ONLINE MUTATION REPORT

Compensatory amplification of mtDNA in a patient with a novel deletion/duplication and high mutant load

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The clinical manifestations of patients with mitochondrial DNA (mtDNA) deletions are quite variable. Kearns-Sayre syndrome (KSS; OMIM #530000), the most commonly recognised phenotype, is characterised by the diagnostic triad of progressive external ophthalmoplegia (PEO, OMIM #555000), onset before 20 years of age, pigmentary retinopathy, and one or more of the following: cerebellar ataxia, cardiac conduction defect, and/or elevated protein concentration of greater than 1 g/L in cerebrospinal fluid.¹ Other phenotypes associated with mtDNA deletions include chronic progressive external ophthalmoplegia, Pearson marrow-pancreas syndrome, Addison disease, diabetes mellitus, cyclic vomiting, deafness, optic atrophy, and renal tubulopathy.¹⁻⁸ KSS typically occurs in adult patients, and the deleted mutant mtDNA is usually not present in blood.⁴ On the other hand, the clinical presentation of mtDNA deletion syndromes in infants and young children is variable and does not necessarily feature the classic symptoms seen in KSS. In severe cases with multisystemic involvement, a high percentage of deleted mutant mtDNA is present in all tissues examined. We describe a 41 year old woman who presented with atypical KSS at the age of 30 years. A novel mtDNA deletion was detected in both muscle and blood specimens of this patient, despite the finding of respiratory chain enzymes in the normal range in muscle samples.

CLINICAL REPORT

A 41 year old female was diagnosed with chronic PEO (CPEO), ptosis, and pigmentary retinopathy at the age of 30 years. The patient was born to a 21 year old, G2P1 healthy mother after an uneventful pregnancy. She had a blood transfusion during the neonatal period for hyperbilirubinaemia, and had an otherwise unremarkable childhood. She also had transient, mild iron deficiency anaemia at approximately 9 years of age. She progressed through a normal puberty and had two normal pregnancies at 29 and 30 years of age. Following her first pregnancy, an ophthalmological examination revealed CPEO and retinopathy. No other diagnostic evaluation was suggested at that time. Eye muscle surgery was performed at 31 years of age, and the eyelids were surgically elevated bilaterally at 34 years. At 40 years of age, she developed left pelvic pain radiating to the lumbosacral spine region. Computed tomography scan of the pelvis and abdomen, magnetic resonance imaging of the lumbosacral spine, and colonoscopy were normal. She was treated with carbamazepine for neuralgia. The patient also began to experience palpitations and chest pain. An electrocardiogram showed very frequent premature ventricular contractions and occasional premature atrial contractions. An echocardiogram was normal except for mild mitral valve prolapse. A treadmill test showed comparatively limited exercise tolerance. She had mildly elevated serum lactate and lactate/pyruvate ratio, but plasma carnitine and urine organic acids were normal.

Because of CPEO, ptosis, and retinopathy, a mitochondrial disorder was suggested. However, blood mtDNA analysis did

Key points

- The most common mitochondrial DNA (mtDNA) deletion syndrome, Kearns-Sayre syndrome, is characterised by adult onset, chronic progressive external ophthalmoplegia, pigmentary retinal degeneration, and at least one of the following: complete heart block, arrhythmia, cerebrospinal fluid protein above 1 g/L, diabetes mellitus, and/or cerebellar ataxia.
- About one third to one half of Kearns-Sayre syndrome patients have a common 5 kb mtDNA deletion. KSS is usually sporadic, and the deletion mutant mtDNA is typically detectable only in muscle.
- Here we report a 41 year old woman who had a normal childhood, puberty, and early adulthood, including two normal pregnancies, but developed chronic progressive external ophthalmoplegia, ptosis, and pigmentary retinopathy at the age of 30 years.
- Muscle histology showed characteristic ragged red fibres, but respiratory enzyme complex assays were normal. Molecular studies revealed a novel mtDNA deletion of 3078 bp between nt8419 and nt11498. The proportion of deleted mutant mtDNA was 92% in muscle, but only at levels detectable by PCR in her blood. A partial duplication mtDNA was detected in both blood and muscle.
- Real-time quantitative PCR analysis revealed an amplification of mtDNA in muscle of approximately ninefold, which probably explains the relatively mild clinical course of our patient despite such a high mutant load.
- This case illustrates the need to perform extensive mtDNA analyses, both qualitatively and quantitatively, in patients with features suggestive of mitochondrial disease, even if the mitochondrial respiratory enzyme complex assays are normal.

