Characterisation of repeat and palindrome elements in patients harbouring single deletions of mitochondrial DNA

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METHODS

Patients

The 18 patients included in this study were clinically diagnosed with CPEO (10 patients) and KSS (eight patients), and there was no evidence of maternal inheritance. CPEO was defined by the presence of ophthalmoplegia, ptosis, and proximal limb weakness. Patients with KSS presented with the invariant triad of: (1) onset before 20 years, (2) progressive external ophthalmoplegia, ptosis, and proximal limb weakness, and (3) pigmentary retinopathy, plus at least one of heart block, cerebellar syndrome, or a protein concentration in cerebrospinal fluid above 100 mg/ml. Muscle biopsies were performed after informed consent.

Molecular genetic studies

Blood DNA was extracted from peripheral blood cells by conventional methods, and muscle DNA was obtained from 10 mg of muscle after treatment with proteinase K and extraction with phenol/chloroform/isoamyl alcohol. Southern blot analysis was performed using 5 µg of total blood or muscle DNA by standard procedures. Each sample was digested with PvuII to linearise the mtDNA and was loaded on a 0.7% agarose gel. After electrophoreses and transfer to a nylon membrane, the mtDNA was linearised and was loaded on a 0.7% agarose gel. Southern blot analysis were submitted to long PCR to map the deletion breakpoints. Three out 12 patients had previous deletions, with two clinical presentations: chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndrome (KSS). Here, we report the molecular characterisation of a series of 18 patients in whom we have identified single deletions of mtDNA. These patients were, as expected, diagnosed with CPEO and KSS. Deletions were identified and measured by Southern blot analysis and were mapped by long polymerase chain reaction (PCR) to locate the deletion breakpoint. We report nine novel deletions and defined their characteristics in terms of sequence of the tandem repeat, presence of palindrome sequences, length of the deleted molecule, and heteroplasmy level in muscle.

RESULTS

Southern blot analysis identified the presence of the so-called 4977 nt “common deletion” in six out of 18 patients (33%). In all patients the deletion was found only in muscle (it was not found in blood). The classic 13 bp tandem repeat flanking the deletion breakpoint was confirmed by specific PCR and the percentages of deleted genome in muscle ranged from 6% to 68%. Twelve patients harboured a single deletion other than 4977. Sequence analysis of the long PCR product allowed us to map the deletion breakpoints. Three out 12 patients had previously reported deletions and nine were novel. Table 1 shows the proportion of mutated genomes in muscle from those patients without 4977 as well as the length of the deleted mtDNA. These novel deletions ranged from 2.3 to 9.4 kb with percentages of heteroplasmy in muscle from 7% to 86%. Fig 1 shows the nucleotide sequence involved in the deletion event. In most cases, these were direct tandem repeats ranging from 5 to 15 bp and there was a high percentage of deleted genome in muscle.

Key points

- We report the molecular findings in a series of 18 patients in whom we have identified single deletions of their mitochondrial DNA (mtDNA).
- From a clinical point of view, patients fall into two categories: chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndrome (KSS).
- After mapping the deletion breakpoints, we report nine novel deletions varying in size and heteroplasmic levels, in which direct tandem repeat elements are involved. Also, several palindrome sequences were identified in three patients, and in one of these flanking direct repeats were not found.
- These findings further expand our knowledge of the mitochondrial genotype in those neuromuscular disorders produced by single deletions of mtDNA.

Abbreviations: CPEO, chronic progressive external ophthalmoplegia; KSS, Kearns-Sayre syndrome; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction.

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proportion of imperfect tandem repeats (50% of cases) which were found in those repeats longer than 10 nucleotides. However, in two patients (patients 1 and 11) the deleted sequence was not flanked by tandem repeat elements. Moreover, several patients harboured palindrome sequences either within or near the deletion breakpoints. These palindromes were found in three patients: patient 8 had a 10 bp tandem repeat plus a 7 bp palindrome sequence upstream of the 5′ end of the breakpoint; patient 11 did not show any tandem repeat, but several palindrome sequences were identified, a 15 bp perfect palindrome and a 6 bp palindrome within the deletion plus a 14 bp palindrome 4 nt from the 3′ end of the breakpoint; and patient 12 had 14 bp palindrome at the 3′ end plus an 8 bp palindrome within the deletion at the 5′ end. Interestingly, patient 1 did not have either repeat elements or palindrome sequences flanking the deletion breakpoint.

