Single mitochondrial DNA deletions in chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndrome (KSS) patients from a multiethnic Asian population

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Abstract

Mitochondrial DNA (mtDNA) deletions are a major cause of chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndrome (KSS). We analyzed single mtDNA deletions in 11 CPEO and one KSS patients by means of Southern blot and long polymerase chain reaction (PCR) assays. The deletion sizes ranged from 3.4 kb to 6.9 kb whereas the heteroplasmy level varied from 18.8% to 85.5%. Two unique deletions sized 4320 bp and 4717 bp were found. This study represents the first genetic screen of mtDNA disorders in Malaysia, and it follows the data seen in other published reports on CPEO and KSS genetic aetiology.

INTRODUCTION

Compared to the approximate 30,000 genes in nuclear DNA, the human mitochondrial DNA (mtDNA) genome has only 16,568 bp and codes for only 37 genes. Nevertheless, defects in mitochondria genome can account for a range of mitochondrial diseases including chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome (KSS), Leber's hereditary optic neuropathy (LHON), mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) and myoclonus epilepsy and ragged-red fibres (MERRF).[1] Mutations in mtDNA can exist as point mutations, duplications, deletions or depletions. However, defects in nuclear genes also play a role in the development of mitochondrial disorders as mitochondrial protein synthesis, complex assembly, mtDNA replication and transcription require nuclear DNA encoded proteins.[2] Some mtDNA mutations are the result of nuclear DNA mutations, for example, *TWINKLE* gene mutations results in multiple mtDNA deletions (if heterozygous) and mtDNA depletions (if homozygous) while DNA polymerase gamma (POLG) mutations cause multiple mtDNA deletions and depletions.[3,4]

CPEO is characterized by eye muscle involvement and the patient presents with bilateral ptosis followed by progressive ophthalmoplegia and frequent retinal pigmentary abnormalities.[5] CPEO can occur as a specific syndrome or be part of another mitochondrial disorder e.g. MELAS or MERRF. Occasionally, there can be systemic involvement and the term CPEO-plus is used. KSS is defined as CPEO with early onset retinal degeneration before the age of 20 years in addition to one of the following characteristics: heart block, elevated cerebrospinal fluid protein or cerebellar ataxia.[6,7] Previous studies have shown that large-scale mtDNA deletions are commonly identified in patients with CPEO and KSS.[8,9] The deleted mtDNA fragments are usually several kilobase pairs in length and are contained within the major arc of two replication origins of light strand and heavy strand.[10] Most deletions are sporadic although some may be familial. Since mtDNA deletions result in different clinical phenotypes, factors including the length, location and heteroplasmy level of a deletion play a role in determining the clinical phenotype and disease severity.[11]

Malaysia is a Southeast Asian country with an ethnically mixed population consisting of Malays and other indigenous races, Chinese and Indians. The genetics of CPEO and KSS in Malaysian patients have not been previously reported. In this study, we present the mtDNA

deletion pattern of a group of Malaysian patients with CPEO and KSS diagnosed clinically and on muscle pathology and analysed the genotype and phenotype correlations.

METHODS

The muscle biopsy databank of the Department of Pathology, University of Malaya Medical Centre (UMMC), Kuala Lumpur, a tertiary referral centre for neuromuscular disorders in Malaysia, was reviewed for cases of CPEO or KSS. Muscle biopsy samples were obtained through open biopsy after the patient's written informed consent for diagnostic purposes. All muscle biopsies were snap frozen in isopentane chilled with liquid nitrogen and kept at -80°C until needed for analyses. The study was approved by the UMMC Medical Ethics committee.

