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A Novel Mitochondrial tRNA^{Phe} Mutation Inhibiting Anticodon Stem Formation Associated with a Muscle Disease

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We have identified a novel mitochondrial (mt) DNA mutation in the tRNA^{Phe}-gene in a patient with an isolated mitochondrial myopathy. This T to C transition at position 618 disrupts a strictly conserved base pair within the anticodon stem of tRNA^{Phe}. Computer analysis showed that the affected base pair is essential for anticodon stem formation of tRNA^{Phe}. The mutant mtDNA was heteroplasmic in skeletal muscle (95% mutant) and peripheral blood cells (20% mutant) from the patient but was undetectable in blood cells from his healthy sister. The patient presented with ragged red fibers and reduced activities of complex I and complex III in skeletal muscle. The T618C mutation described here is the second found in this region. Both mutations affect the same base pair of the tRNA^{Phe} anticodon stem substantiating the pathogenic nature of both mutations.

A number of human diseases have been associated with mtDNA mutations, such as large-scale rearrangements or point mutations within either protein-coding-genes or more commonly, tRNA-genes. Mitochondrial disorders present with a spectrum of neuromuscular and non-neuromuscular symptoms (for review see 1, 2), and several clinical entities have been defined and associated with specific mtDNA mutations, such as Kearns-Sayre syndrome (KSS) (3), mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (4), myoclonus epilepsy with ragged red fibers (MERRF) (5), or myoclonus epilepsy, deafness, ataxia, cognitive impairment, and COX deficiency (6). However, perfect taxonomy is difficult and the same pathogenic mtDNA mutation may give rise to different phenotypes. Tissue-specific accumulation and additional nuclear and mtDNA mutations were discussed to influence the clinical presentation (4).

Myopathy is a common syndromic or isolated symptom of mt disorders, and patients may suffer from general muscle weakness, or symptoms may be very confined as in patients with chronic progressive external ophthalmoplegia (CPEO) (3).

We report on a patient with a novel point mutation in the tRNA^{Phe}-gene who presented with general muscle weakness and pain, ragged red fibers, and respiratory chain deficiencies in skeletal muscle.

MATERIALS AND METHODS

Case history. The family of the patient originates from Western Turkey. The patient, a 36-year-old male with asthenic build, has been under medical observation for muscle weakness and pain for 10 years. At the age of 30 years he had suffered from repeated bronchial infections, and a syncope with fall and consecutive commotio cerebri. At the age of 35 years he was hospitalized with heavy thoracal pain, exercise-induced dyspnea, and vertigo. The actual presentation was due to fatigue, easy exhaustion, general mild muscle weakness and pain, especially in arms and thorax. Severe metabolic acidosis was diagnosed with an acute serum lactate of 14.1 mM (normal resting value 0.63-2.44 mM) that decreased at 6.55 and 4.33 mM the following days. Glucose concentrations in serum and cerebrospinal fluid were normal. Serum creatin kinase activity was not elevated. On examination general strength was reduced (score 4, active movement against resistance), muscle bulk was slightly decreased, Gowers' sign was positive, tendon reflexes were present and symmetrical, and sensibility was intact. His gait was unaffected. He had no signs of polyneuropathy, ataxia, or chronic progressive external ophthalmoplegia and ocular fundi were normal. Electromyography (EMG) of several limb muscles did not show significant alterations. Electrocardiography and electroencephalography detected no abnormality. Computer tomography and magnetic-resonance-imaging (MRI) showed a mild diffuse atrophy but otherwise no alterations of the brain. The patient's muscle weakness is gradually progressive. A skeletal muscle biopsy from the quadriceps femoris and a blood sample were obtained. The patient's mother died during the birth of the third child at the age of 21 years and was described as asthenic with general muscle weakness. The third child died at the age of 8 months due to unidentified reasons. The older sister of the patient is apparently healthy. A blood sample was obtained for analysis of the mt genome. The patient has two healthy children.

Morphological, histological, and biochemical studies. Morphological and histological investigations were performed according to stan-

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dard procedures. Activities of the enzyme complexes of the respiratory chain were determined spectrophotometrically as described previously (7).

