Two Novel Mitochondrial DNA Mutations in Muscle Tissue of a Patient With Limb-Girdle Myopathy

Ann Meulemans, MSc; Boel De Paepe, PhD; Jan De Bleecker, MD, PhD; Joël Smet, BSc; Willy Lissens, PhD; Rudy Van Coster, MD, PhD; Linda De Meirleir, MD, PhD; Sara Seneca, PhD

Author Affiliations: Center for Medical Genetics (Ms Meulemans and Drs Lissens and Seneca) and Department of Pediatric Neurology Universiteit Ziekenhuis Brussel (Dr De Meirleir), Vrije Universiteit Brussel, Brussels, and Departments of Neurology (Drs De Paepe and De Bleecker) and Pediatric Neurology and Metabolism Service (Drs De Paepe and Van Coster and Mr Smet), Ghent University Hospital, Ghent, Belgium.

Background: Defects in the oxidative phosphorylation system can cause a broad spectrum of clinical symptoms ranging from an isolated myopathy to a multisystemic disorder.

Objective: To study and identify the underlying molecular defect in a patient with limb-girdle myopathy.

Design: Biochemical, histochemical, and immunocytochemical analyses were performed in combination with polymerase chain reaction–single-strand conformation polymorphism and restriction fragment length polymorphism–polymerase chain reaction techniques.

Setting: University hospital.

Patient: A 48-year-old woman with limb-girdle myopathy.

Main Outcome Measures: The pathogenic characteristics of the identified nucleotide alterations were defined using single-muscle fiber analysis.

RESULTS: A complex III deficiency was detected using blue native–polyacrylamide gel electrophoresis, while immunocytochemical results showed a mosaic staining pattern for complexes I and IV. After molecular analyses, 2 novel heteroplasmic mitochondrial DNA (mtDNA) nucleotide aberrations, m.5888insA and m.14639A>G, were identified in muscle tissue. Single-muscle fiber analyses demonstrated that cytochrome c oxidase–deficient fibers, compared with cytochrome c oxidase–positive fibers, harbored statistically significantly higher levels of both mtDNA mutations (P < .001, t test).

Conclusions: These results, together with previously defined canonical criteria determining the pathogenic characteristics of mtDNA mutations, suggest that both nucleotide changes are pathogenic mutations. To our knowledge, this is only the third report of the coexistence of 2 pathogenic mtDNA mutations present in different genes within individual skeletal muscle fibers of a patient.

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REPORT OF A CASE

A 48-year-old woman had experienced a slowly progressive limb-girdle muscle weakness for several years. She had mild global facial weakness with mild bilateral ptosis and an asymmetric facial droop. Ocular movements were normal. The weakness most prominently affected the pelvic girdle and shoulder muscles. The patient walked unaided but could not rise from a squatting position and had difficulty climbing stairs. The shoulder girdle was less affected, with mild asymmetric scapular winging and weakness of the deltoid, triceps, and biceps muscles. The patient's daughter (aged 23 years) and brother (aged 43 years) had no notable medical problems. However, her mother (aged 69 years) had a small, set figure and a short neck. Four years previously, her mother had been clinically diagnosed as having multiple sclerosis with diffuse white matter disease as seen on brain magnetic resonance imaging. No muscle biopsy specimen was available for further study, but her mother's symptoms seemed unrelated to an mtDNA disorder.
METHODS

ENZYME ANALYSIS

A muscle biopsy of the left quadriceps femoris from the proband was performed for routine histochemical, immunocytochemical, spectrophotometric, and blue native–polyacrylamide gel electrophoresis (BN-PAGE) analyses as described previously. Following BN-PAGE, complex III activity was visualized using a blotting kit in stable peroxide substrate buffer (1-step TMB [tetramethylbenzidine]; Pierce Chemical, Rockford, Illinois). A green-blue precipitate was produced in the presence of TMB by the action of the peroxidase on the heme in complex III.4

Figure 1. Results of the histochemical, immunocytochemical, biochemical, and molecular analyses. A, Staining with Gomori trichrome showed some ragged-red fibers (a); there were succinate dehydrogenase–hyperstained fibers (b) and numerous cytochrome c oxidase (COX)–deficient fibers (c). Immunocytochemical staining revealed mosaic staining in a few muscle fibers for complex I (20-kDa subunits) (d) and for complex IV (subunit I) (f). In contrast, the results of staining for complex III (core2a) (e) were normal. B, Catalytic staining after blue native–polyacrylamide gel electrophoresis analysis of the patient’s (P) muscle tissue showed decreased complex III activity, while the activities of complexes I, IV, and V were normal compared with control (C) specimens. C, Sequencing electropherograms showing the m.14649A->G nucleotide alteration and the m.5888insA nucleotide aberration in the patient’s muscle tissue (a and b, respectively) and blood (c and d, respectively).
DNA ANALYSIS

Total DNA was prepared from leukocytes, muscle tissue, and cultured skin fibroblasts following standard procedures. Southern blotting techniques were used to investigate the presence of deletions or duplications in the mt genome. All mtDNA-encoded tRNA genes were investigated for nucleotide alterations using polymerase chain reaction–single-strand conformation polymorphism analysis. In case of an aberrant migration pattern, the corresponding gene was subjected to direct sequencing using a sequencing kit system (BigDye Terminator version 1.1 Cycle Sequencing kit on the ABI 3130xl genetic analyzer, Applied Biosystems, Lennik, Belgium) and was compared with the revised Cambridge reference sequence.5

To determine the level of heteroplasmy of identified nucleotide alterations, last hot cycle polymerase chain reaction–restriction fragment length polymorphism was performed as previously described.6 The appropriate primers and restriction enzymes were used.

