Mutations in mitochondrial tRNA genes: a frequent cause of neuromuscular diseases

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ABSTRACT

We have sequenced the tRNA genes of mtDNA from patients with chronic progressive external ophthalmoplegia (CPEO) without detectable mtDNA deletions. Four point mutations were identified, located within highly conserved regions of mitochondrial tRNA genes, namely $tRNA^{Leu(UAG)}$, $tRNA^{Ser(GCU)}$, $tRNA^{Gly}$ and $tRNA^{Lys}$. One of these mutations ($tRNA^{Leu(UAG)}$) was found in four patients with different forms of mitochondrial myopathy. An accumulation of three different tRNA point mutations ($tRNA^{Leu(UAG)}$, $tRNA^{Ser(GCU)}$ and $tRNA^{Gly}$) was observed in a single patient, suggesting that mitochondrial tRNA genes represent hotspots for point mutations causing neuromuscular diseases.

INTRODUCTION

Since the discovery of deleted mitochondrial DNA in patients with mitochondrial diseases by Holt *et al.* (1), many studies confirmed these results in most cases of KSS (Kearns-Sayre-syndrome)(2-5), the Pearsons syndrome (6), some cases of CPEO (chronic progressive external ophthalmoplegia)(7-9) and in the brain of patients with Parkinsonism (10). A recombinational event was suggested to cause these deletions (9,11,12), based on the frequent occurence of direct repeats in human mtDNA (11). The inheritance of these deletions is unknown since most cases with KSS are sporadic (14). In contrast a maternal inheritance was demonstrated for other mitochondrial diseases: LHON (Leber's Hereditary Optic Neuropathy) (15), MERRF (Myoclonic Epilepsy with Ragged Red Fibers disease) (16) and MELAS (Mitochondrial myopathy, Encephalopathy, Lactic Acidosis and Stroke-like episodes) (18,19). In fact, a point mutation in the NADH dehydrogenase subunit 4 gene (nt 11778) was identified and related to LHON (20), whereas a point mutation in the mitochondrial gene for $tRNA^{Lys}$ (nt 8344) was linked to MERRF disease (21), and a point mutation at nucleotide pair 8993 in subunit 6 of mitochondrial ATPase was associated to a new mitochondrial disease (22). The molecular basis of CPEO without mtDNA deletions, however, is still unknown. We have sequenced the tRNA genes of mtDNA from muscle biopsy of a patient with CIPO (Chronic Intestinal Pseudoobstruction with myopathy and ophthalmoplegia) (23) and a patient with CPEO. One new mutation in the $tRNA^{Leu(UAG)}$ (nt 12308, A to G) was identified in both patients, whereas two additional new mutations in the $tRNA^{Ser(GCU)}$ (nt 12246, C to A) and $tRNA^{Gly}$ (nt 10006, A to G) were found in the patient with CIPO only.

EXPERIMENTAL

DNA preparation: Genomic DNA used for PCR amplification was extracted and purified from skeletal muscle biopsies as described by Wallace *et al.* (16).

DNA sequencing: The tRNA genes were sequenced directly using asymmetrically amplified mtDNA templates, oligonucleotides (Applied Biosystems 391 DNA synthesizer) and T7 DNA Polymerase (Pharmacia).The asymmetrically amplified DNA (ssDNA) was prepared according to the amplification protocol of Perkin-Elmer/Cetus from 19 overlapping double stranded PCR fragments encompassing all mitochondrial tRNA genes with the following nucleotide coordinates (24): 247-1677, 1562-4508, 3598-4508, 3598-5918, 4831-6263, 5317-7608, 6795-8022, 7392-10107, 7851-8921, 9628-10728, 9911-11873, 9911-12576, 11711-12576, 11711-14208, 13914-15865, 14243-15153, 14728-725, 15560-16159, 16453-1696. The ssDNA was purified using Centricon™ 30 microconcentrators as described in the purification protocols (Amicon). Sequencing was carried out with internal primers, which were used as supplied without further purification.

