A Novel Mitochondrial Transfer RNA<sup>Asn</sup> Mutation Causing Multiorgan Failure

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**Background:** Mitochondrial cytopathies are a heterogeneous group of disorders with a broad spectrum of clinical symptoms.

**Objective:** To characterize a novel mutation in the transfer RNA<sup>Asn</sup> (m.5728A>G) identified in a 13-year-old boy with multiorgan failure.

**Design:** Biochemical and immunocytochemical studies were performed in combination with transmitochondrial cybrid analysis.

**Setting:** A university hospital. Molecular and biochemical analyses were performed in collaboration between 2 other university hospitals.

**Patient:** Thirteen-year-old boy with multiorgan failure.

**Results:** In the patient's muscle tissue and cultured skin fibroblasts, a combined deficiency of complexes I and IV was found using spectrophotometric analysis and activity staining in the gel following blue native polyacrylamide gel electrophoresis. An identical biochemical profile was seen in transmitochondrial cybrids carrying more than 55% mutant mitochondrial DNA.

**Conclusion:** These data suggest that the m.5728A>G transition is a pathogenic mutation and is the cause of the respiratory chain dysfunction in the propositus.

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**PATIENTS WITH MITOCHONDRIAL ENCEPHALOMYOPATHIES**

Patients with mitochondrial encephalomyopathies are a heterogeneous group with symptoms starting at any age and often including neurological and muscular dysfunctions that can be complicated with cardiac, renal, hepatic, or endocrine involvement. For the synthesis of proteins, mitochondria are dependent on the nuclear and mitochondrial genomes. Therefore, mutations causing mitochondrial disease can be located in the nuclear genome and in the mitochondrial DNA (mtDNA). Today, more than 118 different pathogenic mtDNA point mutations have been identified, and new mutations are being reported. Although the 22 mitochondrial transfer RNA (tRNA) genes represent only a small part of the mitochondrial genome, mutations in these genes are often responsible for mtDNA disease. We studied the molecular pathogenesis of a novel tRNA gene mutation using immunocytochemical, spectrophotometric, blue native polyacrylamide gel electrophoresis (BN-PAGE), and transmitochondrial cybrid cell techniques.

**REPORT OF A CASE**

The patient, a boy born in 1993, was initially seen at the age of 2 years with acute prerenal insufficiency, dehydration, and failure to thrive. He had proteinuria, and focal glomerulosclerosis was diagnosed by renal biopsy. Since birth, the patient was below the third percentile for length and weight, but it was only at the age of 5 years that a growth hormone insufficiency was detected and treated with growth hormone supplementation. Because of mental retardation, he was directed toward special schooling.

At the age of 10 years, he was admitted to the hospital because of progressive ataxia, weakness in distal and proximal muscle groups, regression in his mental status, and frequent generalized seizures. Tendon reflexes were absent because of a myopathy. Plasma and cerebrospinal fluid lactic acid levels were elevated at 34.8 mg/dL (3.86 mmol/L) (reference level, <18.0 mg/dL [<2.0 mmol/L]) and 31.0 mg/dL (3.44 mmol/L) (reference level, <19.8 mg/dL [2.2 mmol/L]), re-
spectively. Histologic examination revealed numerous ragged red fibers after Gomori staining. Cytochrome-c-oxidase–negative fibers were seen after cytochrome-c-oxidase staining. Electron microscopy showed accumulation of subsarcolemmal granularity in some of the muscle fibers. A computed tomographic scan of the brain revealed basal ganglia calcifications. Magnetic resonance imaging showed cerebral and cerebellar atrophy. An electroencephalogram showed continuous generalized epileptic activity. The echocardiogram was normal. There was no family history of neurological disorders or features of other typical mitochondrial disorders.

### METHODS

#### BIOCHEMICAL ASSAYS

Spectrophotometric studies in muscle tissue and in cultured skin fibroblasts from the patient were carried out by means of normalization of the activities relative to citrate synthase as previously described.1,2 Isolation of mitochondria, solubilization of oxidative phosphorylation complexes, BN-PAGE, and catalytic staining in BN-PAGE gels were performed as previously reported.9

#### IMMUNOCYTOCHEMISTRY AND MOLECULAR GENETICS

Immunocytochemistry was performed in fibroblasts as previously described.10 Total DNA was prepared from leukocytes, muscle tissue, and cultured skin fibroblasts using standard techniques.