Between 1999 and 2008, there were a total of 12 patients, 11 of which were diagnosed as CPEO and one as KSS. The diagnosis was based on the presence of clinical features of ptosis and ophthalmoplegia and supported by muscle histological findings of ragged red fibres (RRF) and/ or cytochrome C oxidase (COX) activity deficiency. DNA was extracted from patients' archived muscle tissue using standard phenol-chloroform method for mtDNA analysis.[12]

Southern blot hybridization

MtDNA deletions were examined by Southern blot using *Pvu*II in conjunction with a 16.3-kb mtDNA probe.[13] Probe labeling and chemiluminescent detection were carried out using Amersham Gene Images AlkPhos Direct Labelling and CDP-Star Detection System (GE Healthcare, Buckinghamshire, UK). Relative mutant load was quantified using Spot Densitometry in a gel imaging software (AlphaImager, AlphaInnotech, US). To distinguish duplications from deletions, a second Southern blot was carried out using a different restriction enzyme and probe; *Sna*BI and a probe corresponding to *ND4* gene.

Long PCR

In order to screen the entire mitochondrial genome for large deletions, 6.5-kb and 11.2-kb fragments corresponding to the minor arc and major arc respectively, were amplified with use of two overlapping primer pairs.[14] The two sets of primers were – Set A with primers p318/ p329, which corresponded to the 6.5 kbp fragment, and Set B with primers p1001/ p1004 which

corresponded to the 11.2 kbp fragment. Expand Long Template PCR System (Roche, Germany) was used to amplify the long fragments. The PCR reaction was carried out in a thermal cycler (Veriti 96-Well Thermal Cycler, Applied Biosystems, US) with cycling profiles of 30 cycles of 92°C for 10 s, 54°C for 30 s and 68°C for 5 min; 20 s elongation time was added for each successive cycle in the last 20 cycles; followed by a final elongation at 68°C for 7 min. For the amplification of the 11.2-kb amplicon, 8 min of elongation was used instead. PCR products were checked upon electrophoresis with a 0.8% agarose gel. Smaller fragments indicated a deletion was present.

Restriction mapping of deletions was performed as previously described.[15] The PCR fragment of 11.2 kbp was digested with *Xba*I, *Hin*dIII, and *Bam*HI (NEB, USA) in a reaction tube according to the manufacturer's specifications. Based on the digestive pattern, the deleted region was relatively confined and the flanking region was subsequently amplified with deletion-specific primers prior to direct DNA sequencing. Deletion junction was determined by aligning the sample sequence with the mtDNA reference sequence (AC_000021.2).

RESULTS

Clinical presentations of patients are shown in Table 1. The ages of disease onset ranged from 8 - 40 years (19.6 ± 10.7 years). Six were females (50%). The 11 CPEO patients were characterized by three major symptoms: ptosis in all patients (100%), ophthalmoplegia in 10 (90.9%) and muscle weakness in 4 (36.3%). These included facial, bulbar and truncal muscle weakness. One patient in addition, had sensorineural deafness. The patient with KSS had typical features with the age of onset at 13 years, retinitis pigmentosa, heart block and seizures in addition to CPEO symptoms. On muscle histopathology, RRF was seen in 11 of 12 patients (91.6%). Reduced or absent COX activity was seen in 10 (83.3%) including the patient without RRF.

Either by Southern blot (Figure 1) or long PCR, mtDNA deletions were detected in all patients. No duplication was found. On long PCR, with the Set A primers, a single band of 6.5 kb was obtained in all except for patient C7 (Figure 2), who showed an additional smaller fragment of ~1.8 kb, suggesting an infrequent deletion in the minor arc. With the Set B primers, we found the normal 11.2 kbp fragment as well as smaller fragments in all our patients (Figure 3), indicating

