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Recurrent Myoglobinuria in a Sporadic Patient with a Novel Mitochondrial DNA tRNA^{Ile} Mutation

Valentina Emmanuele¹, Evangelia Sotiriou¹, Maryam Shirazi¹, Kurenai Tanji^{1,2}, Ronald G. Haller³, Katja Heinicke³, Peter E. Bosch⁴, Michio Hirano¹, and Salvatore DiMauro¹

¹Department of Neurology, Columbia University Medical Center, New York, NY

²Department of Pathology, Columbia University Medical Center, New York, NY

³Institute for Exercise and Environmental Medicine, Department of Neurology, VA Medical Center and University of Texas Southwestern Medical Center, Dallas, TX

⁴Department of Neurology, Mayo Clinic Arizona, Scottsdale, AZ

Abstract

The differential diagnosis of myoglobinuria includes multiple etiologies, such as infection, inflammation, trauma, endocrinopathies, drugs toxicity, and primary metabolic disorders. Metabolic myopathies can be due to inherited disorders of glycogen metabolism or to defects of fatty acid oxidation. Primary respiratory chain dysfunction is a rare cause of myoglobinuria but it has been described in sporadic cases with mutations in genes encoding cytochrome *b* or cytochrome *c* oxidase (COX) subunits, and in 4 cases with tRNA mutations. We describe a 39-year-old woman with myalgia and exercise-related recurrent myoglobinuria, who harbored a novel mitochondrial DNA mutation at nucleotide 4281 (m.4281A > G) in the tRNA-isoleucine gene. Her muscle biopsy revealed ragged-red and COX-deficient fibers. No deletions or duplication were detected by Southern blot analysis. The m.4281A > G mutation was present in the patient's muscle with a mutation load of 46% and was detected in trace amounts in urine and cheek mucosa. Single-fiber analysis revealed significantly higher levels of the mutation in COX-deficient (65%) than in normal fibers (45%). This novel mutation has to be added to the molecular causes of recurrent myoglobinuria.

Keywords

mitochondrial myopathy; myoglobinuria; mitochondrial DNA; tRNA^{Ile}; myalgia; elevated serum CK

1. Introduction

Rhabdomyolysis is a potentially life-threatening event characterized by acute breakdown of skeletal muscle fibers with release of muscle cells components into the circulation. Classical findings are limb weakness, muscle pain and swelling, massively elevated serum creatine

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Correspondence: Salvatore DiMauro, MD, 4-424 B, College of Physicians & Surgeons, 630 West 168th Street, New York, NY 10032; tel. 212 305 1662; sd12@columbia.edu.

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kinase (CK) levels, and gross pigmenturia due to increased excretion of myoglobin. Myoglobin is a 17.8-kDa protein that is excreted in the urine only when the renal threshold is exceeded, and is grossly visible as brown (i.e. tea- or cola-colored) urine when serum concentration surpasses 100 mg per deciliter [1]. Acute kidney shutdown is a potential complication of massive myoglobinuria, regardless of the etiology. Myoglobinuria requires an extensive differential diagnosis, including trauma, infections, electrolyte disturbances, exposure to drugs and toxins, and primary metabolic disorders. Recurrent episodes of rhabdomyolysis and myoglobinuria are usually seen in patients with metabolic myopathy due to impaired glycogen metabolism or fatty acid oxidation [2]. The circumstances triggering the episodes of myoglobinuria (strenuous vs prolonged exertion, fasting) may help identify the underlying metabolic myopathy. As “metabolic myoglobinuria” is associated with blocks in energy metabolism, it is surprising that defects of the mitochondrial respiratory chain, the “business end” of ATP production, are relatively rare causes of myoglobinuria. Most reported cases were due to point mutations or microdeletions in the cytochrome *b* gene or in genes encoding cytochrome *c* oxidase (COX) subunits. Notably, all these cases were sporadic [3–8]. Mutations in tRNA genes have been associated with rhabdomyolysis only in four patients, of whom three harbored the m.3243A > G MELAS mutation in tRNA^{Leu(UUR)} [9–11] and one harbored the m.4298G > A transition in tRNA^{Ile} [12]. A mutation in tRNA^{Phe} (m.606A > G) had been considered pathogenic by Chinnery *et al.* [13] in a patient with exercise intolerance and myoglobinuria before they discovered the true pathogenic mutation in the gene encoding subunit II of COX (COX II) [4].

