Tissue dependent co-segregation of the novel pathogenic G12276A mitochondrial tRNA<sub>Leu(CUN)</sub> mutation with the A185G D-loop polymorphism

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Most of the known disease associated point mutations in mitochondrial DNA (for overviews see Wallace and Shoubridge and Molnar) affect mitochondrial tRNA genes leading to impaired translation of the mitochondrial encoded subunits of the oxidative phosphorylation complexes I, III, IV, and V. Among these, three different point mutations in the mitochondrial tRNA<sub>Leu(CUN)</sub> gene have been identified so far. These rare mutations were reported only in individual studies and found to be associated with skeletal muscle disorders, including chronic progressive external ophthalmoplegia (CPEO),<sup>7</sup> isolated skeletal myopathy,<sup>8</sup> and chronic fatigue syndrome. However, the detailed pathogenic mechanisms of these tRNA point mutations leading to mitochondrial dysfunction in skeletal muscle have remained elusive.

From a diagnostic point of view it is noteworthy that the mitochondrial genome is highly variable, which in turn leads to difficulties in proving the pathogenic role of any particular heteroplasmic mtDNA mutation.<sup>9</sup> Therefore, it has become common practice to provide correlation between the mutation load (degree of mtDNA heteroplasmy) and the biochemical pathology (for example, cytochrome c oxidase (COX) negativity) in individual muscle fibres or cybrids.

Here, we report a novel heteroplasmic point mutation in the mitochondrial tRNA<sub>Leu(CUN)</sub> gene associated with a mild form of CPEO. This mutation causes, due to its location in the highly conserved DHU-stem, a severe decrease in the steady state levels of this tRNA, explaining the impaired translation of mitochondrial encoded proteins. Interestingly, this mutation strictly co-segregates with one allele of a heteroplasmic D-loop polymorphism. This causes pseudo-correlation of the neutral polymorphism with a pathological biochemical phenotype and might be responsible for routing of the pathogenic mutation into skeletal muscle.

METHODS

Case description

A 28 year old female patient presented with a history of mild exercise intolerance starting in early childhood. At the age of 13 years she developed chronic progressive bilateral ptosis and restriction of extraocular eye movements. Apart from mild CPEO, neurological examination was normal. Further medical history was uneventful. She has two healthy brothers aged 16 and 13 years. Her mother, who died in a traffic accident, was reported to be free of neuromuscular symptoms. The study was conducted following the guidelines of the University of Bonn ethical commission and informed consent was obtained from all investigated subjects or their parents.

Muscle histology

Consecutive cryostat sections of muscle biopsy (6 μm) were stained for Gomori’s trichrome, succinate dehydrogenase (SDH), myosin ATPase, and COX as described by Dubowitz.<sup>7</sup>

Key points

- We present the coexistence of two heteroplasmic mitochondrial DNA point mutations in a patient with a mild form of chronic progressive external ophthalmoplegia (CPEO).
- In a muscle biopsy showing abundant ragged-red fibres we identified a novel heteroplasmic G12276A mutation, residing in the mitochondrial tRNA<sub>Leu(CUN)</sub> gene, as well as a previously described heteroplasmic D-loop polymorphism (A185G). Both mutations were also present in fibroblasts, buccal mucosa, and blood, although at much lower levels than in skeletal muscle. Northern blot analysis demonstrated a 50% reduction of tRNA<sub>Leu</sub> (CUN) in the patient’s skeletal muscle in comparison to controls, thus explaining the general failure of mitochondrial translation. A severe defect of oxidative phosphorylation was found in muscle fibres harbouring high loads of the G12276A and A185G mutations. Single-fibre PCR and allele specific PCR revealed that three allelic combinations of the two heteroplasmic mutations were present.
- The genetic and biochemical analysis of our CPEO case demonstrates that the origin of the dramatic impairment of respiratory chain function in the patient’s muscle lies in the instability of the mitochondrial tRNA<sub>Leu(CUN)</sub> gene, a consequence of the heteroplasmic G12276A mutation. The pseudo-correlation of the neutral 185G D-loop polymorphism with the pathological biochemical phenotype can be explained by a cosegregation of the pathogenic 12776A allele with the 185G allele. The apparent contradiction between dramatic biochemical and mild clinical phenotype is probably due to the muscle specific segregation of the pathogenic mutation that might be routed by the associated allele of the D-loop polymorphism.

