry Test

The patient underwent an exercise test on a cycle ergometer. The patient has a slight enlargement of the ascending aorta, and is missing and aortic valve, and for that reason he was not pushed to his limit during exercise testing. Instead he performed a cycle test at 69% of VO_2 max. We withdrew a blood sample before, during, and after the bicycle test for lactate analysis.

Results

Muscle Biopsies and Blood Tests

Biochemical analyses revealed an isolated complex I defect in the muscle of the family members (Table 1). Histochemical data from the proband and his sister showed a severe myopathic muscle with internal nuclei in almost all fibers and an increased variability in fiber diameter (Fig. 1a). The muscle biopsy of the aunt also showed multiple centrally nucleated fibers (11%). There were no dystrophic changes or histological signs of congenital myopathy in any of the biopsies. No COX-negative or ragged red fibers were found in any of the family members (Fig. 1b–d). The blood samples of the proband and his sister showed increased values of CK (1,100 and 953 U/l

Table 1 Activities of mitochondrial enzymes in skeletal muscle

	Proband	Sister	Daughter	Aunt	Normal values
Complex I	0.10	0.09	0.08	0.19	0.34 ± 0.09 (0.18–0.58)
Complex II	0.52	0.24	0.41	0.42	0.39 ± 0.07 (0.32–0.62)
Complex III	1.50	0.68	1.49	1.05	1.20 ± 0.21 (0.75–1.71)
Complex IV	5.3	3.3	4.6	5.0	3.8 ± 0.7 (2.5–5.7)
Citrate synthase	239	345	307	212	309 ± 83 (181–468)
Complex I/II	0.19	0.39	0.20	0.46	0.87 ± 0.21 (0.48–1.36)

Enzyme activity is expressed as milliunits per milliunits citrate synthase. Citrate synthase activity is expressed as milliunits per milligrams protein. Normal values are from 29 age-matched controls and expressed as mean $\pm$ SD (ranges in parentheses)

Fig. 1 Muscle sections from the proband (**a–d**) and a control (**e–h**). (**a**) H&E dye showing multiple centrally nucleated fibers in the muscle fibers of the proband. (**b–d**) Gömöri trichrome, COX, and SDH

showing no COX-negative or ragged red fibers in the muscle fibers of the proband. The white bar is 50 μ m

(normal values: 35–210 U/l)) and myoglobin (139 and 144 μ g/l (normal values: 19–49 μ g/l)). The sister had normal lactate values and the blood sample of the niece and aunt showed normal values. An acyl-carnitine analysis of the proband was in the normal range. Western blot analysis showed that the proband had normal amounts of ND1 protein in the muscle (data not shown).

DNA Analysis and HUMARA Assay

PCR and direct sequencing of the *MT-ND1* genes from the blood showed that all family members were homoplasmic for a novel m.4087A>G mutation (Fig. 2). The mutation

was found homoplasmic in the muscle and blood of the proband, his maternal aunt, his sister, and her daughter. Since the mutation was found to be homoplasmic in four generations, and in two embryonically different tissues (blood and muscle), we did not find it necessary to test for the mutation in other tissues. The mutation predicts a threonine to alanine substitution at position 261 (p.T261A). The threonine at position 261 is not highly conserved, and the physiochemical difference between threonine and alanine is only moderate. No other mutations were detected in the seven complex I mtDNA genes.

To exclude a possible X-linked inheritance of an unknown mutation, the sister of the proband was assessed

m.4087 A>G

I: 1 Maternal grandmother (blood)

II: 1 Mother (blood)

2 Maternal aunt (blood + muscle biopsy)

III: 1 Proband (blood + muscle biopsy)

2 Sister (blood + muscle biopsy)

IV: 1 Niece (blood + muscle biopsy)

2 Nephew (blood)

Fig. 2 Family pedigree. All shown family members carried 4087 A>G mtDNA mutation in *MT-ND1*

with the HUMARA assay. She displayed a slightly biased ratio (18:82), which is not uncommon, arguing against an X-linked inheritance.

Bicycle Ergometry Tests

During the bicycle ergometry test, the proband reached a VO_2 level of 22 ml $\text{O}_2/\text{kg}/\text{min}$ at a workload of 64 watt. Since the patient cycled at predicted 69% of VO_2 max, VO_2 max was calculated to be 32 ml $\text{O}_2/\text{kg}/\text{min}$, which is 60% of expected for age (Swain et al. 1994). The lactate levels in the blood sample before and at the end of cycling were 0.6 and 2.7 mmol/l, respectively. On another occasion his blood lactate levels at rest had been slightly elevated (2.7 mmol/l).