Table 1: Clinical, histological and genetic findings of the 12 patients

Patient	Age at presentation/ sex	Age at onset	Race	Ophthalmoplegia	Ptosis	Muscle weakness	Other manifestations	RRFs/ COX activity on muscle biopsy	Deletion size (bp)	Deletion junction	Direct repeat (bp)	Heteroplasmy level (%)
K1	13/M	ND	Malay	+	+	+	Retinitis pigmentosa, heart block, seizures, myopathic facies	+/ Reduced	6926	7506:14433	5	23.2
C1	18/F	8	Javanese	+	+	-	-	+/ Normal	4329*	10290:14260*	9/10	85.5
C2	18/M	13	Chinese	+	+	-	-	+/ Reduced	4977	8482:13460	13	48.5
C3	20/F	10	Chinese	+	+	+	Dysphagia, facial and truncal weakness	+/ Reduced	4977	8482:13460	13	73.6
C4	28/M	17	Chinese	+	+	-	-	+/ Loss	4977	8482:13460	13	63.3
C5	33/F	ND	Chinese	+	+	-	-	+/ Loss	4717*	10721:15439*	No	40.2
C6	33/M	12	Indian	+	+	-	-	+/ ND	4977	8482:13460	13	56.8
C7	39/F	32	Chinese	-	+	+	Weakness of facial, skeletal and bulbar, intermittent diplopia	-/ Loss	4681	470:5152	8	18.8
C8	41/M	24	Indian	+	+	-	Fatigue in chewing, neck muscle weakness	+/ Reduced	4977	8482:13460	13	57.7
C9	46/F	40	Indian	+	+	-	-	+/ Loss	4977	8482:13460	13	41.0
C10	51/M	20	Malay	+	+	+	Dysphagia, nasal speech, dyspepsia, bilateral cataract, sensory neuronal deafness, squint	+/ Reduced	4977	8482:13460	13	42.2
C11	57/F	ND	Chinese	+	+	-	-	+/ Loss	3399	8034:11434	11	50.2

* Unreported deletions; (-) absent; (+) present; (ND) not determined

Table 2: Breakpoint sequence homology in the 49nt sequence flanking the 5' and 3' breakpoints as analysed with the ClustalW software. Regions that show sequence homology are highlighted in bold. Patients C2, C2- C4, C6, C8- C10 all share the common 4,977-bp mtDNA deletions. Patient C5 did not have any direct repeats flanking the deleted site therefore, the letters underlined in C5 indicate the flanking nucleotides around the breakpoints of the deleted regions.

| Patient	49 nucleotides around 5' breakpoint	49 nucleotides around 3' breakpoint	ClustalW2 Score (% Homology at breakpoint)
K1	GGTTTCAAGCCAACCCCATGGCCT**CCAT**-GACTTTTTCAAAAAGGTATTA	CACCAAGACCTCAACCCCTGACCCC**CATG** CCT-CAGGATACTCCTCAATA	57.0
C1	CCCTCCTTTTACCCCTACCATGAG**CCCTACAAA**-CAACTAACCTGCCACT	AATAGGGAGAAGGCTTAGAAGAAAAA**CCCCA-CAAAC**CCCATTACTAAACCC	26.0
C2, C3 C4, C6, C8, C9, C10	AATATTAAACACAAACTACCACCTA**CCTCCCTCAC-CAAAG**CCCATAAAA	CTCAAAACCATACCTCTCACTTCAA**CCTCCCT-CACC**ATTGGCAGCCTAG	55.0
C5	CCTACTAGTCTCAATCTCCAACACAT**A**TGGCCTA-GACTACGTACATAAC	ACTACACAATCAAAGACGCCCTCGG**C**TTACT-TCTCTTCCTTCTCTCCTT	38.0
C7	CATTATTTTCCCCTCCCACTCCCA**TACTACTA**ATCT CATCAATACAACC	ACTTAAACTCCAGCACCACGACCCT**ACTAC-TA**TCTCGCACCTGAAACAA	57.0
C11	GACAATGCGAGTAGTACTCCCGATT**GAAGCCCCCAT**-TCGTATAATAATTA	TTATGACTCCCTAAAGCCCATGTCG**AAGCCCCCA-TC**GCTGGGTCAATAG	44.0
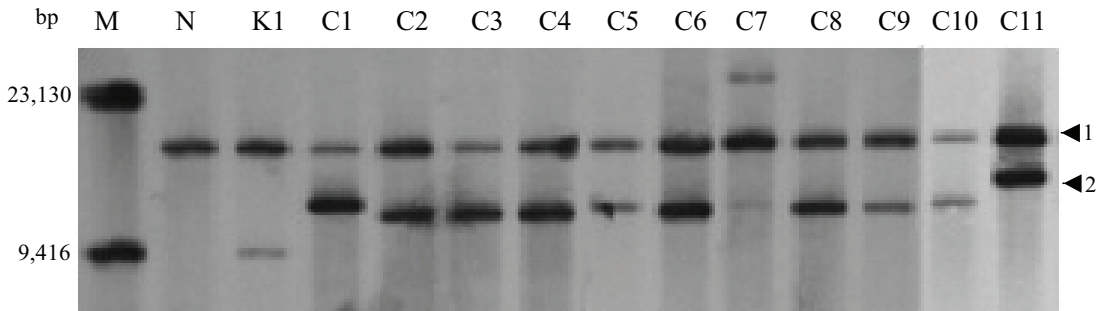
Figure 1. Autoradiogram of Southern blot hybridization analysis. Genomic DNA was digested with PvuII and probed with a near full-length probe. M, Lambda-HindIII DNA Marker. N, normal control; patient K1 with KSS, patients with CPEO: C1-C11. Two populations of mtDNA are present in all the patient samples except C7, one corresponding to the normal-length mtDNA (16.6 kb) (indicated by arrowhead 1) and another faster-migrating band indicates rearranged mtDNA molecule (indicated by arrowhead 2). The migrating rate for rearranged mtDNA is different in patients K1, C1, and C11, suggesting uncommon deletion. Patient C7 demonstrated three bands of different electrophoretic mobilities.