Quantitative single-fibre analysis of histochemical COX and SDH activities using fibre specific grey level determinations of 12 bit video images was performed as described previously.<sup>7</sup> In brief, video images of identically stained adjacent sections were acquired with monochromatic illumination at 625 nm (for SDH, with a DIF 625 double interference filter) or at 450 nm (for COX, with a DIF 450 double interference filter) using an IX-70 microscope (Olympus, Tokyo, Japan).

Abbreviations: COX, cytochrome c oxidase; CPEO, chronic progressive external ophthalmoplegia; CS, citrate synthase; SDH, succinate dehydrogenase.
encompassed with a 12 bit high resolution CCD camera (model Spot RT, Diagnostic Instruments, Burroughs, MI, USA). The image analysis was performed using the MetaMorph software package (Universal Imaging, West Chester, PA, USA).

For the calculation of activity ratios the individual average single-fibre grey value readings were converted into absorbance values (ΔA) using the formula: ΔA = log10(grey value of background/grey value of fibre).

The linearity of the histochemical COX and SDH staining reactions (up to 40 min developing time for both COX and SDH) was tested in control experiments. For immunohistochemistry we used the mouse monoclonal antibodies against subunit I of human COX and the 15 kDa protein of human complex I (product of the NDUF5 gene) (Molecular Probes, Eugene, OR, USA). The immunochemical reaction was developed with DAB using a peroxidase labelled anti-mouse secondary antibody (Dianova, Hamburg, Germany). Fibre types were distinguished based on the myosin ATPase stainings at pH 4.6 and pH 9.3.

**Enzyme activities**

The activities of rotenone sensitive NADH:CoQ1 oxidoreductase, COX, and citrate synthase (CS) in skeletal muscle homogenates were measured spectrophotometrically as described previously.

**mtDNA mutation analysis**

Genomic DNA was extracted from 10 ml aliquots of EDTA anticoagulated blood using a salting out method20 or from 30 mg skeletal muscle specimens with the QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany). The mitochondrial genome was pre-screened for mutations using an automated denaturing high pressure liquid chromatography (DHPLC) instrument (WAVE System, Transgenomic, Omaha, NE, USA). Direct sequence analysis of 28 PCR fragments amplified from the whole mitochondrial genome was carried out on an automatic sequence analyser (ABI 377; Applied Biosystems, Foster City, CA, USA).

For the detection of the G12276A point mutation mismatched primers were designed to introduce either a novel MboI restriction site in the wild type allele (5′-CTAGGACCA ATGGATACGCTGAT-3′, mismatch underlined) or a Tsp509I site in the mutant (5′-CTAGGACCAATGGATACGCTGAT-3′). Either of these primers was used together with primer 5′-GAGGATAGCTGT-3′ (12119F) or 5′-ATTACCGGTGTTCCTCTGG-3′ (resulting in a 182 bp product that was subsequently digested with the corresponding restriction endonuclease. Restriction fragments were separated on 10% polyacrylamide gel, and visualised by SYBR Green I staining (Sigma-Aldrich, Steinhem, Germany). Proportions of wild type and mutant mitochondrial DNA were estimated from band intensities using Scion Image analysis software. The 185 polymorphism was detected in a similar manner, creating either a HinP1 restriction site in the presence of “G” at position 185 by primer 5′-TTTAAACACCTTTAGAATGATGTGCCG-3′, or a HpyCH4IV site in the presence of “A” by primer 5′-TTTAAACACCTTTAGAATGATGTGCCG-3′ together with primer 5′-CACCTTATATAA CGACTAGC-3′.

