ONLINE MUTATION REPORT

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

A Solano, J Gámez, F J Carod, M Pineda, A Playán, E López-Gallardo, A L Andreu, J Montoya

J Med Genet 2003;40:e86(<http://www.jmedgenet.com/cgi/content/full/40/7/e86>)

Single deletions of mitochondrial DNA (mtDNA) were the first pathogenic mutations to be identified in human mtDNA. In a seminal paper, Holt *et al*¹ reported the presence of single deletions of the mitochondrial genome in patients presenting with mitochondrial myopathies, and since then, the field has experienced enormous progress. To date, 97 different deletions have been reported in MITOMAP, the main international database for mtDNA related disorders (www.mitomap.org), and most of these deletions are associated 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.

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; 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 electrophoresis and transfer to a nylon membrane, the membrane was hybridised with a 1.3 kb PCR generated probe encompassing the *16S RNA* and *ND1* genes (nt positions 3130–4407),² which was labelled with digoxigenine (DIG-High Prime, Boehringer-Mannheim, Mannheim, Germany).

Those samples in which single deletions were identified by Southern blot analysis were submitted to long PCR to map the deletion breakpoints. Briefly, the whole mitochondrial genome was amplified using the following primers: forward (5'-TCTATCACCTATTAACCACTACGGGAGCT-3'), and reverse

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 heteroplasmy levels, in which direct tandem repeat elements are involved. Also, several palindrome sequences within or near the deletion breakpoints 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.

(5'-CGGATACAGTTCACCTTAGCTACCCCCAAGTG-3') using LA-*Taq* (Takara Shuzo, Japan). The long PCR product was sequenced using internal primers in an automatic sequencer (ABI Prism 310, Perkin-Elmer, CA) using Big-Dye chemistry. The deletion breakpoints were identified by comparing with the reference sequence for the human mtDNA.²

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 of 12 patients had previously reported deletions^{3–5} 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

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

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 those is also shown (positions 12112 to 13059, dotted rectangle).

occur than intergenomic recombinations. Shoffner *et al*¹¹ 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*¹² 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*¹³ 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.¹⁴ 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*,¹⁵ 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*¹⁶ 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 only 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 than mechanisms other than the classic direct repeat hypotheses could be involved in the pathogenesis of the mtDNA single deletion phenotype.

ACKNOWLEDGEMENTS

This study was supported by grants from the Spanish Ministry of Health (FIS 00/0797), Diputación General de Aragón (P032–2000), and Spanish Network for Mitochondrial Disorders (G03–011). AS was the recipient of a fellowship from the Mexican Government (CONACYT-119894/121963).

Authors’ affiliations

A Solano, A Playán, E López-Gallardo, J Montoya, Departamento de Bioquímica y Biología Molecular y Celular, Universidad de Zaragoza, Zaragoza, Spain

J Gámez A L Andreu, Centre d’Investigació en Bioquímica i Biologia

Molecular, Hospital Universitari Vall d’Hebron, Barcelona, Spain

F J Carod, Serviço de Neurologia, Hospital Sarah, Brasília, Brasil

M Pineda, Servei de Neuropediatria, Hospital Sant Joan de Déu, Barcelona, Spain

Correspondence to: Dr A L Andreu, Centre d’Investigació en Bioquímica i Biologia Molecular, University Hospital Vall d’Hebron, P Vall d’Hebron 119–129, 08035 Barcelona, Spain; tandreu@hg.vhebron.es

REFERENCES

- 1 **Holt IJ**, Harding AE, Morgan-Hughes JA. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature* 1988;**331**:717–19.
- 2 **Anderson S**, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. *Nature* 1981;**290**:457–65.
- 3 **Yuzaki M**, Ohkoshi N, Kanazawa I, Kagawa Y, Ohta S. Multiple deletions in mitochondrial DNA at direct repeats of non-D-loop regions in cases of familial mitochondrial myopathy. *Biochem Biophys Res Commun* 1989;**164**:1352–7.
- 4 **Johns DR**, Rutledge SL, Stine OC, Hurko O. Directly repeated sequences associated with pathogenic mitochondrial DNA deletions. *Proc Natl Acad Sci USA* 1989;**86**:8059–62.
- 5 **Rotig A**, Bourgeron T, Chretien D, Rustin P, Munnich A. Spectrum of mitochondrial DNA rearrangements in the Pearson marrow-pancreas syndrome. *Hum Mol Genet* 1995;**4**:1327–30.
- 6 **Moraes CT**, DiMauro S, Zeviani M, Lombes A, Shanske S, Miranda AF, Nakase H, Bonilla E, Werneck LC, Servidei S. Mitochondrial DNA deletions in progressive external ophthalmoplegia and Kearns-Sayre syndrome. *N Engl J Med* 1989;**320**:1293–9.
- 7 **Holt IJ**, Harding AE, Cooper JM, Schapira AH, Toscano A, Clark JB, Morgan-Hughes JA. Mitochondrial myopathies: clinical and biochemical features of 30 patients with major deletions of muscle mitochondrial DNA. *Ann Neurol* 1989;**26**:699–708.
- 8 **Mita S**, Rizzuto R, Moraes CT. Recombination via flanking direct repeats is a major cause of large-scale deletions of human mitochondrial DNA. *Nucleic Acids Res* 1990;**18**:561–7.
- 9 **Schon E**, Rizzuto R, Moraes CT, Nakase H, Zeviani M, DiMauro S. A direct repeat is a hotspot for large scale deletions of human mitochondrial DNA. *Science* 1989;**244**:346–9.
- 10 **Efstathiadis A**, Posakony JW, Maniatis T, Lawn RM, O'Connell C, Spritz RA, DeRiel JK, Forget BG, Weissman SM, Slightom JL, Blechl AE, Smithies O, Baralle FE, Shoulders CC, Proudfoot NJ. The structure and evolution of the human beta-globin gene family. *Cell* 1980;**21**:653–68.
- 11 **Schoffner JM**, Lott MT, Voljavec AS, Soueidan SA, Costigan DA, Wallace DC. Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with mtDNA deletion: A slip-replication model and metabolic therapy. *Proc Natl Acad Sci USA* 1986;**79**:7952–6.
- 12 **Buroker NE**, Brown JR, Gilbert TA, O'Hara PJ, Beckenbach AT, Thomas WK, Smith MJ. Length heteroplasmy of strurgeon mitochondrial DNA: an illegitimate elongation model. *Genetics* 1990;**124**:157–63.
- 13 **Rocher C**, Letellier T, Copeland WC, Lestienne P. Base composition at mtDNA boundaries suggests a DNA triple helix model for human mitochondrial DNA large-scale rearrangements. *Mol Genet Metab* 2002;**76**:123–32.
- 14 **Trinh TQ**, Sinden RR. The influence of primary and secondary DNA structure in deletion and duplication between direct repeats in *Escherichia coli*. *Genetics* 1993;**134**:409–22.
- 15 **Saiwaki T**, Shiga K, Fukuyama R, Tsutsumi Y, Fushiki S. A unique junctional palindromic sequence in mitochondrial DNA from a patient with progressive external ophthalmoplegia. *Mol Pathol* 2000;**53**:333–5.
- 16 **He L**, Chinnery PF, Durham SE, Blakely EL, Wardell TM, Borthwick GM, Taylor RW, Turnbull DM. Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR. *Nucleic Acids Res* 2002;**30**:e68.