deletions were present.

Following restriction analysis using *Xba*I, *Hin*dIII, and *Bam*HI, the region flanking the deletion breakpoints were amplified and subjected to sequencing to ascertain the deletion size and location. These are summarized in Table 1. Deletion size ranged from 3.4 to 6.9 kb, the common 4977 bp deletion between nucleotide 8482 and 13460 was seen in 7 patients (58.3%). Three deletions (6926 bp between nt 7506 to nt 14433, 4681 bp between nt 470 to nt 5152, and 3399

bp between nt 8034 to nt 11434 in Patients K1, C7 and C11, respectively) have been previously reported. Two deletions (4329 bp between nt 10290 to nt 14260, and 4717 bp between nt 10721 to nt 15439 in Patients C1 and C5, respectively) have not been reported on MITOPMAP (www.mitomap.org). In the CPEO patients, nine to 11 genes were affected by deletions while in the KSS patient, the large 6926-bp deletion removed 17 genes from the mtDNA (Figure 4). This deletion has previously been described in
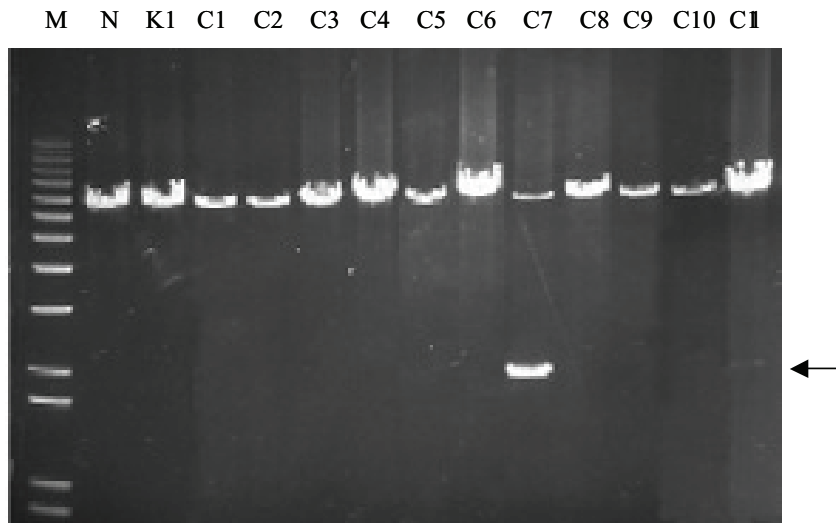