Restriction enzyme analysis: Fragments of mitochondrial tRNA genes were amplified by 'mispairing PCR' as described (25). In short, this method applies one primer which binds its 3'end next to the mutated nucleotide. The primer includes nonpairing nucleotides in order to generate a new restriction site in the amplified fragment, including the position of the mutated nucleotide. This allows to distinguish between mutated and wild type mtDNA after restriction enzyme digestion. The sequences

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of the primers used for PCR were: CPEO-Ser-For: 5'-GAACTGCTAACTCATGCCCCCGAGT, nt 12221–12245; CPEO-Leu-Rev: 5'-CTTTTATTTGGAGTTGCACCAGAATT, nt 12309–12334; CPEO-Gly-For: 5'-GGGTCTTACTCTTTTAGTTTAA, nt 9984–10005; Gly-Rev: 5'-GTAGTAAGGCTAGGAGGGTG, nt 1088–10107; Lys-For: 5'-CCCCTCTAGAGCCCACTGTAAAGC, nt 8282–8305; MERRF-Lys-Rev: 5'-GGGGCATTTCACTGTAAAGAGGTGCCGG, nt 8345–8372. Forward primers (For) are homologous to the heavy strand, reverse primers (Rev) to the light strand of human mtDNA, according to Anderson *et al.*(24). Underlined nucleotides differ from the Cambridge sequence and create new restriction sites. Amplification of mtDNA was performed as described in the amplification protocol of Perkin-Elmer/Cetus and purified from agarose gels by electroelution. The fragment amplified with CPEO-Ser-For and CPEO-Leu-Rev (113 bp) created two new restriction sites: digestion with *Eco RI* cleaved the mutated DNA (*Fig.3*, $tRNA^{Leu}$) into two fragments of 89 bp and 24 bp, whereas digestion with *Hinf I* cleaved only the wild type DNA (*Fig.3*, $tRNA^{Ser}$) into two fragments of 90 bp and 23 bp. The

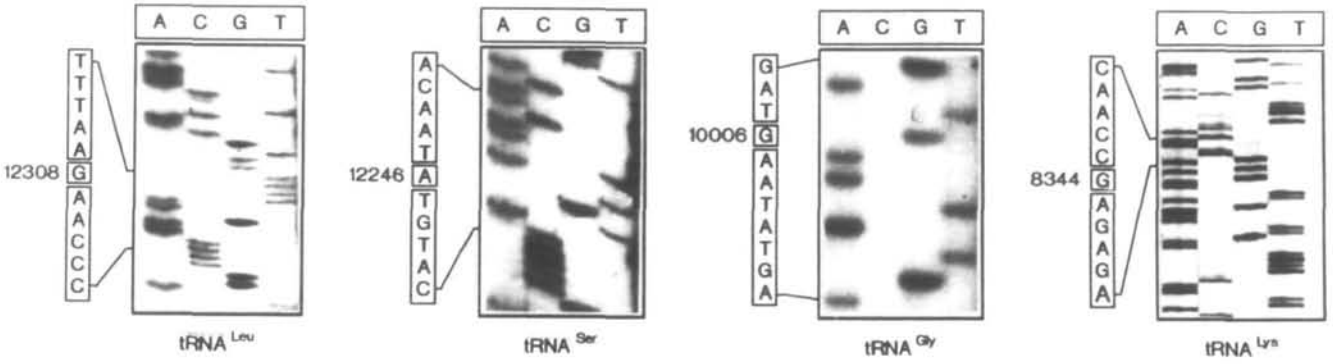

Fig. 1. Sequences around point mutations in mitochondrial tRNA genes of patients with mitochondrial myopathy.

