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The frequency of common mitochondrial DNA mutations in a cohort of Malaysian patients with specific mitochondrial encephalomyopathy syndromes

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Abstract

A cohort of Malaysian patients with clinico-pathological diagnosis of three specific mitochondrial encephalomyopathy syndromes comprising of mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonus epilepsy with ragged-red fibers (MERRF) and Leigh syndrome were studied to determine the frequency of their common mitochondrial DNA mutations. The 'hot-spot' point mutations for MELAS, MERRF and Leigh syndrome were screened. In the absence of common point mutations, screening of large-scale deletions as well as sequencing of $tRNA^{Leu}$ and $tRNA^{Lys}$ genes were performed. Of 22 patients studied, nine m.3243A>G mutations, four m.8344A>G mutations, one m.8993T>G mutation and one deletion were identified (65% detection rate). While the m.3243A>G mutation was closely associated with MELAS, the m.8344A>G was more heterogenous, being seen in one MERFF, two isolated mitochondrial myopathies and one Leigh syndrome patient. Screening for m.8993T>G in Leigh syndrome has a low yield as unsurprisingly Leigh syndrome has considerable genetic heterogeneity.

INTRODUCTION

Mitochondrial encephalomyopathies are a group of clinically and genetically heterogeneous disorders characterised by degeneration and dysfunction of high energy-requiring tissues such as muscle, owing to a deficiency in the mitochondrial respiratory chain. The mitochondrial respiratory chain system is made up of about 87 protein subunits, forming 5 multiprotein complexes, of which 13 are encoded by the mitochondrial genome and the rest by nuclear genome.[1] In addition, the mitochondrial genome contains 22 tRNAs and two rRNAs genes involved in mitochondrial transcription and translation.[2] Since the identification of the first mtDNA mutation in pedigrees with Leber hereditary optic neuropathy (LHON)[3], mtDNA pathogenic mutations have increasingly been described.[4]

Several mtDNA mutations are typically described for specific clinical syndromes e.g. mitochondrial encephalopathy, lactic acidosis and stroke-like syndrome (MELAS) associated with m.3243A>G mutation, myoclonic epilepsy with ragged-red fibres (MERRF) with m.8344A>G, and Leigh syndrome with m.8993>C/G mutations.[1] However, different mtDNA mutations can result in the same clinical syndrome while a specific mutation can result in different clinical phenotypes.

A definitive molecular diagnosis in a suspected mitochondrial disorder is important in confirming the diagnosis and identifying the specific genes and mutations. This may vary in different populations. The spectrum of the common mtDNA mutations and their phenotype correlation in Malaysian patients with mitochondrial diseases has not been previously described. In this paper, we describe the frequency of the common mtDNA mutations in a group of patients with clinico-pathological diagnosis of three specific mitochondrial encephalomyopathy syndromes.

METHODS

A retrospective study of archived muscle biopsy and blood samples referred to the Department of Pathology, University of Malaya Medical Centre between 1996 and 2008 revealed 22 patients with clinic-pathological diagnosis of mitochondrial

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encephalomyopathy. Of these, 11 were diagnosed as MELAS, 3 as MERRF and 6 as Leigh syndrome. Patients were diagnosed as MELAS if they had at least 2 of the following clinical features of stroke-like episodes, encephalopathy with seizures or dementia or recurrent headache/vomiting as well raised serum lactate or the presence of ragged-red fibres (RRF) on muscle biopsy.[5] Patients were diagnosed as MERRF if they had 2 of the following clinical features myoclonus, epilepsy or ataxia as well as raised serum lactate or RRF on muscle biopsy.[6] On the other hand, Leigh syndrome patients were selected primarily according to their clinical features because muscle histopathological changes are often absent. This included psychomotor delay, hypotonia, ataxia and lactic acidosis and/or neuroimaging changes in the basal ganglia, brainstem and cerebellum.[7] In addition another 2 patients had isolated muscle weakness with RRF on muscle biopsy suggesting mitochondrial myopathy.

A systematic molecular genetic analysis to look for the common mutations based on the clinical diagnosis was carried out (Figure 1). The study protocol was approved by the Medical Ethics Committee of University Malaya Medical Centre (reference number: 697.14).

DNA extraction

Total genomic DNA was isolated from eight sections (8 μm) of frozen muscle tissue using standard phenol/ chloroform extraction and ethanol precipitation method.[8] DNA was dissolved in distilled water and stored at -20˚C.