Figure 2. Amplification with primer set p318/ p329. M, 1 kbp Plus DNA Ladder; N, normal control; patient K1 with KSS, patients with CPEO: C1-C11. Arrow indicates normal mtDNA fragment of 6.5 kb. Patient C7 yielded two fragments of 6.5 kb and ~1.8 kb suggesting a deletion of ~4.7 kb within the minor arc.
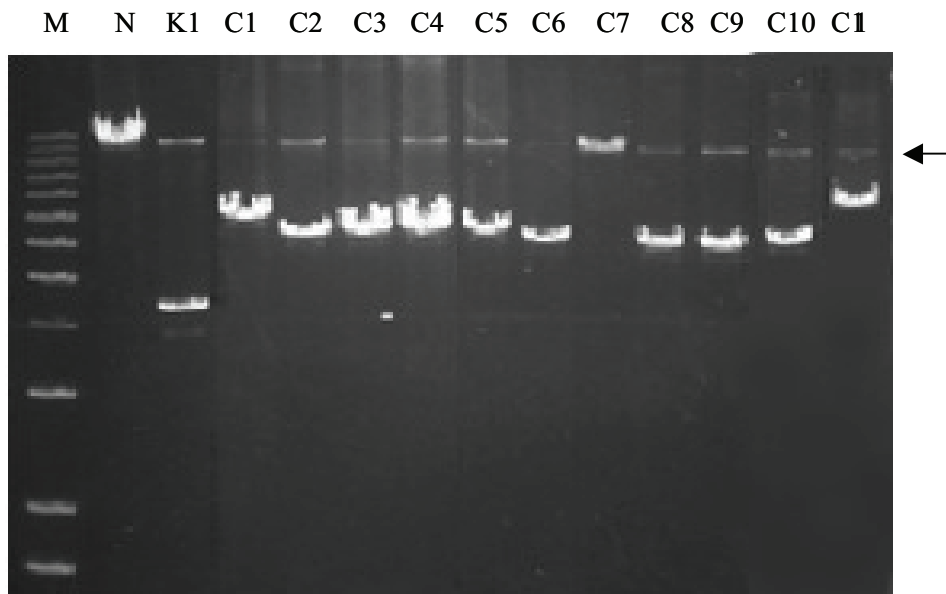
Figure 3. Amplification with primer set p1001/ p1004. M, 1 kbp Plus DNA Ladder; N, normal control; patient K1 with KSS, patients with CPEO: C1-C11. Arrow indicates a normal mtDNA fragment of 11.2 kb. Bands of lower molecular weight suggest the presence of deletions. Deleted bands are more prominent than the wild-type bands, owing to the preferential amplification of the smaller fragments.

another KSS patient (16). Direct repeats flanking the deletion breakpoints of 5-13 bp were found in 11 of 12 patients (91.7%). One patient (Patient C1) had an imperfect repeat while another had no flanking repeats (Patient C5, Figure 4).

We found that deleted mtDNAs coexisted with wild-type mtDNAs in a heteroplasmic state in all cases (Figure 1). By using Alphaview Denso Spot software, proportion of deleted mtDNA was quantitated. The levels of heteroplasmy ranged from 18.8-85.5% and did not appear to correlate with the severity of phenotype. The patient with CPEO who harbored the highest proportion (85.5%) of deleted mtDNA had only ophthalmoplegia and ptosis with no additional features. On the other hand, the patient with KSS had a lower percentage (23.2%) of deleted mtDNA.

The sequences surrounding the deleted regions were analysed for sequence homology using the ClustalW software, and were found to show an increased degree of sequence homology in most cases, ranging from 26% to 55% (Table 2).

DISCUSSION

Using established Southern blot and long PCR techniques, we found deletions in mtDNA in

our 12 patients with CPEO and KSS, with the common 4977 bp deletion in 7 patients (58.3%) previously reported but rarer deletions in 3 patients (including the single KSS patient) and 2 patients with previously unreported deletions.