**Allele specific PCR**

Primers specific for each of the two alleles of 185 (5′-CACCATTAAGTGATGTCCG[CT/T]-3′) were used together with primer 12119F to amplify a 4.7 kb PCR fragment comprising the 12276 polymorphic site. The PCR program included the following steps: 95°C for 5 min; 25 cycles of 95°C for 30 s and 68°C for 8 min; and finally 72°C for 7 min. A 1 μl sample of the 25 μl reaction was applied in a second round of mismatched PCR performed as described above. In allele specific real time PCR reactions primers 5′-GGCTTTCTCAATTTAAAGA-3′ and 5′-ACACTGATTTTACATAATGGG-3′ were used together with the TaqMan probe FAM-CCCTCCCA AATTGGTTCACCTCA-BHQ1 for assessing the amounts of the mutant 12276A allele. As a reference, the overall amounts of mitochondrial DNA were determined with primers 5′-CAGGTTCATGCGCCATCTTAGAT-3′ and 5′-GGGCA TTTCCTGAAGAGGTTTG-3′ together with TaqMan probe VIC-CACCCTCTT(DABCYL)ACCCCCTCTAGAGCC Cap. The thermocycler was programmed as follows: 95°C for 10 min; and 40 cycles of 95°C for 15 s and 62.5°C for 30 s.

**RESULTS**

**Histological and biochemical consequences of a novel mitochondrial tRNA mutation**

Histological analysis of a skeletal muscle biopsy from a patient with the 12276A point mutation revealed abundant ragged-red fibres (asterisks, fig 1A) as well as multiple SDH positive fibres (fig 1B) that were negative for COX staining (circles, fig 1D). Furthermore, immunostaining showed decreased immunoreactivity for the mitochondrial encoded COX subunit I (fig 1C) in these fibres. Additionally, the immunostaining for the nuclear encoded 15 kDa complex I subunit was also lower indicating decreased stability of the entire complex (fig 1E). In accordance with the very high proportion of affected fibres, a severe reduction of CS activity in skeletal muscle homogenates was observed (fig 1F).
To establish a correlation between biochemical abnormalities and G12276A mutation loads in individual muscle fibres, we determined the levels of heteroplasmy in DNA samples isolated from single fibres. As shown in fig 3, fibres harbouring less than 75% of the mutant mitochondrial DNA exhibited normal COX/SDH ratios. However, mutation loads above the 80% threshold level resulted in severe deficiency of COX activity, in both type I and type IIa fibres.

The G12276A mutation leads to mitochondrial tRNA<sub>Leu(CUN)</sub> instability

The G12276A change in the tRNA<sub>Leu(CUN)</sub> gene resides in a highly conserved nucleotide position, which is likely to have a crucial role in forming the DHU-stem (fig 2C). The hypothesis that this particular mutation seriously disturbs the structure of the tRNA<sub>Leu(CUN)</sub> was further underlined by Northern blot analysis. In comparison to the nuclear encoded 5S rRNA (which serves as a loading control), we observed a selective 50% reduction of the amount of tRNA<sub>Leu(CUN)</sub> in total skeletal muscle RNA (fig 2D). The decrease of tRNA<sub>Leu(CUN)</sub> was even more pronounced when compared to the level of the other mitochondrial tRNA for leucine, tRNA<sub>Leu(UUR)</sub>. No relevant changes in the level of the ND1 transcript were found (data not shown). Since the Leu(CUN) codon is present at a high frequency in most protein coding mitochondrial genes, low levels of the tRNA<sub>Leu(CUN)</sub> readily explain the general failure of translation of mitochondrial encoded proteins.

The D-loop polymorphism A185G co-segregating with the G12276A mutation

As outlined above, sequence analysis of the patient's mitochondrial DNA revealed the presence of a previously described heteroplasmic polymorphism (A185G), located in one of the two hypervariable regions (HVR2) of the D-loop (fig 4A). Genetic analysis of skeletal muscle from the patient showed that approximately 60% of the mitochondrial DNA carried the 185G allele, whereas fibroblasts carried approximately 6%, and buccal mucosa and blood less than 3% of this allele (fig 4B, lanes 2–5, 9). The presence of 185G in blood at very low levels was further confirmed by allele specific PCR (data not shown). Similar to the patient, DNA samples from blood of the patient's two brothers contained a minor fraction of the 185G allele in a predominantly 185A background (fig 4B, lanes 6, 7, 10, 11). Since the examined family belongs to a subgroup of the J<sup>+</sup> haplogroup characterised by the presence of the 185A allele, we refer to this polymorphism as A185G and not G185A, despite the fact that the 185G allele is more frequent in the general population.