Classic mtDNA mutations such as deletions, duplications, and the NARP 8993 mutation were screened using Southern blotting techniques, while denaturing gradient gel electrophoresis was used to analyze the tRNA⁰⁰ and tRNA⁰⁰⁰ genes for nucleotide alterations. The other 20 tRNA genes were screened for nucleotide alterations using polymerase chain reaction single-stranded conformational polymorphism analysis,11 and fragments with aberrant migration patterns were subjected to direct sequencing using the BigDye Terminator version 1.1 Cycle Sequencing kit on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, Calif).

#### CELL CULTURES

Human skin fibroblasts were cultured in medium 199 with Earle salt and L-glutamine (A & E Scientific, Marcq, Belgium) supplemented with 20% fetal bovine serum and antibiotics. Transmitochondrial cybrids were obtained by fusion of enucleated fibroblasts from the patient using rho 0 cells as previously described.12

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### RESULTS

A combined deficiency of complexes I and IV was detected in muscle and in cultured skin fibroblasts from the propositus using spectrophotometric analysis (Table). In mitochondria isolated from muscle tissue, BN-PAGE followed by activity staining in the gel showed a decreased intensity in the band for complex I and a minor decrease in the band for complex IV. The intensities of the bands corresponding to complexes III and V were normal, but the band corresponding to complex II was increased compared with the control sample (Figure 1A). The same technique applied to cultured skin fibroblasts revealed low to normal intensities of the bands corresponding to complexes I and IV, while the bands corresponding to complexes II and V were comparable to those of the control. Immunocytochemical staining of the patient’s fibroblasts using a specific antibody against complex I (20-kDa subunit) showed a mosaic pattern (Figure 1B). Negative cells were observed alongside normally staining fibroblasts. Immunocytochemical staining for complex II (lp subunit), complex III (core 2), complex IV (1 subunit), and complex V (α subunit) demonstrated normal patterns. Routine analysis identified no classic mutations, deletion or duplication. However, a thorough investigation of the tRNA genes identified a novel A→G transition at nucleotide position 5728 in the tRNA⁰⁰⁰ gene of muscle mtDNA of the patient. Mutation loads in the patient’s blood, muscle tissue, and fibroblasts were found to be 50%, 97%, and 50%, respectively. No mutation was detected in the blood of the patient’s healthy mother, 2 siblings, or maternal grandmother. Unfortunately, muscle tissue from the mother and the siblings was unavailable for analysis. The mutation was also absent in more than 100 healthy control samples and in more than 100 patients suspected of having a mitochondrial encephalomyopathy.

To study the effect of the m.5728A→G mutation on oxidative phosphorylation, transmitochondrial cells were created. Homoplasmic mutant cybrid cell lines were undetected during evaluation. Five cybrid lines were selected for further studies. Four heteroplasmic cell lines harbored mutant mtDNA (86%, 82%, 75%, and 55%), while the fifth cell line contained 100% wild-type mtDNA. The fifth cell line was used as a control cell line in our

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**Table. Respiratory Chain Activities in Different Tissues Using Spectrophotometry**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Complex I/CS</th>
<th>Complex II/CS</th>
<th>Complex II + III/CS</th>
<th>Complex III/CS</th>
<th>Complex IV/CS</th>
<th>CS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>0.44</td>
<td>0.67</td>
<td>0.65</td>
<td>0.75</td>
<td>0.77</td>
<td>458</td>
</tr>
<tr>
<td>Control samples (n = 30)</td>
<td>0.60 ± 0.05</td>
<td>0.71 ± 0.06</td>
<td>0.69 ± 0.07</td>
<td>0.74 ± 0.10</td>
<td>0.90 ± 0.06</td>
<td>214 ± 61</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>0.28</td>
<td>0.59</td>
<td>0.70</td>
<td>0.58</td>
<td>0.81</td>
<td>133</td>
</tr>
<tr>
<td>Control samples (n = 30)</td>
<td>0.49 ± 0.07</td>
<td>0.64 ± 0.06</td>
<td>0.73 ± 0.09</td>
<td>0.72 ± 0.09</td>
<td>0.92 ± 0.04</td>
<td>123 ± 40</td>
</tr>
</tbody>
</table>

Abbreviation: CS, citrate synthase.