not reveal any mtDNA deletion or common point mutations. Muscle histology showed mild to moderate fibre size variation, type 2 fibre predominance, increased lipid, and numerous ragged red fibres (fig 1A). Electron microscopy showed mitochondrial proliferation with numerous, variably shaped mitochondria containing paracrystalline arrays

Abbreviations: CPEO, chronic progressive external ophthalmoplegia; CS, citrate synthase; KSS, Kearns-Sayre syndrome; mtDNA, mitochondrial DNA; PEO, progressive external ophthalmoplegia; rt-qPCR, real time quantitative polymerase chain reaction; SDH, succinate dehydrogenase

(fig 1B). Despite these findings, mitochondrial respiratory chain complex activities, including NADH dehydrogenase, succinate dehydrogenase (SDH), NADH-cytochrome c reductase, cytochrome c oxidase, and citrate synthase (CS) were all in the normal range (table 1).

The patient has been treated with coenzyme Q₁₀, vitamin B complex, and carnitine supplementation since several months before the muscle biopsy. The patient's two daughters, age 11 and 12 years, have no known health problems at this time, although the parents have noted some mild eyelid droopiness in their older daughter. Our patient has not given consent for further molecular testing of her children.

MATERIALS AND METHODS

Total DNA was extracted from muscle and blood according to published protocols.^{9–10} Mutational analysis of mtDNA for common point mutations and deletions was performed as previously reported.^{11–12} The location and the size of the deletion were determined by Southern analysis and polymerase chain reaction (PCR), followed by sequencing.⁵ The probes used for Southern analysis of mtDNA deletion and duplication were generated by PCR with four pairs of overlapping primers covering nucleotides (nt) 3361 to 15863 (probe I) for the detection of wild type (fig 2A), deletion mutant (fig 2B), and partially duplicated (fig 2C) mtDNA. Another PCR product, nt9104 to 11150 (probe II), was generated for the detection of the wild type (fig 2A), and partially duplicated (fig 2C) mtDNA. The Southern probe for 18S rRNA gene was generated by PCR with forward primer, 18S540F (5'-TTTCGAGGCCCTGTAATTGG-3'), and reverse primer, 18S1548R (5'-CGCTGAGCCAGTCAGTGTA-3') (Genbank accession number U13369). MtDNA was digested with *Eag*I, *Hind*III, or *Sna*BI under the conditions recommended by the manufacturer (New England Biolabs, Beverly, MA, USA). The restriction fragments were separated by electrophoresis on a 0.6% agarose gel and transferred to a positively charged nylon membrane (Zeta-Probe, Bio-Rad, Hercules, CA, USA). The membrane was hybridised with α -³²P-dCTP labelled probe prepared with *Rediprime*TM II random prime labelling system (Amersham Biosciences Inc, NJ, USA). The percentage of deleted mtDNA was analysed densitometrically on a Macintosh computer using the NIH Image program (<http://rsb.info.nih.gov/nih-image>). The primers used for the PCR detection of the deletion were mtF8295 and mtR11757. Those used for the detection of wild type mtDNA were mtF11091 and mtR11757. The PCR conditions were those described previously.^{11–12} DNA sequencing was carried out with a BigDye Terminator Cycle Sequencing Ready Reaction Kit analysed on an ABI 377 autosequencer (both Applied Biosystems, Foster City, CA, USA). The locations of the oligonucleotide primers were indicated by the number of the nucleotide at the 5' end with forward (F) or reverse (R) direction. The numbering of the nucleotides was based on GenBank sequence (accession number NC001807) and also in the mitomap database (<http://www.mitomap.org>).

Real time quantitative PCR (rt-qPCR) analysis of mtDNA copy number was performed in duplicate using 1× *Taq*Man Universal PCR Master Mix (ABI P/N 4304437), on an ABI-Prism Sequence Detector System 7700 and analysed with SDS software (version 1.7).¹³ Two mtDNA probes in the undeleted regions were used for the measurement of mtDNA proliferation. Briefly, the primers for rt-qPCR analysis of mtDNA were mtF3212/mtR3319 (tRNA-leu region) and mtF16498/mtR32 (D-loop region). Each primer was 20 nucleotides long starting from the 5' of the nucleotide position of the forward (F) or the reverse (R) primer. Those for the nuclear DNA (nDNA), microglobulin gene (β 2M

Figure 1 Muscle histochemistry and electron micrograph. (A) Cryosection of quadriceps muscle biopsy showing numerous ragged red fibres and prominent variation in myofibre sizes (modified Gomori trichrome, × 200). (B) Electron micrograph demonstrating subsarcolemmal accumulation of numerous mitochondria, some of which are enlarged and contain crystalloid inclusions. Original magnification × 17600.