Discussion

Here, we report a family with a novel, homoplasmic m.4087A>G mutation in *MT-ND1*, which expands the genotypic spectrum of mtDNA-related diseases and provides a new phenotype for aberrations in *MT-ND1* and for homoplasmic mtDNA mutations in general. We have sequenced the *MT-ND1* gene in an excess of 80 samples from an ethnically matched population and never encountered the m.4087A>G change before. Moreover, m.4087A>G is absent from MitoMap.org, a highly comprehensive database compiling all published mtDNA variations.

The symptoms of myopathy and exercise intolerance in affected family members, and the ophthalmoplegia in the

proband, are all common symptoms in mitochondrial myopathies. Less common are painful exercise-induced muscle cramp accompanied by high levels of CK and episodic myoglobinuria and rhabdomyolysis. Recurrent episodes of myoglobinuria have only been described in a few other mitochondrial myopathy cases, particularly in cases with mutations in genes encoding structural complex III and IV genes and some mtDNA-tRNA genes (Vissing et al. 2013). Recurrent episodes of myoglobinuria caused by mutations in mtDNA complex I subunit genes have not been described before, and neither has a homoplasmic mtDNA mutation with a myopathic phenotype. Only one (heteroplasmic) mtDNA mutation of a complex I gene (m.11832G>A mutation in *MT-ND4*) has been reported to cause myopathy, but not myoglobinuria (Taylor and Turnbull 2005).

The *MT-ND1* mutation was present in all family members, and accordingly complex I activity in skeletal muscle was significantly decreased in all tested individuals (22–52% of mean control when expressed as a ratio relative to complex II activity) and muscle histology showed myopathic changes. An acyl-carnitine analysis of blood from the proband showed normal values, indicating intact beta-oxidation of fatty acids. A western blot showed normal amounts of ND1 protein. Since complex I is a very large protein complex, and we only targeted it with a single antibody, the finding of normal protein expression does not exclude a complex I deficiency. In fact, biochemical analyses showed a clear defect of complex I activity in the muscle of all tested family members.

The proband performed a bicycle ergometry test with a calculated VO_2 max of 32 ml $\text{O}_2/\text{kg}/\text{min}$. This is 60 % of what is expected in age-matched controls (Astrand 1960) showing that the patient has exercise intolerance and supports an oxidative defect. The lactate levels before and at the end of cycling were in the normal range. We have previously showed that lactate measurements cannot be used as a reliable test to diagnose mitochondrial myopathy (Jeppesen et al. 2003).

The pedigree could theoretically be compatible with an X-linked mode of inheritance, and we therefore carried out the HUMARA assay to assess for potential biased X-chromosome inactivation. The sister to the proband displayed a slightly biased ratio (18:82), which is not uncommon. Thus, the HUMARA assay does not support an X-linked mode of inheritance.

It is common to see a great variability in phenotype among affected family members with homoplasmic mtDNA mutations, e.g., in LHON, where only 50% of males and 10% of females develop impaired vision, indicating that nuclear genetic factors play an important role in the clinical expression (Taylor and Turnbull 2005), as well as environmental factors (cigarette smoking,

alcohol, and certain drugs) (Yu-Wai-Man et al. 2011). Other homoplasmic complex I mtDNA mutations are associated with a spectrum of phenotypes from subtle symptoms to severe multisystem disorders. As an example, the homoplasmic m.14459G>A mutation in the *MT-ND6* gene can cause LHON, dystonia, and Leigh-like disease but can also be found in asymptomatic individuals within the same family (Wong 2007). The family in our study expresses a new phenotype for homoplasmic mutations in *MT-ND1* ranging from mild myopathic symptoms and reduced complex I activity to myopathic features on muscle biopsy and recurrent episodes of myoglobinuria, rhabdomyolysis, and muscle cramps.

Synopsis

We present a novel maternally inherited *MT-ND1* mutation associated with exercise intolerance and myoglobinuria.

Compliance with Ethics Guidelines

Competing Interest

Dr. Vissing has received research and travel grants from Genzyme Corporation and serves on the scientific global advisory board for Pompe disease for Genzyme Corporation.

Jabin Rafiq, Morten Duno, Elsebet Østergaard, Kirstine Ravn, Christoffer Vissing, and Flemming Wibrand declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients before inclusion in the study.

Details of the Contributions of Individual Authors

Mrs. Rafiq – study concept and design, acquisition of data, and drafting of the paper

Dr. Vissing – study concept and design, acquisition of data, study supervision, and critical revision of the manuscript

Dr. Duno – analysis and interpretation and critical revision of the manuscript

Dr. Wibrand – analysis and interpretation and critical revision of the manuscript

Dr. Østergaard – acquisition of data, analysis, and interpretation of data

Mr. C.R. Vissing – acquisition of data, analysis, and interpretation of data

Dr. Ravn – acquisition of data, analysis, and interpretation of data

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