*Specific activity is expressed as nanomoles of substrate per minute per milligram of protein. All other data are expressed as the logarithm of respiratory chain complex activity divided by the logarithm of citrate synthase activity. Control sample ratios are given as mean ± SD.
studies. A decrease of the complex I and IV bands following activity staining in the BN-PAGE gel was only detected in the cybrid lines with at least 75% mutant mtDNA. The cybrids with 55% mutant mtDNA and with 100% wild-type mtDNA had normal intensities compared with the control (Figure 1A). Immunocytochemical staining revealed a mosaic pattern for complex I in fusion cells with at least 75% mutant mtDNA, while in the other 2 cybrid lines a normal staining pattern was found (Figure 1B).

**COMMENT**

In a 13-year-old boy with multiorgan failure, a novel point mutation was identified changing an A>G at position 5728 in the tRNA\textsubscript{Asn} gene. Spectrophotometric assays and activity staining in BN-PAGE gels revealed a decrease of complex I and IV activity in muscle tissue and in cultured skin fibroblasts. Immunocytochemical staining in fibroblasts showed a mosaic pattern for complex I.

Although some of the mitochondrial tRNA genes (eg, tRNA\textsubscript{Gly}, tRNA\textsubscript{Leu(UUR)}, and tRNA\textsubscript{Asn}) are known to be mutational hotspots, so far only 3 heteroplasmic mutations in the tRNA\textsubscript{Asn} gene have been described, to our knowledge. All 3 mutations (m.5692T>C, m.5698G>A, and m.5703G>A) are clinically associated with chronic progressive external ophthalmoplegia and with mitochondrial myopathy. The nucleotide change seen in our patient is the second base of the amino acid stem of the tRNA\textsubscript{Asn} gene (Figure 2). A conserved adenine is changed into a guanine, leading to a disturbed Watson-Crick base pairing (G-T). Only 3 (cat, baboon, and orangutan) of 31 other mammalian species have a guanine at position 5728. Although pathogenic mutations most often affect highly conserved nucleotides, exceptions have been reported. In muscle tissue from the propositus, almost 100% of the mtDNA was mutated, while in blood and in fibroblasts the mutation load was only 50%. The mutation was undetected in the blood of the proband’s healthy mother, 2 siblings, or grandmother. Therefore, most likely the mutation arose de novo.
novel in the germline of the developing embryo. Moreover, the mutation was undetected in more than 100 healthy controls. Unfortunately, blood only (no muscle) from the mother was available for analysis. In her blood, the mutation was undetected, but it is possible that traces of mutant mtDNA remained undetected using polymerase chain reaction single-stranded conformational polymorphism analysis.

Determination of the pathologic significance of novel variants is of great importance to the family concerned. For this reason, transmitochondrial cybrids were created, and 5 colonies were selected for further biochemical and immunocytochemical studies. One of the colonies was homoplasmic for wild-type mtDNA, whereas the other 4 colonies contained different percentages of mutated mtDNA (86%, 82%, 75%, and 55%). Blue native polyacrylamide gel electrophoresis followed by activity staining in the gel revealed a decrease of complexes I and IV in cybrid cells with at least 75% mutant mtDNA. In the homoplasmic wild-type cell line and the cell line with 55% mutated mtDNA, normal signals for all the complexes were detected. The mosaic pattern observed after immunocytochemical staining in cultured skin fibroblasts from the propositus was also seen in the cybrid cell lines carrying at least 75% mutant mtDNA, whereas the homoplasmic wild-type cells and the cell lines with only 55% mutated mtDNA showed normal staining.

Based on our biochemical results and in agreement with previously published criteria, the m.5728A>G mutation qualifies as being pathogenic. It is a heteroplasmic A>G transition, with a high level of affected mtDNA in muscle tissue and with only 50% mutant mtDNA in blood and in fibroblasts. Neither the patient’s healthy mother, both healthy siblings, nor the maternal grandmother carried the mutation. The mutation is located in a conserved region of the acceptor stem of the tRNA<sup>Asn</sup> gene and was absent in healthy controls. Biochemical defects were only detected in cybrids with a mutation load of at least 75%.

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