To investigate whether mutations A185G and G12276A had occurred in a random or sequential order, we determined the allelic distribution of both mutations in 24 muscle fibres (fig 5). A wide range of heteroplasmy was detected for both mutations. We found three main populations of fibres: (i) the mutant 12276A allele was primarily found in fibres with a high percentage of the 185G allele (fig 5, upper right corner); (ii) fibres with a high percentage of the 185A allele were found to exclusively contain the 12276G wild type allele (fig 5, lower left corner); and (iii) certain muscle fibres also contained the 185G allele in combination with the wild type 12276G (fig 5, upper left corner). However, the fourth theoretically possible combination, 185A/12276A, was not detected suggesting a sequential order of early mutation events.

To exclude an experimental bias in single-fibre PCR studies, we set up a method to detect combinations of the two heteroplasmic mutations in bulk muscle DNA preparations from our patient. Using allele specific PCR and restriction analysis we found that the PCR fragments amplified by a 185G specific primer contained about 85% of...
the mutant 12276A allele and approximately 15% of the wild type 12276G allele. PCR products from the 185A specific amplification contained only the wild type 12276G allele (inset, fig 5). These findings are in full agreement with the single-fibre PCR data, thus confirming that the pattern of heteroplasmy observed in the patient’s muscle was created by a mixture of three distinct mitochondrial genomes.

The presence of three distinct mitochondrial genomes in diseased skeletal muscle prompted us to correlate the genetic findings with the results of our biochemical analysis. An important consequence of this non-random segregation of two heteroplasmic mutations was the observation that the mutation load of the pathogenic 12276A allele (fig 3) as well as the mutation load of the 185G D-loop polymorphism (fig 4C) showed an inverse correlation with COX enzyme activity in individual fibres. The distribution of the data points in fig 4C appears similar to that of a pathogenic mutation causing COX negativity above a high threshold level, although one has to note the existence of a few fibres with 100% 185G and normal COX activity. Since the 185G allele is the wild type genotype in the vast majority of the human population, this inverse genotype biochemical phenotype relationship has to be considered as a “pseudo-correlation”.

**DISCUSSION**

In the present study we describe the co-existence of two heteroplasmic mitochondrial DNA point mutations in a patient with chronic external ophthalmoplegia. One of the heteroplasmic mutations, A185G, is a well know polymorphism located in the non-coding D-loop region. Most of the human population carries “G” at this position, although the 185A genotype is also accepted as a normal sequence variation reported to be associated with at least two separate mitochondrial haplogroups (J*, U5). Therefore, it is very unlikely that the A185G mutation is directly involved in the biochemical defect responsible for the patient’s mitochondrial cytopathy. Our genetic and biochemical data suggest that the disease is caused by the other heteroplasmic mutation, G12276A, for the following reasons. (i) The G12276A mutation was found only in the affected individual, and was not detected in healthy family members, and is not present in the general population. (ii) The mutation is located at a highly conserved position in the DHU-stem of the mitochondrial tRNAlue(CUN). In accordance with this fact, the steady state levels of tRNAlue(CUN) were found to be decreased in total skeletal muscle to a degree that corresponded to the mutation load of the 12276A allele in bulk tissue. (iii) As expected in a case of a pathogenic mitochondrial tRNA mutation, a combined complex I/COX deficiency was observed in fibres with high loads of the G12276A mutation.

Interestingly, relative amounts of the “G” allele of the heteroplasmic D-loop polymorphism, A185G, also showed a reverse correlation with the activity of oxidative phosphorylation in individual muscle fibres, although it was not as closely linked as the 12276A allele. The apparent accumulation of the 185G allele in COX deficient muscle fibres is due to the non-random segregation of the two heteroplasmic mutations; the pathogenic 12276A phenotype was exclusively found in association with 185G and not with 185A. In the case presented here, the available biochemical data clearly suggest the nature of the genetic defect leading to disease, and it is therefore easy to see which of the two heteroplasmic mutations is responsible for the biochemical phenotype. However, less characterised heteroplasmic polymorphisms co-segregating with mild pathogenic mutations might confuse the picture when tracing back the root of mitochondrial disease.