Here, we report a sporadic patient with myalgia and exercise-induced recurrent myoglobinuria, who harbored a novel mtDNA mutation (m.4281A > G) in the tRNA isoleucine (tRNA^{Ile}) gene (*MTT1*).

2. Case report

A previously healthy 39-year-old woman presented with a 3-year-history disabling daily myalgia, which required the application of fentanyl patches. She had always been very active without neuromuscular symptoms. She served in the Navy for two years and had no problems during basic training at the age of 23 years. In the two years prior to evaluation, she experienced several episodes of pigmenturia after intense physical exercise. Her exercise regimen included using an elliptical machine for 50 minutes followed by circuit training and rock climbing three times a week. Serum CK during the episodes was higher than 10,000 U/l (normal value <176 U/l). The first 2 episodes required hospitalization and intravenous hydration. After each episode, the acute muscle pain subsided over hours, but the patient still had intermittent spontaneous pain in calf and back muscles, and CK levels fluctuated from mildly elevated to normal. At the time of the first episode, the patient was on a weight reduction diet. The attacks of myoglobinuria were not preceded by infection, trauma, or electrolytes disorders. Urinary drug screening was positive for opiates and benzoids after the first episode. Past medical history was unremarkable for neuromuscular symptoms. She suffered from depression. Her mother had chronic myalgias, but no documented episodes of myoglobinuria. Two elder brothers and four sisters had no neuromuscular symptoms.

At the time of referral, neurological examination was normal. Serum CK was mildly elevated (277 U/l). Thyroid function tests were normal. HIV antibodies were negative. Motor and sensory nerve conduction studies were normal. Electromyography revealed minimally short duration motor unit potentials in proximal arm, leg and paraspinal muscles, compatible with myopathy.

2.1 Methods

Histochemical studies of muscle using 8-mm-thick sections were carried out as described [14]. Biochemical analysis was performed in 10% muscle extracts as previously described [15].

Maximal and submaximal cycle exercise testing was performed with measurement of gas exchange, cardiac output and calculation of systemic arteriovenous oxygen levels as previously described [16]. Serum CK was determined at baseline and serially following exercise testing.

Total DNA was extracted from skeletal muscle, urinary sediment, and cheek mucosa using the Puregene® DNA purification kit (Gentra System). Southern blot analysis of the patient's skeletal muscle mtDNA excluded the presence of large-scale rearrangements. All 22 mtDNA tRNA genes were sequenced using suitable oligonucleotide primers. The resulting PCR fragments were subjected to direct sequencing with the ABI PRISM Dye Terminator Cycle Sequencing (Ready Reaction Kit) and 3130xl Genetic Analyzer (Applied Biosystem, Perkin-Elmer, Foster City, CA).

2.2. Results

Histochemical analysis of a gastrocnemius muscle biopsy revealed 1–4 ragged-red fibers with the Gomori trichrome stain or ragged-blue fibers with the succinate dehydrogenase (SDH) stain. There were 15.8% COX-negative fibers. These findings were consistent with a primary mitochondrial myopathy.

Spectrophotometric enzyme analysis of a muscle extract showed normal activities of respiratory chain complexes and cytrate synthase (Table 1).

Maximal and submaximal cycle exercise tests were performed using a stationary bicycle to measure the effects of exercise stress. Blood pressure, heart rate, oxygen uptake, cardiac output, lactate and lactate/pyruvate ratio at rest and during exercise were normal. Peak oxygen uptake, 28.4 ml/kg/min, and peak levels of extraction of available oxygen from blood (~14ml/dl) were normal; and serum CK at baseline and following exercise was normal.