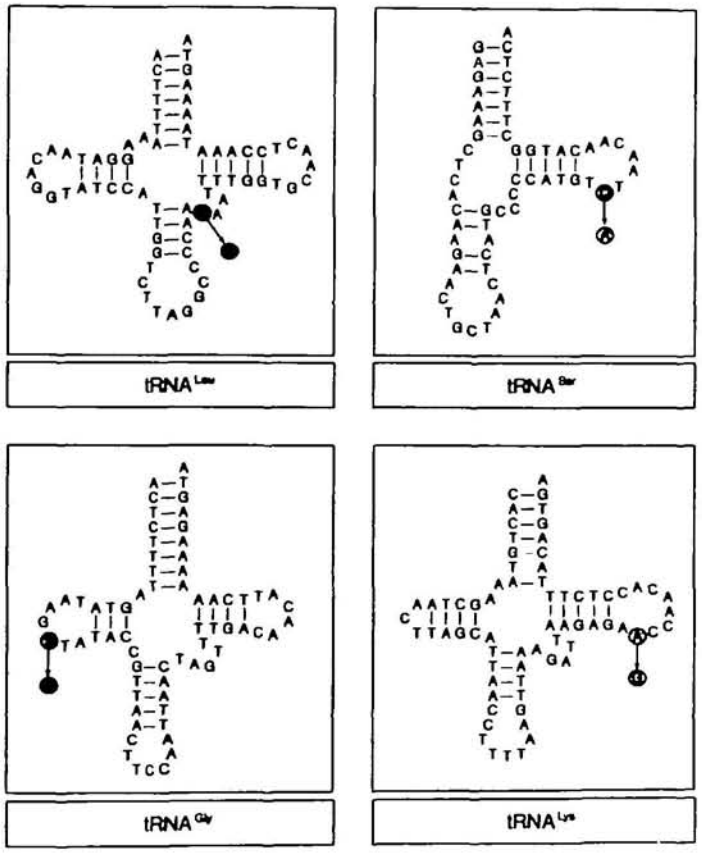

Fig. 2. Proposed clover leaf structures of mitochondrial tRNA genes for leucine (TAG), serine (GCT), glycine (TCC) and lysine (TTT). The exchanged nucleotides are indicated by shaded circles. The arrow points to the mutated nucleotide found in patients (see *Fig.4*).

Fig. 3. Homology of mitochondrial tRNA genes from different species. The DNA sequences from human (Hu), rat (Ra), mouse (Mo), bovine (Bo) and Xenopus laevis (Xe) as well as the alignments were taken from Sprinzl *et al.* (26), whereas the data from Atlantic cod (AC) were taken from Johansen *et al.* (27). The boxed nucleotides indicate the position of the mutation observed in patients with mitochondrial diseases.