Genetic analysis. Total DNA was extracted from peripheral blood cells and skeletal muscle via spin columns (Qiagen). Large-scale rearrangements were excluded by long polymerase chain reaction (PCR) (8). Conventional PCR amplification was performed as described previously (8). Restriction fragment length polymorphism (RFLP)-analysis was used to rule out the nt 8993 mtDNA mutations. The mtDNA regions encompassing the 22 tRNA genes were PCR-amplified using the following primer pairs corresponding to the Cambridge sequence (9): FA (516-534), FB (768-749), VA (1522-1541), VB (1782-1763), LA (3149-3168), LB (3372-3353), IA (4218-4237), IB (4516-4497), W1A (5451-5469), W1B (5736-5717), W2A (5677-5697), W2B (5950-5931), SA (7177-7199), SB (7830-7810), KA (8167-8186), KB (8498-8479), GA (9931-9950), RB (10553-10534), HA (12010-12028), HB (12412-12394), EA (14599-14618), EB (14922-14904), TA (15787-15806), TB (16148-16127). Single strand conformation polymorphism (SSCP) analysis of the amplified PCR products was performed on native 12% polyacrylamide gels (12% acrylamide/piperazine-diacrylamide (84:1), 7% glycerol) using a two-buffer system. Direct sequencing of the PCR products was performed on an ABI 377 DNA Sequencer (Applied Biosystems, PE) using the ABI PRISM BigDye Terminator Cycle Sequencing Kit.

For haplogroup affiliation primer pair D1 (16110-16129), D2 (261-242) was used for PCR amplification and direct sequencing. Testing of the specific branching positions (7028, 15904, 12705, 12308/12372, 4216, 10398) was performed as described (10).

For diagnostic analysis of the T618C mutation, the mutated mtDNA region was amplified by primer pair FA, FB, digested using *Dde*I, separated on a 4% agarose gel (NuSieve, FMC), and visualized by ethidium-bromide staining.

Quantification of mutated mtDNA. Mutated DNA was quantified by fluorescence-based detection on an ABI 377 DNA Sequencer (ABI, PE). PCR amplifications were performed as for diagnostic analysis with the exception of using primer FA labeled with 6FAM. The amplified products were digested with *DdeI* overnight. Serial dilutions were resolved on a 4% denaturing polyacrylamide gel and analyzed with the GeneScan computer program (ABI, PE).

Computer analysis. Folding of the mutant $tRNA^{Phe}$ was analyzed with the Zuker algorithm (11) of the mfold program (GCG of the University of Wisconsin).

RESULTS

Histological and ultrastructural examinations. Histological analysis of the muscle biopsy showed significant subsarcolemmal mitochondrial accumulations, which appeared as typical ragged red fibers. No abnormal lipid storage or abnormal glycogen pattern was found. There were only little signs of myopathic changes, such as few internalized nuclei. There was no evidence for neurogenic lesions, as e.g. fiber type grouping, fiber atrophy, or target fibers. Electron microscopy showed aggregated subsarcolemmal mitochondria which presented with typical intramitochondrial paracrystalline type I inclusions located in the intracristal space.

Biochemical studies. Analysis of respiratory chain was performed using frozen skeletal muscle. Citrate synthase (CS) activity was elevated, compatible with mitochondrial proliferation. The activity of complex I was markedly decreased at 20% of the lowest reference value when referred to muscle weight whereas complex III was only slightly reduced. The activities of complex I and complex III were reduced to 10% and 45%, respectively of the lowest reference value when related to the activity of CS.

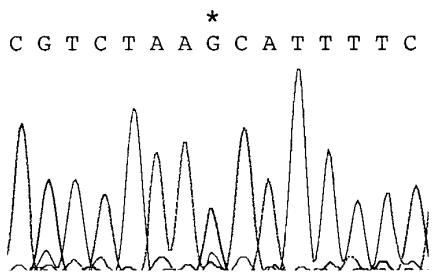

FIG. 1. Sequence chromatogram of the region encompassing the T618C mutation (asterisk) from skeletal muscle of the patient (reverse complement).