In the two cases with the unreported deletions, the deleted regions overlapped to a certain degree with the commonly deleted regions, encompassing the *ND3 (in C1)*, *ND4L*, *ND4* and *ND5* genes. In both patients C1 and C5, the deleted regions extended further to encompass the *ND6* gene, while *MTCYB* was also deleted in patient C5. Mutations in *ND6* have been reported in Leigh syndrome, MELAS, LHON and LHON/dystonia syndrome (www.mitomap.org), while mutations in *MTCYB* have been associated with severe exercise intolerance and myopathy in most cases, but can also account for several other disorders including respiratory complex III deficiency[17], mitochondrial encephalopathy, cardiomyopathy, septo-optic dysplasia and multisystem disorders.[18] However in patient C5, the only symptoms were ophthalmoplegia and ptosis with no other obvious manifestations.

The patient with KSS had the largest deletion of 6926 bp and this most likely accounted for the more severe phenotype viz. earlier age of onset and

Patient	5'	Deletion region	3'	Affected genes
K1	$\overline{5}$ CCCATGGCC TC**CATG** tRNA-S	$\overline{5}$ [ACTTTTTCAAAAAGG.........CCCCTGACCC**CATG**] 6,926 bp (7507)	CCTCAGGATACTCCT (14433) ND6	TS1, TD, CO2, TK, ATP8, ATP6, CO3, TG, ND3, TR, ND4L, ND4, TH, TS2, TL2, ND5, ND6
C1	TACCCCTACCATGAG ND3	$\overline{9/10}$ [**CCCTACAAAC**AACTA.........AGGCTTAGAAGAAAA] 4,329 bp (10291)	$\overline{9/10}$ **CCCCACAAACCCCAT** (14620) ND6	ND3, TR, ND4L, ND4, TH, TS2, TLS, ND5, ND6
Common deletions: C2, C3, C4, C6, C8, C9, C10	$\overline{13}$ **CTACCTCCCTCACCA** ATPase8	$\overline{13}$ [AAGCCCATAAAAATA.........CA**ACCTCCCTCACCA**] 4,977 bp (8483)	TTGGCAGCCTAGCAT (13460) ND5	ATP6, CO3, TG, ND3, TR, ND4L, ND4, TH, TS2, TL2, ND5
C5	TCAATCTCCAACACA ND4L	[TATGGCCTAGACTAC.........CAAAGACGCCCTCGG] 4,717 bp (10722)	CTTACTTCTCTTCCT (15439) Cytb	ND4L, ND4, TH, TS2, TL2, ND5, ND6, TE, CYB
C7	$\overline{8}$ ACTCCCAT**ACTACTA** HVS3	$\overline{8}$ [CCGCCCATCCTACCC.........ACGACCC**TACTACTA**] 4,681 bp (479)	TCTCGCACCTGAAAC (5160) ND2	TF, RNR1, TV, RNR2, TER, TL1, ND1, TI, TQ, TM, ND2
C11	$\overline{11}$ GATT**GAAGCCCCCAT** COII	$\overline{11}$ [TCGTATAATAATTAC.........TGTC**GAAGCCCCCAT**] (8035)	CGCTGGGTCAATAGT (11434) ND4	CO2, TK, ATP8, ATP6, CO3, TG, ND3, TR, ND4L, ND4