A novel m.4281A > G transition was found in the tRNA^{Ile} gene of the patient's skeletal muscle mtDNA (Fig. 1A). This mutation disrupts a phylogenetically highly conserved site in the dihydrouridine stem (DHU stem) (Fig.1B,C). To assess the mutation load, we performed restriction fragment length polymorphism analysis (RFLP) on DNA extracted from muscle, cheek mucosa, and urinary sediment. The m.4281A > G transition generates a *Bse*RI restriction site. After digestion of a PCR-generated 127 base-pair (bp) fragment containing the mutation, mutant mtDNA was cut into two fragments (90 bp and 38 bp). The digestion products were run in a 15% non-denaturing acrylamide gel and subjected to SYBR Gold® Nucleic Acid Gel Stain (Invitrogen) to quantify the proportion of mutant mtDNA. PCR-RFLP showed that muscle of the proband contained 46% mutant genomes, whereas urinary sediment and cheek mucosa had only traces of mutant mtDNA. The mutation was not detected in mtDNA extracted from urinary sediment or cheek mucosa from the patient's unaffected mother. To prove the pathogenicity of the mutation, we performed single-fiber PCR analysis. After histochemical staining with succinate dehydrogenase (SDH) and COX, single COX-positive and COX-negative muscle fibers were isolated from 30µm thick sections, using a P.A.L.M Microbeam Laser System (P.A.L.M. Microlaser Technologies, Bernried, Germany) and collected in sterile tubes containing 40µl distilled water. Single fibers were then processed and analyzed by PCR-RFLP analysis as previously described. The proportion of mutant mtDNA was 65.49±7.13% (mean ±SD) in 10 COX negative

fibers, and $44.91\% \pm 7.84\%$ (mean $\pm$ SD) in 10 COX positive fibers. This difference was statistically significant ($p < 0.05$, Mann-Whitney test) (Fig. 2).

3. Discussion

We describe a patient with myalgia and recurrent myoglobinuria triggered by intense exercise. The metabolic causes of myoglobinuria fall into three groups: disorders of glycogen metabolism, defects of fatty acid oxidation, and defects of the mitochondrial respiratory chain [2]. In our patient, muscle biopsy revealed an underlying mitochondrial myopathy and directed our attention to the respiratory chain. Most cases with recurrent myoglobinuria and mitochondrial myopathy have been associated with mutations in subunits of complex III or complex IV. However, biochemical analysis of muscle extracts in our patient failed to show any specific respiratory chain complex deficiency and was, in fact, essentially normal. This finding led us to sequence all 22 tRNA genes of the mitochondrial genome because a few patients with myoglobinuria did harbor mutations in these genes. We found a novel heteroplasmic m.4281A > G transition in *MTT1*. This mutation fulfills canonical criteria for pathogenicity. First, the mutation was heteroplasmic and predominant in skeletal muscle, the only clinically affected tissue. Second, single fibers analysis showed a statistically significant correlation between histochemically COX-negative fibers and mutation load. Third, the change has not been reported as a neutral polymorphism in the mitochondrial genome databases (<http://mitomap.org> and <http://www.genpat.uu.se/mtDB/>). Fourth, the mutation disrupts an evolutionary highly conserved base pair located in the D-stem of tRNA^{Ile}, and it is likely to have functional importance. This is further supported by previous reports: a T > C transition at nucleotide position 4274, which disrupts the same base-pair in the DHU stem, caused chronic progressive external ophthalmoplegia in one case [17] and motor neuron disease in another [18]. Studies of the molecular mechanism by which the m.4274T > C mutation impairs mitochondrial protein synthesis have shown reduced efficiency of the mutant tRNA^{Ile} to undergo post-transcriptional modification, as for the other *MTT1* mutations [19]. In particular, this substitution impairs the efficiency of aminoacylation [20, 21].

Among human mitochondrial tRNA genes, tRNA^{Ile} is a recognized hot spot: to date, 13 pathogenic point mutations have been associated with different clinical phenotypes. Two features prevail with *MTT1* mutations: cardiomyopathy [22–26] and ophthalmoplegia. [27, 28] However, there is great clinical variability, with no obvious correlation between position of the mutations within the tRNA secondary structure and phenotype. Clinical heterogeneity is evident even within the same family: for example, the m.4284G > A transition caused cardiomyopathy, ophthalmoplegia, progressive spastic paraparesis, or complex neurological features in different family members [29]. Other clinical presentations have been described, including CPEO and multiple sclerosis [30], pure myopathy [31], progressive encephalopathy [32], occipital stroke before age 45 [33], encephalocardiomyopathy [34], and motor neuron disease [35]. A putative pathogenic role has been proposed for the m.4291T > C mutation in the metabolic syndrome, characterized by hypertension, hypercholesterolemia, and hypomagnesemia [36]. More relevant to our case, Crimi et al. described a 38-year-old man with a three-year history of rhabdomyolysis (serum CK levels up to 20,000 U/l), myoglobinuria, and diffuse myalgias after prolonged fasting or vigorous exercise. He harbored an m.4298G > A transition in the anticodon stem of tRNA^{Ile}, affecting the aminoacylation efficiency [37]. Similar to our patient, the mutation load was 48.7% in muscle, but almost undetectable in his blood or in the blood from his unaffected mother. However, in our patient the presence of the mutation in trace amounts in accessible non-muscle tissues makes it more plausible that the mutation was generalized, but – due to skewed heteroplasmy – it surpassed the pathogenic threshold only in muscle. This would