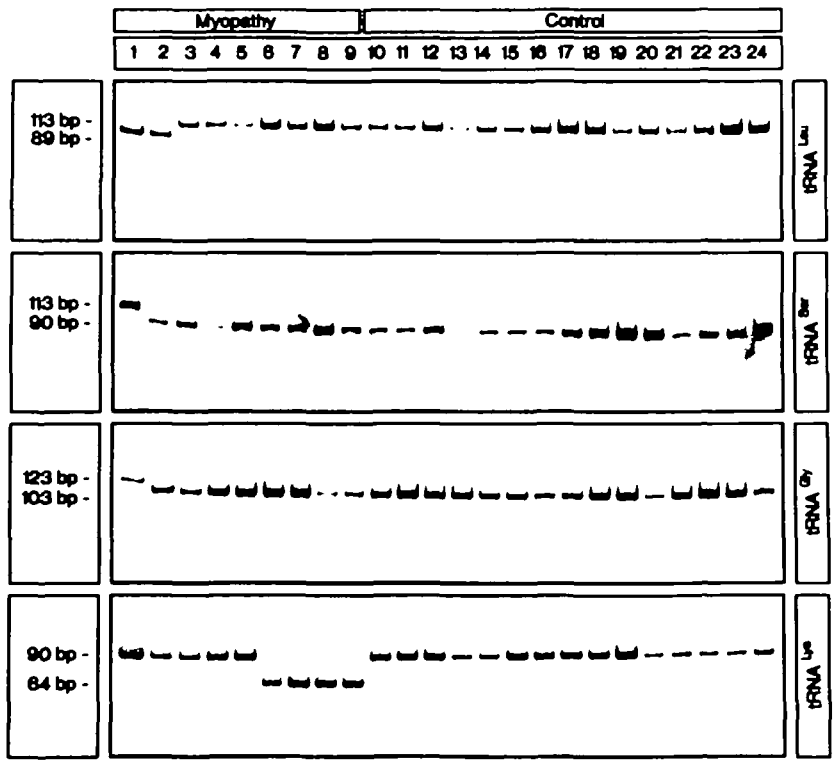

Fig. 4. Restriction enzyme analysis of tRNA gene fragments amplified by mispairing PCR. The panel shows the results of restriction enzyme analysis from 9 patients with mitochondrial myopathy and 15 control individuals. Template DNA was extracted and amplified from skeletal muscle biopsies (lanes 1–12, 16–18 and 20), platelets (lanes 13, 14), HeLa cells (lane 15), placenta (lane 16), heart muscle (lane 19) and blood (lanes 21–24). Lane 1: patient with Chronic Intestinal Pseudoobstruction with myopathy and ophthalmoplegia (23), male, age 49; lane 2: CPEO (29) male, age 32; lane 3: myopathy (29), female, age 42; lane 4: CPEO (personal communication M. Johnson), male, age 49; lane 5: ptosis and myopathy (29), male, age 50; lane 6: ptosis and myopathy (29), male, age 48; lane 7: MERRF (25), female, age 40; lane 8: MERRF (25), male, age 15; lane 9: MERRF (25), female, age 11; lanes 19, 20: patients with cystic fibrosis (personal communication J. Horst); lanes 21–24: patients with β-thalassemia (personal communication J. Horst).

second fragment amplified with CPEO-Gly-For and Gly-Rev was digested with *Dra I* and mutated DNA was cleaved into two fragments of 103 bp and 20 bp (*Fig.3*, $tRNA^{Gly}$). The third fragment was amplified with Lys-For and MERRF-Lys-Rev and digested with *Nae I*. The mutated DNA was cleaved into two fragments of 64 bp and 26 bp (*Fig.3*, $tRNA^{Lys}$). The digestion products were separated on an 8% polyacrylamide gel and silver stained as described by Perbal (28). Fragments smaller than 30 bp in length are not shown.

RESULTS

Fig.1 shows the 3 new identified mutations found in mtDNA of a patient with CIPO ($tRNA^{Leu(UAG)}$, $tRNA^{Ser(GCU)}$ and $tRNA^{Gly}$) and a patient with CPEO ($tRNA^{Leu(UAG)}$ only) together with the transition mutation in the $tRNA^{Lys}$ (nt 8344, A to G) recently identified to cause MERRF disease (21,25). The positions of the point mutations in clover leaf structures of the tRNA's are presented in *Fig.2*. Since the function of individual nucleotides in tRNA's is largely unknown, the consequences of these mutations on the function of the tRNAs remains to be investigated. However, as shown in *Fig.3*, all mutations occur at positions which are highly conserved between species during evolution. The mutated nucleotides found in the tRNA genes of patients do not occur at this position in any of the 6 species. In order to elucidate that the mutations observed are not polymorphic variations of normal mtDNA, we analyzed the mitochondrial genomes of 9 patients with mitochondrial myopathies as well as 15 controls by mispairing PCR (25). Three different fragments were amplified with primers creating new restriction sites adjacent to the four mutations. As shown in *Fig.4*, the patient with CIPO presented three point mutations in tRNA genes (namely genes coding for $tRNA^{Leu(UAG)}$, $tRNA^{Ser(GCU)}$, $tRNA^{Gly}$), suggesting that mitochondrial tRNA genes are hotspots for mutations leading to neuromuscular diseases. In two additional patients, clinically classified as myopathy (*Fig.4*, lane 3) or CPEO (*Fig.4, lane 4)*), both, mutated (lower band) and wild-type mtDNA (upper band) of the $tRNA^{Leu(UAG)}$ was found in each patient. In another patient with ptosis and myopathy (*Fig.4*, lane 5) none of the 4 described mutations in tRNAs were detectable. We therefore assume that additional mutations of mitochondrial tRNAs may occur in these patients. Surprisingly a patient, clinically characterized as ptosis and myopathy (*Fig.4*, lane 6) (29) showed the transition mutation in the $tRNA^{Lys}$ gene (nt 8344, A to G), previously linked to MERRF disease (21). This result indicates that different clinical symptoms could arise from the same mutation of mtDNA. None of the controls (*Fig.4*, lanes 10–24) showed any of the 4 mutations in the mitochondrial tRNA genes.