The fact that only three allelic combinations of the two heteroplasmic mutations were detected in the patient’s muscle indicates that the two mutations were generated in sequential order. First, the 185G allele appeared on the original 185A genotype, and then the pathogenic tRNA mutation occurred on
a 185G mitochondrial DNA molecule. Although high levels of both mutations were found only in the patient’s muscle, both mutations were also detected in other investigated tissues. Therefore, the pathogenic mutation must have happened at a very early stage of development or possibly during oogenesis. The D-loop heteroplasmy must have already existed at this time, which is in accordance with the presence of the D-loop heteroplasmy in family members (though they lack the pathogenic mutation). A sequential order of mutational events as the source of a double-heteroplasmic pattern seems evident (and in fact the few previous studies addressing double-heteroplasmy also showed the presence of triplasmy). However, in light of a recent report demonstrating recombination-like events in human mitochondrial DNA, it remains unclear why a random distribution of the two heteroplasmic mutations (quadruplasmy, that is the presence of all four theoretical allelic combinations) cannot be observed in our patient. One possible explanation could be that the heteroplasmic mutations segregated quickly to separated mitochondrial compartments and therefore the different genomes did not have the chance to come into physical contact. Alternatively, one could speculate that recombination-like events might be restricted to certain early periods of development and the second (pathogenic) mutation was generated only after such a phase of dynamic changes.

Our histological and biochemical findings in skeletal muscle clearly indicate that the G12276A mutation has serious consequences for tRNA Leu(CUN) stability and mitochondrial protein synthesis. The mutation loads in individual muscle fibres that are required for the loss of COX activity (around 80%) are lower than those established for other, more frequently encountered mitochondrial mutations, such as MERRF (above 85%) and MELAS (between 90% and 95%). Moreover, the percentage of COX negative and ragged-red fibres in muscle is unusually high (21% and 16%, respectively). However, these dramatic biochemical effects are in obvious contradiction with the mild clinical phenotype. One plausible explanation could be that the heteroplasmic mutations segregated quickly to separated mitochondrial compartments and therefore the different genomes did not have the chance to come into physical contact. Alternatively, one could speculate that recombination-like events might be restricted to certain early periods of development and the second (pathogenic) mutation was generated only after such a phase of dynamic changes.

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Figure 5  Co-segregation of G12276A and A185G. Plot of mutation loads of G12276A and A185G in 24 single fibres. Error bars represent the SEM of data obtained from two separate RFLP analyses using different restriction endonucleases. The mutant 12276A allele was primarily found in fibres with a high percentage of the 185G allele (upper right corner). Fibres with a high percentage of the 185A allele were found to exclusively contain the 12276G wild type allele (lower left corner). Certain muscle fibres contained also the 185G allele in combination with the 12276G wild type allele (upper left corner). Inset: Left lane, molecular weight marker. Lanes G, A: Amounts of 12276A (mut) and 12276G (wt) in allele specific PCR products amplified from the patient’s skeletal muscle DNA with primers specific for 185G (lane G) and 185A (lane A), respectively.

explanation to resolve this contradiction would be tissue specific segregation of the pathogenic mutation. In full agreement with the patient’s symptoms which are restricted to skeletal muscle, high loads of the G12276A mutation were found only in muscle and not in other investigated tissues. Since skeletal muscle has a high glycolytic capacity able to produce sufficient ATP even under the condition of a serious disturbance of oxidative phosphorylation, mitochondrial mutations might escape to present severe clinical manifestations if they predominantly accumulate in muscle. It seems to us very tempting to speculate that the co-segregating D-loop allele could have an influence on the tissue distribution of the pathogenic mutation in our patient. Individuals with predominantly 185A genotype in blood usually show high levels of 185G in skeletal muscle (see Khrapko et al25; G Zsukra, unpublished data), thus muscle specific accumulation of 185G seems to be a general phenomenon. Filosto et al26 demonstrated that several other polymorphic sites in the D-loop region show muscle specific accumulation of one allele in patients with muscle restricted mitochondrial disease. Similarly, Battersby and Shoobridge23 showed in heteroplasmic mice that different mitochondrial genotypes that cannot be distinguished phenotypically might differentially accumulate in specific tissues. This would raise the possibility that neutral D-loop polymorphisms could route associated pathogenic mutations to specific tissues, thus having an important influence on the clinical manifestation of an individual mitochondrial disease.

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ELECTRONIC-DATABASE INFORMATION