imply that the mutation loads in accessible tissues from our patient's mother were below the limit of detection.

To our knowledge, myoglobinuria had been associated with mtDNA tRNA mutations only in 3 other patients, all of whom harbored the m.3243A > G MELAS transition in tRNA^{Leu(UUR)}. The first patient was a 33-year-old woman with typical MELAS but also with acute peripheral neuropathy and rhabdomyolysis [9]. The second patient was a 43-year-old man with MELAS and nocturnal myalgia [10], and third patient was a 43-year-old woman with MELAS, who had one episode of myalgia, as well as elevated serum myoglobin and CK levels, consistent with rhabdomyolysis [11].

The pathogenesis of rhabdomyolysis could involve ATP depletion in myocytes, leading to altered calcium homeostasis and calcium overload, persistent contraction, energy depletion, activation of calcium-dependant proteases and phospholipases, resulting in muscle breakdown [38, 39]. However, in contrast with this hypothesis, there is no apparent correlation between severity of the oxidative defect and rhabdomyolysis in mtDNA-related myopathies, which is illustrated by our patient, who had essentially normal mitochondrial function by both enzymatic and physiological criteria. In conclusion, tRNA-linked disorders cover a wide range of symptoms and the relationship between mutations and clinical phenotypes is complex; a single mutation can lead to different phenotypes, and different mutations to the same phenotype. This study adds m.4281A > G to the list of point mutations in *MTTI*, and underlines the importance of sequencing the entire mtDNA in patients with mitochondrial myopathy and recurrent myoglobinuria, after more common causes have been excluded.

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Figure 1. Novel m.4281A > G transition in tRNA^{Ile} gene

(A) Electropherogram of DNA sequence. Adenine at nucleotide 4281 was substituted by guanine.

(B) Nucleotide sequences showing interspecies similarity of the mitochondrial tRNA^{Ile} DHU stem. The base-pair involving the nucleotide at position 4281 (in bold) is strictly conserved among species. The mutation in our patient is highlighted with a bold red letter.

(C) tRNA^{Ile} cloverleaf secondary structure. Red dots indicate nucleotides with known mutations. A red circle highlights the novel m.4281A > G transition in the tRNA^{Ile} DHU stem.

Figure 2. Single-fiber PCR assay

Percentage of mutant mtDNA in COX-positive and COX-negative muscle fibers by single-fiber PCR analysis. Each bar-graph shows the mean mutant load (as percentage of total mtDNA) of 10 fibers $\pm$ SD. The difference is statistically significant ($p < 0.05$, Mann-Whitney test).

Table 1

Mitochondrial enzyme activities in muscle.

Enzyme (Complex)	Patient	Controls (mean $\pm$ SD)
NADH-cyt <i>c</i> reductase (I+III)	0.72	1.02 $\pm$ 0.38
NADH dehydrogenase (I)	30.43	35.5 $\pm$ 7.07
Succinate-cyt <i>c</i> reductase (II+III)	0.71	0.70 $\pm$ 0.23
Cytochrome <i>c</i> oxidase (IV)	2.34	2.80 $\pm$ 0.52
Succinate dehydrogenase (II)	1.22	1.00 $\pm$ 0.53
Citrate synthase	9.39	9.88 $\pm$ 2.55

Activity expressed as μ moles/min/g. Controls are means $\pm$ standard deviations, n = 78. NADH, nicotinamide adenine dinucleotide-dehydrogenase. Cyt *c* reductase, cytochrome *c* reductase.