gene), were β 2MF542 (5'TGCTGTCTCCATGTTTGATGTATCT 3') and β 2MR627(5'TCTCTGCTCCCCACCTCTAAGT3'). The nucleotide position number is based on Genbank sequence accession number NM004048. Position 1 is where the RNA starts. The PCR products were 108, 104 and 86 bp for mtDNA

Table 1 Respiratory enzyme complex activities		
Enzyme complex	Patient	Control
Complex I		
NADH dehydrogenase	10.83	14.74 (4.48)
Complex I/III		
NADH cytochrome c reductase	0.67	0.81 (0.20)
Complex II		
Succinate dehydrogenase	0.82	0.87 (0.21)
Complex II/III		
Succinate cytochrome c reductase	0.93	1.03 (0.31)
Complex IV		
Cytochrome c oxidase	2.72	2.43 (0.70)
Citrate synthase	13.52	15.74 (4.44)

Controls are expressed as mean (SD).
Enzyme units are expressed as μ mol/min/g of tissue.

Figure 2 Diagram showing mitochondrial DNA deletion, partial duplication, restriction sites, and location of probes I and II. (A) Wild type, full length mtDNA; (B) deletion nt8420–11498 mutant mtDNA; (C) partially duplicated mtDNA.

tRNA-leu region, mtDNA D-Loop region, and nDNA respectively. The *Taq*Man probes; 6FAM-5'-TTACCGGGCTCTGCC ATCT-3'-TAMRA, 6FAM-5'-CTTAAATAAGACATCAGATGG ATCAC-3'-TAMRA and VIC-5'-TTGCTCCACAGGTAGCTCTA GGAGG-3'-TAMRA, were labelled at the 5' end with a fluorescent reporter, 6FAM, 6FAM, or VIC, for mtDNA tRNA-leu region, mtDNA D-Loop region, and nDNA, respectively, while the 3' ends were labelled with a quencher, TAMRA. During the amplification, the increase in fluorescence was recorded in real time at the extension step of every cycle. Real time PCR conditions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds denaturation at 95°C, 60 seconds annealing/extension at 60°C. Both the mtDNA and the nDNA were amplified under the same PCR conditions. Standard DNA for the mtDNA tRNA-leu region, mtDNA D-loop region, and the nDNA β 2M gene were generated from cloned PCR products of mtF3212/mtR3319, mtF16498/mtR32, and β 2MF542/ β 2MR627. Serial dilutions were made and the rt-qPCR reactions were performed to construct the standard curve using the threshold cycle number, C_T , and the copy number of mtDNA and β 2M DNA template in the reaction mixture.¹³ As there are multiple copies of mtDNA per nucleus in muscle, the original DNA template solution was properly diluted to 0.2–2 ng/ μ l, and 1 μ l was used in a 25 μ l reaction mixture for rt-qPCR measurements in order to obtain optimal results.

Mitochondrial respiratory chain enzymes were measured in 5% (w/v) muscle homogenates as previously described: NADH dehydrogenase;¹⁴ NADH cytochrome c reductase;¹⁴ succinate dehydrogenase;¹⁵ succinate cytochrome c reductase;¹⁵ cytochrome c oxidase;¹⁶ citrate synthase.¹⁷ Enzyme activities are expressed as μ mol/min/g of tissue (wet weight).

RESULTS

Southern analysis revealed an mtDNA deletion of about 3.0 kb in the muscle specimen but not in the blood (fig 3A). The percentage of deleted mtDNA (2.4 kb band versus 5.4 kb band of *Hind*III digest) was calculated to be approximately 92% in this patient's muscle sample. The possibility of mtDNA duplication was also investigated by Southern analysis of the *Sna*BI digest. A linearised partially duplicated mtDNA band was detected (fig 3B and 3C, l-dup). Figs 3B and 3C were duplicated DNA blots. Blot B was hybridised

with probe I, which detected all mtDNA species, and blot C was hybridised with probe II, which detected only the DNA species containing the deleted regions. *Sna*BI has a single cleavage site at nt10736 that is within the deleted region of the mtDNA. Therefore, *Sna*BI digestion will produce a linearised DNA band that will hybridise with the probe generated from the deleted region (probe II, nt9104–11150) only if duplication (joining of the intact mtDNA with the mutant mtDNA) is present. As the wild type full length mtDNA comprised only about 8% of the total mtDNA, the amount of full length mtDNA available for duplication was very low. Thus, it showed as a very light band (fig 3C, l-dup) under *Sna*BI digestion. This faint band was masked by the large amount of the circular deletion form of the mutant mtDNA that was not digested by *Sna*BI (fig 3B). The c-del band shown in *Eag*I digest was a result of incomplete digestion by *Eag*I. *Eag*I cleaves the mtDNA at nt2567 and linearises both the wild type and the deleted mtDNAs. The l-nl and l-del bands shown in the uncut lanes were probably due to mechanical shearing during DNA isolation (figs 3B and 3C). The top bands of muscle specimen in *Sna*BI and uncut lanes are probably concatamers.