DISCUSSION

The effect of the described point mutations on the biological properties of the tRNA's is unknown. The described mutations in the tRNA genes of mtDNA from patients with mitochondrial myopathies, however, could cause the clinically symptoms for the following reasons:

1. A decreased synthesis of mitochondrial coded proteins was recently found in cultured fibroblasts from MERRF-patients, containing the point mutation at nt 8344 in the mitochondrial gene for $tRNA^{Lys}$ (30).

2. All 4 described mutations concern highly conserved nucleotides of tRNA genes (*Fig. 3*; see also Sprinzl *et al.* (26) for a compilation of tRNA genes).

3. In none of 17 controls, including 2 patients with KSS (not shown) and 6 patients with known nuclear mutations (cystic fibrosis and β-thalassemia), was any of the 4 mutations detected.

4. The mitochondrial translation system functions with a minimum of 22 tRNAs. Therefore any defective tRNA will affect mitochondrial protein synthesis.

In individual mitochondria both, mutated and wild-type mtDNA could occur,because each mitochondria contains 3–10 mtDNA molecules (31). A defective mitochondrial protein synthesis may only become manifest after all mtDNA molecules of a mitochondrium are mutated (32). A variation in the ratio mutated/wild-type mtDNA was found in different members of a family with MERRF (21), and in different tissues of patients with MERRF (30). Even family members without symptoms were shown to carry the mutated mtDNA, although to a smaller extent (30). A certain quantity of mutated mtDNA molecules seems necessary until cellular energy production becomes limited (energetic threshold) and clinical symptoms manifest. The mechanism of propagation of mutated versus normal mtDNA is unknown. In addition to the segregation of mutated mtDNA during cell division (33), propagation of mutated mtDNA will also occur in individual cells (or fibres) of nondividing tissues (e. g. skeletal muscle), since the continuous turnover of mtDNA is independent of the turnover of nuclear DNA (34). A tissue specific energetic threshold will be reached in different cells or tissues at different percentages of mutated mtDNA resulting in a variety of clinical symptomes of mitochondrial diseases. In addition to the described mutations, any variation of mtDNA leading either to a defective tRNA by point mutation or to the lack of a tRNA by deletion of mtDNA will impair mitochondrial protein synthesis and decrease the generation of ATP by oxidative phosphorylation.

The molecular basis of frequent mutations in tRNA genes of mtDNA is unknown. Two explanations may be suggested: i) The single stranded D-loop intermediate during mtDNA replication could favour the formation of clover leaf structures at the tRNA genes, increasing the error rate of mitochondrial γ-polymerase at regions with secondary structure. ii) A mutation in a nuclear gene coding for a mitochondrial protein involved in mtDNA replication could increase the error rate during duplication, as has been suggested for a patient with KSS (35).

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