Single-muscle fibers, stained for cytochrome c oxidase (COX) activity, were isolated using laser-capture microdissection (PixCell II laser-capture microscope, Arcturus, Mountain View, California) as previously described.7 Total DNA was isolated from these single-muscle fibers as previously reported.6 The percentage of mutant mtDNA was quantified using last hot cycle polymerase chain reaction–restriction fragment length polymorphism analysis. Both polymerase chain reactions, detecting m.5888insA or m.14639A>G, were performed separately on part of the lysate of each captured single fiber.

RESULTS

ENZYME ANALYSIS

Histochemical analysis revealed an abnormal variation in muscle fiber diameter. A mild type 1 fiber predominance was seen; few fibers were necrotic or regenerating. Numerous COX-deficient fibers were present, and most of them were also succinate dehydrogenase hyperstained. A few fibers were COX-deficient and succinate dehydrogenase hyperstained and showed ragged-red changes after Gomori trichrome staining.

Immunocytochemistry showed a mosaic staining pattern in about 20% of the muscle fibers for complex I (20-kDa subunits) and for complex IV (subunit I) (Figure 1 A). Results of staining for complexes II, III, and V were normal.

Spectrophotometric analysis of the skeletal muscle tissue of the patient revealed activities of the mt respiratory chain enzymes within the control ranges. However, activity staining following BN-PAGE showed decreased complex III activity and the presence of complex V subcomplexes (Figure 1B).

DNA ANALYSIS

Southern blotting analysis did not detect large deletions or duplications. However, analyses of the mtDNA-encoded tRNAs revealed the following 2 novel and heteroplasmic mtDNA aberrations in the patient’s muscle tissue: an adenine nucleotide insertion, m.5888insA, located in the tRNA^Tyr gene, and a nucleotide alteration, m.14639A>G, which changes a leucine amino acid into a serine residue in the ND6 gene. Less than 1% of both mutant alterations were present in the patient’s fibroblasts, and they were undetectable in the patient’s blood or in blood and fibroblasts from the patient’s mother. Both mutations were absent in more than 100 healthy control subjects.

Single-muscle fiber analysis showed a direct correlation between the levels of mutant mtDNA and impairment of COX function for both nucleotide alterations, but the contrast between COX-positive and COX-deficient fibers was more pronounced for the ND6 gene mutation (Figure 2). The COX-positive muscle fibers
uncharged, unlike the other amino residues at that position (phenylalanine [F], isoleucine [I], leucine [L], serine [S], and valine [V]).

Although it is not a conserved amino acid, the m.14639A>G mutation leads to a seriously altered amino residue (serine [S]) that is smaller, polar, and uncharged, unlike the other amino residues at that position (phenylalanine [F], isoleucine [I], leucine [L], serine [S], and valine [V]).

Table. Overview of the Pathogenicity Scoring System for Mitochondrial DNA-Encoded Transfer RNA (tRNA) and Complex I Mutations

<table>
<thead>
<tr>
<th>Criterion</th>
<th>m.5888insA</th>
<th>m.14639A&gt;G</th>
</tr>
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<tbody>
<tr>
<td>Biochemical defect</td>
<td>Only in muscle 2/2</td>
<td>Only in muscle 8/10</td>
</tr>
<tr>
<td>Functional studies (single-fiber analysis)</td>
<td>3/3</td>
<td>7/7p</td>
</tr>
<tr>
<td>Previously reported</td>
<td>0/2</td>
<td>0/5</td>
</tr>
<tr>
<td>Heteroplasmy</td>
<td>2/2</td>
<td>5/5</td>
</tr>
<tr>
<td>Segregation of variant with disease within a family</td>
<td>7/2b</td>
<td>7/3b</td>
</tr>
<tr>
<td>Evolutionary conservation of the base</td>
<td>2/2</td>
<td>3/10</td>
</tr>
<tr>
<td>Histochemical evidence</td>
<td>2/2</td>
<td>...</td>
</tr>
<tr>
<td>Total Score</td>
<td>11/15</td>
<td>23/40</td>
</tr>
</tbody>
</table>

a Data are reported as points.
b No muscle biopsy sample of the mother, daughter, or any other maternally related individual was available.
c Only a criterion for mitochondrial tRNA mutations.