Fig 4A illustrated the primers (mtF8295 and mtR11757) used in PCR to localise the deletion. The deletion was readily detected in muscle but it was present at a much lower level in blood (fig 4B, lane 2). Subsequent sequence analysis of the junction fragment revealed a novel 3078 bp deletion between nt8419 and 11498 (fig 4C). The deleted region of mtDNA encodes the genes for ATPase 8, ATPase 6, COX III, tRNA Gly, ND3, tRNA Arg, ND4L, and ND4. There was a direct repeat of 5 bp, ACACT, flanking the deleted region, indicating that the deletion boundary may lie either between nt8420–8424 or nt11498–11502 (fig 4C).

Because the proportion of the deleted mtDNA was high (92%), and the respiratory enzyme complex activities were all within the normal range (table 1), it was suspected that the total number of mitochondrial genomes might have been amplified. Southern analysis revealed that the mtDNA in this patient's muscle was proliferated compared with normal muscle controls (fig 5A, row I). In order to measure the mtDNA proliferation more accurately, rt-qPCR analysis was performed using two mtDNA probes in tRNA-leu and D-loop regions (fig 5A, rows II and III). Using two different probes

Figure 3 Southern blot analysis of mtDNA deletion. (A) *Hind*III digest of patient's muscle DNA hybridised with mtDNA probe I (nt3361–15863). In addition to the normal 5.4 kb band, the patient had a 2.4 kb band, suggesting a heteroplasmic deletion of approximately 3 kb region. nl, normal; p, patient. (B) *Eag*I and *Sna*BI digest of patient's blood (B) and muscle (M) DNA hybridised with probe I (nt3361–15863). c-nl, circular normal; l-dup, linear duplicate; c-del, circular deletion; l-nl, linear normal; and l-del, linear deletion. (C) Same as B, but hybridised with probe II (nt9104–11150).

should prevent any differences in PCR efficiency. Consistent results were obtained with these two probes. Six normal muscle specimens were used as controls. The mtDNA content in muscle varies among different individuals, consistent with our previous finding.¹³ As the mtDNA content increases about 3–4-fold from birth until 5 years of age,¹³ patient 8, a 1.4 year

old child, was excluded from the analysis. The mean (SD) mtDNA/nDNA ratio of the normal control was 611 (199). The mtDNA/nDNA ratio of the proband was 5311 (68). Thus, the mtDNA in the proband's muscle was amplified approximately ninefold. Because our patient had ~8% of wild type mtDNA in the muscle, with a ninefold amplification, the total

Figure 4 Detection of mtDNA deletion by PCR. (A) Diagram showing the positions of primers used for the amplification of the junction fragment and the size of the PCR products. (B) Agarose gel electrophoresis of PCR products. Lane M, 100 bp marker; lanes 1–4, PCR with primers mtF8295 and mtR11757 for the detection of the deletion junction; lanes 5–8, PCR with primers mtF11091 and mtR11757 for the detection of undeleted wild-type mtDNA; lanes 1 and 5, DNA from muscle; lanes 2 and 6, DNA from blood; lanes 3 and 7, DNA from blood of normal control; lanes 4 and 8, no DNA template control. (C) Electropherogram of the DNA sequences at the deletion junction region and the sequence alignment of the junction fragment with the wild-type mtDNA sequences, showing the 5 bp direct repeat.

Figure 5 Measurement of mtDNA proliferation by Southern blot and real time quantitative PCR. A. 0.15 µg of total muscle or blood DNA was digested with *Eag* I. Lane 1, proband's muscle DNA; lane 2, proband's blood DNA; lanes 3–8, muscle DNA from normal individuals of 43, 91, 23, 80, 70, and 1.4 years of age. Row I, intensity ratio of mtDNA/18S rRNA gene; Row II, rt-qPCR results of mtDNA/nDNA copy number ratio with mtDNA tRNA-leu probe; row III, rt-qPCR results of mtDNA/nDNA copy number ratio with mtDNA D-loop probe; row IV is the average of ratios from these two measurements. (B) Correlation of results from Southern and rt-qPCR analyses.

amount of the wild type mtDNA would be about 72% of the normal level. Fig 5B shows the correlation between the results from Southern and rt-qPCR analyses.