**DISCUSSION**

Usually, single deletions of mtDNA present with a variable range from about 2–8.5 kb and are mostly located in an 11 kb region between the *ND5* and *ATP8* genes. The most common deletion of human mtDNA is a 4977 deletion (the so called common deletion) that ranges from nt position 8470 (in the *ATP8* gene) to nt 13447 (in the *ND5* gene). The deletion is flanked by a 13 bp direct repeat and it represents most patients with CPEO. In our series, we have found a similar frequency of the 4977 deletion (33%) and, as expected, all patients presented with CPEO. The presence of direct tandem repeat elements flanking the deletion breakpoints has been proposed as a genetic criterion to classify mtDNA single deletions. According to this classification, single deletions may be divided into two groups. Class I deletions (representing about 70% of patients) are flanked by direct repeats of variable length, and class II deletions are either not flanked by repeats, or if repeats are present, these are located imprecisely relative to the breakpoints. The mechanism by which single deletions are produced is not well understood and several explanations have been proposed. Unequal intergenic crossover as well as slipped mispairing are two proposed models explaining the mechanism of mtDNA deletion during mtDNA replication, and the presence of direct repeats flanking the deletion breakpoints suggest that intramolecular recombinations produced by the repeated sequence are more likely to

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**Table 1** Molecular characteristics of single mtDNA deletions in patients with CPEO or Kearns-Sayre syndrome (KSS) (number of mispairings (mp) in non-perfect repeats are shown in parentheses)

**Figure 1** Sequences involved in lost mtDNA. Numbers below sequences define mtDNA positions at breakpoints of the deleted regions (within brackets). Bold indicates direct repeats (perfect or imperfect), while palindromes are shown in italics and are underlined.
occur than intergenomic recombinations. Shoffner et al. proposed a "slip replication" model in which the single parental H strand anneals erroneously with the L strand at the direct repeat site leading to the deletion of the H strand loop. On the other hand, Buroker et al. have proposed an "illegitimate elongation" model in which the elongating D loop strand is displaced by branch migration of the parental H strand forming base pairs with the complementary L strand, allowing the liberated D loop strand an erroneous misalignment at direct repeat sites. Elongation of the shortened daughter H strand followed by replication of the L strand would lead to deletion of the molecule. A recent "pyrimidine content" hypothesis by Rocher et al. suggests novel molecular bases for mtDNA rearrangements through the distributive nature of the DNA polymerase gamma, at the level of direct repeats. The proposed model suggests the formation of a DNA triple helix between a G rich neosynthesised direct repeat in the heavy strand and the base paired homologous pyrimidine rich direct repeat in the light strand. Interestingly, our data are consistent with this hypothesis as our patients had a high pyrimidine content in the repeat element (ranging from 60% to 80% with an average of 70%). The three hypotheses require the presence of tandem repeat elements which are found in most deletions reported to date as well as in the present series. However, in a few patients, repeat elements are not found. In our series, patients 1 and 11 had no tandem repeats flanking the deletion breakpoint but palindrome sequences either within or near the breakpoint were found in patient 11. Therefore a putative "palindrome sequence" model would explain the deletion mechanism in those patients in whom repeat elements are not found. This hypothesis is supported by the finding of certain palindrome DNA sequences with the potential to form DNA hairpin structures that might stabilise the misalignment of direct repeats and have a high frequency of deletion. Palindrome sequences are common in the mtDNA genome. However, in our patients they are located very close to the deletion breakpoint suggesting that they could be involved in the deletion mechanism. Similar findings were reported by Saiwaki et al., who identified a patient harbouring a 5.3 kb single deletion in whom a 17 bp palindrome and 5 bp tandem repeats were found on both sides of the breakpoint.

Here, we report nine novel deletions that have been mapped to identify the breakpoints (fig 1). Remarkably, in those patients in whom a direct repeat was not evident, there were several palindrome sequences near or within the deletion breakpoints. Therefore, a combination of these four mechanisms, slip replication, illegitimate elongation, pyrimidine content, and palindrome sequence, could occur.

In a recent report, He et al. described a real time PCR based method to measure single and multiple deletions of mtDNA. After analysing their own series of patients, they suggested that one region in the ND4 gene (nt 12087–12170) is deleted in 82% of patients, while another region in the ND1 gene (3485–3553) is deleted in 6% of cases, so they proposed the simultaneous amplification of these sequences to measure the proportion of deleted genomes. Our mapping results agree with their finding (fig 2) as in all cases 12087–12170 is totally or partially included in the deleted genomes, while 3485–3553 is never deleted. This suggests that a real time PCR based strategy could be a good alternative to Southern blot analysis in the routine diagnosis of patients with single deletions of mtDNA.

Extensive mapping studies of patients with single deletions of mtDNA should be performed to clarify the underlying mechanisms involved in the generation of the deletion event during mtDNA replication. Although the presence of repeat elements seems to explain most cases, there is growing evidence suggesting that mechanisms other than the classic direct repeat hypotheses could be involved in the pathogenesis of the mtDNA single deletion phenotype.

ACKNOWLEDGEMENTS

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Figure 2 Linear representation of human mitochondrial DNA, and schematic localisation of the reported CPEO and KSS associated deletions. The common region to all of these is also shown (positions 12112 to 13059, dotted rectangle).
REFERENCES