Genetic analysis. The clinical features, and the histological and biochemical results from the patient's muscle biopsy supported the diagnosis of a mt myopathy and prompted us to analyze the mitochondrial genome. Large-scale rearrangements of mtDNA were ruled out using long PCR (8). We screened all 22 mitochondrial tRNA genes using modified SSCP analysis followed by direct sequencing of polymorphic SSCP-fragments. For haplogroup affiliation the D-loop region nt 16110-261 was sequenced and tested for specific branching positions of the mtDNA European tree (10). We detected the following polymorphic changes from the reference Cambridge sequence (9): T58C, C64T, T146C, T196C, G513A, 522CAdel, C7028T, T16136C, and C16355T. These polymorphic changes are compatible with haplogroup D^{7028} (10) and with haplogroup 1 (12) that was defined as the most frequent haplogroup in Western Turkey with a frequency of 55% (12). The patient also carried a non-described G to A mutation in the non-coding region V at position nt 8292 which was supposed to be a polymorphism. All these mutations were apparently homoplasmic. In addition, we detected a novel point mutation T to C at nt 618 in the mitochondrial $tRNA^{Phe}$-gene (Fig. 1), which changes a nucleotide in the anticodon stem disrupting a strictly conserved base pair (Fig. 2). We used the Zuker algorithm for RNA folding to analyze the structure of the mutated $tRNA^{Phe}$. According to the computer analysis the mutated $tRNA^{Phe}$ does not form a stable anticodon stem. The mutation was present in heteroplasmic state in the patient's skeletal muscle and blood but was not detected in blood from the healthy sister (Fig. 3). The T618C mutation was quantified by RFLP analysis using a fluorescent primer for PCR. The proportion of mutant mtDNA was 95% in skeletal muscle and 20% in blood. We did not detect any mutant mtDNA in blood from the sister even with sensitive fluores-

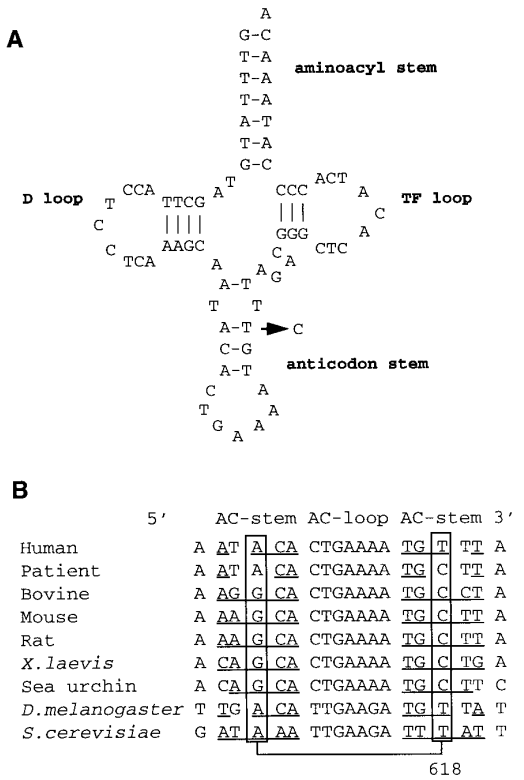

FIG. 2. (A) Proposed secondary structure of the human mitochondrial tRNA^{Phe} and (**B**) comparison of mitochondrial tRNA^{Phe} among different species according to Sprinzl et al. (13). Base pairs are underlined. The T618C mutation is indicated.

cence analysis. The mutation was not reported in literature in normal controls and was absent in 140 unrelated controls, in 20 patients with mitochondrial disease, and in 20 patients suffering from myopathy of unidentified origin.

DISCUSSION

We have identified a novel point mutation which probably inhibits anticodon stem formation of mitochondrial tRNA^{Phe}. The patient presented with a mitochondrial myopathy characterized by general muscle weakness and pain. Morphologically, there were numerous ragged red fibers and ultrastructural mitochondrial abnormalities, and biochemically, we found decreased activities of complex I and III.