DISCUSSION

We identified a novel mtDNA deletion, which constituted 92% of the muscle mtDNA of a 41 year old woman who presented with atypical KSS at 30 years of age. Although the histochemical and electron microscopic studies of our patient's muscle biopsy revealed abnormalities consistent with an underlying mitochondrial cytopathy, the respiratory chain enzyme activities were all within the normal range. The molecular finding of an mtDNA deletion at 92% heteroplasmy in this patient's muscle specimen, however, confirmed the diagnosis of an mtDNA deletion disorder. We demonstrated with rt-qPCR that this patient's mtDNA/nDNA ratio was approximately nine times higher than that of the normal control, consistent with the observation of mitochondrial proliferation from histochemical and ultrastructural studies. The discrepancy between the high level of mutant load (92%) and normal level of respiratory chain enzyme activities may be explained in theory by mitochondrial proliferation. With ninefold total mtDNA amplification and

8% intact mitochondrial genome heteroplasmy, the patient would have about 72% of normal mitochondrial genome content in the muscle, which might be enough to mask enzyme deficiencies in the mitochondrial respiratory chain. This also could explain the relatively mild and late onset of disease in our patient despite such a high mutant load. It should be noted that the high mutant load and the mtDNA proliferation was observed in skeletal muscle. As other tissues were not available for examination, it would be difficult to predict the multisystemic pathology.

The age of onset of KSS is usually before 20 years of age. Our patient did not manifest KSS until the age of 30 years. Also atypical was the finding of a novel 3 kb deletion rather than the common 5 kb deletion. Although most KSS cases are sporadic and the deleted mtDNA is usually only present in muscle, our patient does have deleted mtDNA in her blood at levels detectable by PCR. More extensive studies using *TaqMan* probes for rt-qPCR in the deleted and nondeleted regions revealed an approximately 3% deleted mutant mtDNA in the patient's blood specimen (data not shown). RFLP analysis of a *Sna*BI digest also revealed a low level of partially duplicated mtDNA.

Our patient's mother was asymptomatic; however, one of her two daughters was reported to have droopy eyelids. There are reports on the intergenerational transmission of duplicated mtDNA, but not deleted mtDNA alone. It is a general practice to inform women who harbour sporadic mtDNA deletions that there is a minimum risk of transmission to their offspring.¹⁸ However, in our case, the presence of partially duplicated mutant mtDNA in the blood and muscle implies a high risk for transmission of deleted mtDNA to offspring. Therefore, this case highlights the need for comprehensive mtDNA mutation analysis in potentially affected family members in order to provide accurate genetic counselling.

Testing for maternally inherited mitochondrial disorders presents special challenges. Molecular testing may reveal a possible susceptibility to disease, but does not predict the future development of the disease with certainty. Furthermore, precise counselling is difficult, even if an mtDNA mutation is identified, because of variability in disease expression secondary to tissue specific heteroplasmy. Although screening studies, such as cardiology, audiology, and ophthalmology evaluations, may be beneficial in some cases in detecting early involvement of a given organ system, there is no effective therapy for the majority of these disorders. In the absence of proven presymptomatic therapy, testing children for maternally inherited conditions with onset in adulthood should only be performed by experienced centres after careful consideration of the potential risks and benefits to the patient and family.

The electron microscopic ultrastructural analysis revealed mitochondrial proliferation in this patient's muscle biopsy. However, the activity of CS, a Krebs' cycle enzyme used as a marker for mitochondrial content, was within the normal range. These observations suggest that the apparent increase in the number of mitochondria is probably due to the amplification of the mitochondrial genome with a disproportionate increase in mitochondrial encoded respiratory chain proteins in the absence of increased nuclear encoded enzyme proteins such as CS or SDH. The normal respiratory chain enzyme activities in spite of the high degree of mutant heteroplasmy may also be explained by the cofactor therapy at the time of biopsy.¹⁹ The normal activities of CS and SDH with histochemical evidence of mitochondrial proliferation highlights the difficulties in arriving at an underlying diagnosis of mitochondrial disease, even in patients with highly suggestive clinical features. This case illustrates the importance of using all available laboratory studies including

histochemical/structural, biochemical, and molecular methods for the diagnosis of mitochondrial disorders. Negative results from one test should not discourage the utilisation of other studies.

MtDNA duplication in the case of mtDNA deletion syndrome has been reported.^{20, 21} We investigated the proliferation of mitochondrial genomes associated with mtDNA deletion syndrome. This case underscores the importance of measuring both the degree of mutant heteroplasmy and the total copy number of mitochondrial genomes for the explanation or prediction of disease outcome of patients with mtDNA deletion syndrome.

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