We describe a patient with proximal myopathy in whom 2 novel mutations, m.5888insA and m.14639A>G, were identified in the mt genome; both mutations were heteroplasmic and were present in low levels, 27% and 33%, respectively, in homogenate muscle tissue of the patient. One mutation was located close to the 5' end in the conserved aminoacyl acceptor stem of the mt tRNA^Tyr gene, where an extra adenine was inserted in a stretch of 4 adenines located at positions 5885 through 5888 (Figure 3A). A mutation at this position may affect the stability of the tRNA and the overall efficiency of protein translation, eventually resulting in a defect of OXPHOS function. So far, only 2 pathogenic mutations have been described in the mt tRNA^Tyr gene, to our knowledge. The first mutation, the heteroplasmic m.5874A>G mutation, was present only in the muscle tissue of a patient who experienced exercise intolerance, limb weakness, and isolated complex III deficiency.1 The second mutation was reported in a patient with a heteroplasmic nucleotide deletion, m.5885delA, located in the same adenine stretch as in our patient.10 Again, this single-nucleotide deletion was detected only in muscle tissue. This patient was initially seen with myopathy, exercise intolerance, and chronic progressive external ophthalmoplegia. Muscle biopsy results revealed similar histochemical findings as in our patient. However, the biochemical results were different. Using blue native–polyacrylamide gel electrophoresis, we detected only an isolated complex III deficiency, while the other patient showed a combined deficiency of complexes I, II + III, and IV. This might be explained by our patient's pathogenic mutation load being below the threshold level, causing general deficiency in complexes I through IV. The fact that immunocytochemical staining revealed mosaic patterns for complexes I and IV in only a few of the muscle fibers supports this hypothesis.

The second heteroplasmic m.14639A>G mutation in our patient was located in the ND6 gene. In this gene, encoding a subunit of complex I, more than 10 pathogenic mutations have been described.11 Indeed, defects in mtDNA-encoded complex I genes are increasingly being recognized as important causes of OXPHOS disease, but most have been identified in association with Leber hereditary optic neuropathy.2 Although this nucleotide change is not located in a conserved region of the ND6 protein, a large nonpolar and hydrophobic leucine residue at position 12 is substituted for a small polar and uncharged serine residue, thereby changing side-chain hydrophobicity and volume. In other mammalian species...
In our experience, the finding of a combined OXPHOS deficiency, especially of complexes I and IV, together with a mosaic pattern following immunohistochemical and immunocytochemical staining, as well as the presence of sub-complexes of complex V on BN-PAGE, presents a strong argument in favor of an mtDNA defect. Given the polymorphic nature of mtDNA, it is difficult to determine the pathological significance of mt nucleotide changes. However, based on previously defined canonical criteria, specific scoring systems for determining the pathogenic characteristics of a given sequence variant in mtDNA have been developed, one for mutations in 1 of 7 mtDNA-encoded complex I genes and another for mt tRNA mutations. In both scoring systems, the use of functional studies to provide evidence regarding pathogenicity is considered important. In our patient, both mutations were present only in muscle tissue, so single-muscle fiber analysis was used to address the levels of heteroplasmy of the mt tRNA\(^{\text{TSV}}\) and ND6 gene mutations independently in the same single-fiber lysate. Results of this functional study show a strong correlation between abundance of ragged-red fibers, COX deficiency, and mutant mtDNA for both mutations. Because the 2 mtDNA mutations were present in almost equal amounts in COX-deficient fibers, these findings do not allow us to evaluate the functional consequences of the mt tRNA\(^{\text{TSV}}\) or ND6 gene mutation separately or to demonstrate the importance and effect of one mutation over the other. Both mtDNA mutations were distributed in almost equally high amounts in COX-deficient cells, although 5 of 12 COX-positive cells harbored apparently low amounts (0%-3%) of mutant m.14639A>G mtDNA compared with the amount of the m.5888insA mutation. This is consistent with the absence of both mtDNA mutations on each mtDNA molecule. Considering these results with the other criteria for pathogenic mtDNA mutations, the m.5888insA and m.14639A>G nucleotide aberrations can both be considered novel pathogenic mutations (Table). The presence of more than 1 pathogenic mtDNA mutation is not often encountered and, to our knowledge, has been reported only twice. The first report describes a patient harboring an m.3243A>G tRNA\(^{\text{Glu}}\) gene point mutation in muscle mtDNA and a single-deletion mutation, while the second report describes the spontaneous generation of a suppressor of the m.3243A>G tRNA\(^{\text{Glu}}\) gene mutation in an in vitro hybrid culture.

In conclusion, our findings of severe COX deficiency and high levels of the mt tRNA\(^{\text{TSV}}\) and ND6 gene mutations within single-fiber lysates are consistent with the implication of both mutations in OXPHOS impairment in our patient. The 2 mutations may act in synergy. Future reports of patients with the presence of only 1 of these mtDNA mutations might shed more light on the relative pathogenic characteristics of the individual nucleotide changes.

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Correspondence: Sara Seneca, PhD, Center for Medical Genetics, Vrije Universiteit Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium (lgenesas@az.vub.ac.be).


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REFERENCES