The following features substantiate the pathogenic nature of the T618C mutation. It was absent in 140 unrelated controls, 20 patients with mitochondrial disease, and 20 patients suffering from myopathy of unidentified origin. The mutation disrupts a strictly conserved base pair in the anticodon stem of tRNA^{Phe}, suggesting that the loss may be deleterious. In contrast to other species human mt tRNA^{Phe} harbors non-pairing bases in the anticodon stem which create a bulge (Fig. 2). Computer analysis of mutant tRNA^{Phe} showed that the T618C mutation that is located next to the anticodon bulge destabilizes and destroys anticodon stem formation. Thus, the T618C mutation probably severely affects secondary and tertiary structure of tRNA^{Phe} and is likely to destroy its function.

In addition, the mutation was associated with clinical symptoms and—though this is not obligatory—it was heteroplasmic. It was present in high proportion in the affected tissue skeletal muscle (95%) and in lower proportion in blood (20%) (Fig. 3). This is in accordance with other pathogenic mutations which present with substantially lower degree of heteroplasmy in rapid-turnover tissue such as blood (14).

The patient's clinical symptoms were confined to skeletal muscle, suggesting that skeletal muscle was the only tissue carrying a proportion of mutant mtDNA above the pathogenic threshold level. This underlines the recessive nature of the tRNA^{Phe} mutation.

The origin of the mutation in the family remains unclear. The patient's healthy sister did not carry any detectable mutant mtDNA in blood. The patient's mother was described to be of weak constitution like the patient but no tissue was available for genetic analysis. The mother may have carried the T618C mutation or the mutation may have arisen spontaneously in the patient's maternal germline or during his early embryogenesis.

Recently, Chinnery et al. (15) described a patient with acute rhabdomyolysis and myoglobinuria who carried a mutation in the tRNA^{Phe}-gene. This A606G mutation affects the same base pair within the anticodon stem as the T618C mutation detected in our patient. The fact that both mutations which were found in two unrelated cases affect the same base pair of tRNA^{Phe}, further affirms the pathogenic role of both tRNA^{Phe} mutations. The A606G mutation changes the Watson-Crick A-U base pair into a weak G-U non-Watson-Crick pair that still allows anticodon stem formation. This

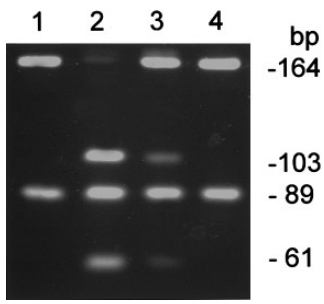

FIG. 3. PCR-RFLP analysis of the T618C mutation. A 253 bp fragment of a normal control is cut into a 164 and 89 bp fragment. In the presence of the T618C mutation the 164 bp fragment is cut into a 103 and 61 bp fragment. Control (lane 1), skeletal muscle (lane 2) and blood (lane 3) from the patient, and blood from the patient's sister (lane 4).

suggests that it may be less deleterious to tRNA structure than the T618C mutation. Functional studies on mutant mt tRNAs have shown that mutations may change tertiary structure and modification of the tRNA (16, 17) leading to a marked reduction in tRNA steady-state levels, impaired aminoacylation, and processing (16, 18, 19), thereby affecting mt protein synthesis.

A number of mt tRNA point mutations have been associated with isolated mt myopathy, such as A3243G (4), T3250C (20), A3302G (21), G7497A (6), A12320G (22), or G15990A (23). Functional molecular and biochemical studies on mutant $tRNA^{Phe}$ have to be performed to pin down the exact pathogenic mechanism of the $tRNA^{Phe}$ mutation and to understand tissue-specific accumulation and expression.

The case described here further underlines the role of mt tRNA mutations as a possible cause of mt myopathies and illustrates that as a result of thorough clinical, biochemical, and genetic investigations virtually all mt tRNA genes emerge as candidate genes for pathogenic mtDNA mutations.

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