Figure 4. **Deletion junctions.** Sequences are shown in direction 5'-3'. Sequences in the brackets have been removed by deletion. Deletion sizes are given between dashed lines below the deleted regions. Nucleotides highlighted in **bold** demonstrate direct repeats precisely adjacent the deletion junctions. Genes interrupted by deletion are shown below the sequence. All deletion regions except C5 are flanked by two direct repeats that are postulated to predispose mtDNA to deletion formation. Genes affected by the deletions are shown in the column on the right.
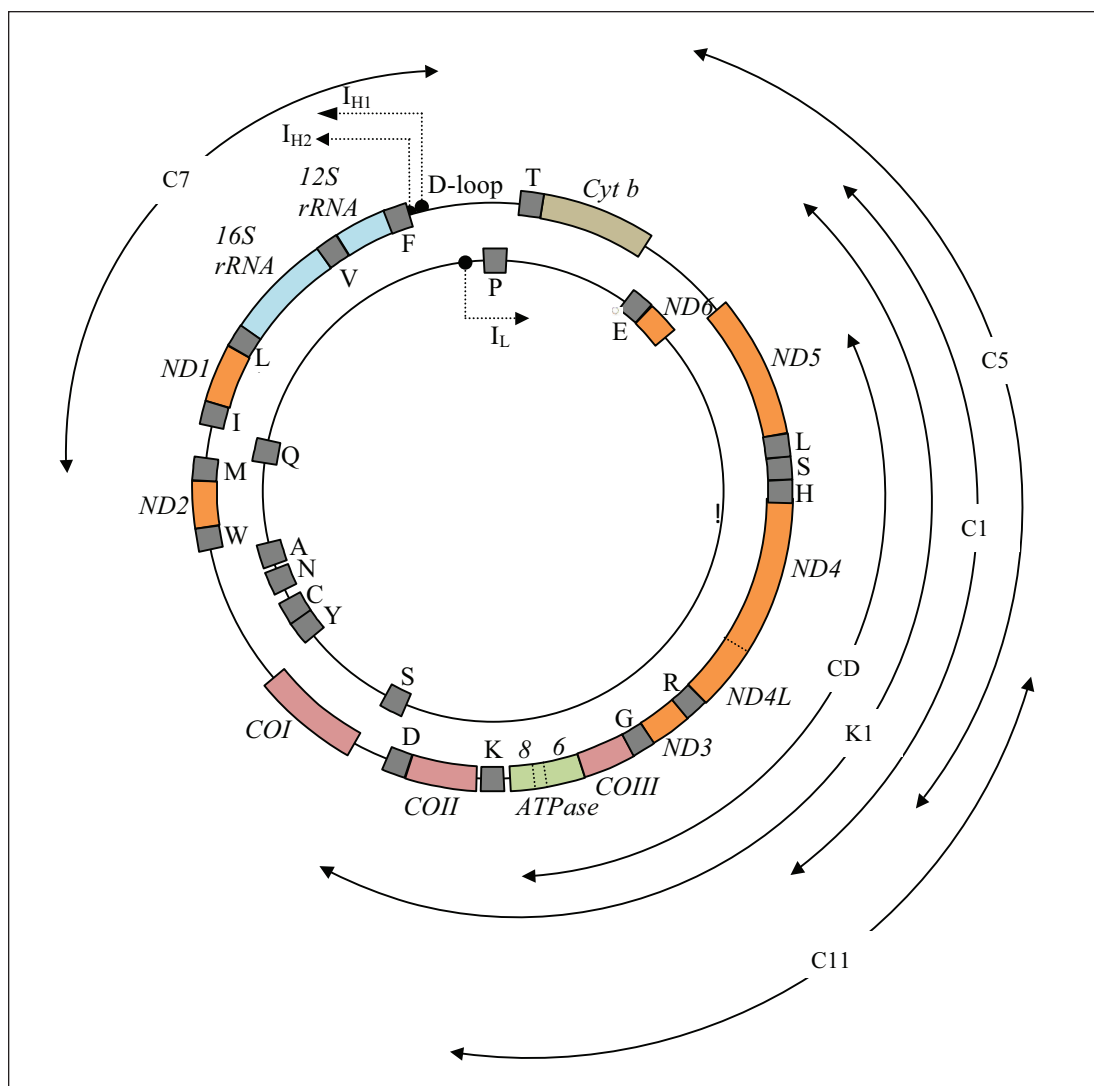
33

Figure 5. Deletion sites. The consensus common deletion is denoted by CD. The human mitochondrial genome encodes for 13 structural subunits (denoted in orange, tan, pink and green), two ribosomal RNAs (blue) and 22 transfer RNAs (grey). A 4329-bp unreported deletion in patient C1 encompasses nine genes with breakpoints in the ND3 and ND6. In patient C5, a 4717-bp unreported deletion spans nine genes, which extending from ND4L to Cyt b. All deletions confined to the major arc except C7 deletion.

multi-systemic involvement. On the other hand, he harboured only 23.2% of deleted mtDNA, which is at the lower end of the expected 20-80% range for KSS.[19]

Therefore, although deletion size correlated with disease severity, the percentage of heteroplasmy did not. This is consistent with previous findings that suggest that KSS patients tended to have larger deletions but that the proportion of heteroplasmy in skeletal muscle did not correlate with severity.[11]

We were able to map all deletions to the major arc between the 2 origins of replication except in 1 patient (Patient C7, Table 1 and Figure 5). The distribution of deletions on the major arc is consistent with the previously reported pathogenic deletions.[10] Patient C7 had a 4,681-bp deletion in the minor arc, which removed the rRNA genes, major heavy strand promoter (HSP) and minor HSP. This deletion has been previously described in a Turkish man with CPEO, who had 21% of deleted mtDNA.[20] The 3399-bp deletion found in

another our CPEO patients (Patient C11, Table 1 and Figure 5) was previously reported in a patient with oculopharyngeal weakness.[21] However, the latter also harbored a pathogenic homoplasmic T5814C mutation, which might explain her pharyngeal weakness. Our patient did not have this clinical or genetic feature.

In 10 out of 12 cases, the deletion junctions were flanked precisely by two perfect direct repeats ranged from 5-13 bp. This is usually explained by the suggestion that the formation of mtDNA rearrangements occurs due to either slipped replication or illegitimate recombination via direct repeats.[22] In our present study, patients C1 and C5 represent the minor group of deletions with imperfect direct repeat and no repeat, respectively and their deletions (Figure 4) have not been reported to date. Therefore, in their case, the cause of mtDNA deletion may occur via a different, as yet unknown mechanism. A study by Yamashita and colleagues also identified patients with incomplete direct repeats longer than 13 bp with one mismatch.[11] A study of 263 deletions showed the uniform distribution of repeats throughout the mitochondrial genome.[10] They reason that since the major arc is more prone to deletions, there may be a common mechanism of deletion formation, which is associated with replication and irrespective of the repeats. Based on our data, we concur that a repetitive sequence represents a predictor of a potential site of mtDNA deletion in most cases.

Recently, Sadikovic *et al*. proposed that apart from just direct repeats flanking regions of mtDNA deletions, the sequence around the deleted sites also showed a high degree of sequence homology.[23] We thus tested for sequence homology around our deleted regions to see if this was the case in our cohort. Using the ClustalW software, we analysed 49nt of our patients' mtDNA sequences for any evidence of sequence homology. We found that in most cases, there was an increase in sequence homology within the close proximity of the breakpoint, averaging 50%. Patient K1 who had the largest deletion, showed the highest degree of sequence homology (57%). Patient C7 also shared the same high degree of sequence homology as KI, even though he had a smaller deletion. Interestingly, patient C5 who did not have any direct repeats flanking the 4.7kbp deleted site had the lowest degree of sequence homology of all (38%). The study by Sadikovic and colleagues suggest that younger patients have lower sequence homology than older affected age groups.[23] Unfortunately, we do not have any record of an exact age of onset for our patient C5 to compare with their data. Although we have fewer patients than Sadikovic *et al*., nonetheless our study adds evidence to the indication that breakpoint sequence homology is a feature of mtDNA deletions.

In conclusion, our study found single mtDNA deletions in Malaysian patients with CPEO/KSS. The common 4977 bp mutation was seen in the majority but found in addition 2 unreported deletions which do not fit into the current idea of how mtDNA deletions occur. Documentation of deletions is crucial to identify these possible factors based on the molecular, biochemical and clinical correlation.

ACKNOWLEDGEMENTS

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