PCR/ RFLP analysis

Mitochondrial DNA encompassing the mutated

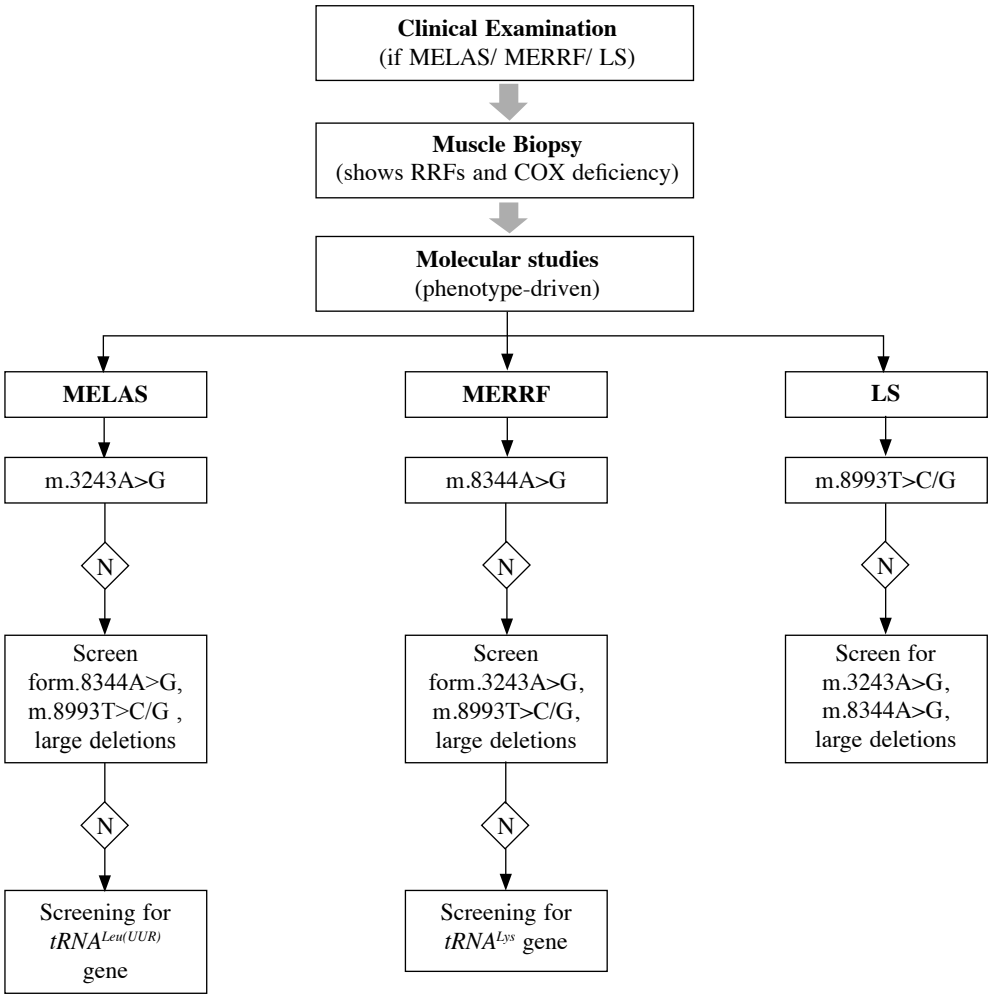

Figure 1. Flowchart summarises the steps for the screening of mitochondrial encephalomyopathies. N, negative test result

Table 1: Clinical data of 11 patients with MELAS features

Patient	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
Age/Sex	4/M	9/F	18/M	18/F	20/M	23/M	28/M	29/M	29/F	31/M	52/F
Age at onset	2/52	8	14	ND	16	21	28	ND	17	28	23
Race	Malay	Murut	Chinese	Chinese	Chinese	Chinese	Chinese	Indian	Chinese	Kadazan	Chinese
Family history	-	+	-	-	-	-	-	-	-	+	-
Stroke-like episodes	-	-	+	-	-	-	+	-	+	-	+
Seizures	-	+	+	-	+	+	+	+	+	+	+
Hearing loss	-	+	+	-	+	+	+	+	-	-	-
Cardiomyopathy	-	+	-	-	-	-	-	-	-	-	-
Short stature	-	-	-	+	-	-	-	-	+	-	-
Myopathy	-	+	-	+	+	-	+	+	-	-	-
Migraine-like headache	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	ND
Vomitting	+	ND	+	ND	ND	+	ND	ND	ND	ND	ND
Dementia or mental retardation	-	-	+	-	-	+	-	+	+	-	+
Visual impairment	+	+	-	-	+	+	-	+	+	+	+
Ptosis	-	+	-	+	-	-	-	-	-	-	-
Opthalmoplegia	-	+	-	+	-	-	-	-	-	-	-
Other manifestations	Nominal aphasia, hyperreflexia	-	-	-	-	Hemiparesis	-	-	-	-	-
CSF lactate (1.1-2.4 mmol/L)	ND	ND	ND		ND	ND	ND	ND	ND	ND	ND
Serum lactate (0.6-2.4 mmol/L)	6.1	ND	1.5		2.84	4.6	4.0	ND	ND	1.8	ND
Neuroradiological abnormalities	ND	Basal ganglia calcification, cerebral atrophy, focal lesions	Occipital infarct	-	Occipital infarct	Occipital infarct	Cerebral infarct	Atrophy, infarct	Cerebral infarct	Occipital infarct	Global cerebral atrophy
RRFs in muscle	+	+	+	+	+	+	+	+	+	+	+
COX activity	Increased	Reduced	ND	-	ND	ND	Reduced	Reduced	Normal	ND	Reduced
m.3243A>G	+	+	+	+	+	+	+	+	+	-	-

M, Male; F, Female; +, Present; -, Absent; ND, Not determined

Table 2: Summary of symptoms in 11 MELAS patients

Symptom(s)	Number of cases	Percent (%)
Seizures	9	82
Visual impairment	8	73
Hearing loss	6	55
Occipital brain infarct	6	55
Myopathy	5	45
Dementia or mental retardation	5	45
High lactate level	4	36
Stroke-like episodes	4	36
Vomiting	3	27
Ocular myopathy	2	18
Short stature	2	18

sites 3243, 8344 and 8993 were amplified with primers pairs spanning np 3130-3149/ 3423-3404, 8155-8176/ 8345-8366 and 8839-8857/ 9017-8998 (courtesy of National Institute of Neuroscience, Tokyo), (GenBank accession number: NC_012920) respectively.[9,10] PCRs were carried out in a 25 µl final reaction volume containing 1 × *Taq* buffer, 2.5 mM Mg^{2+}, 200 µM of each dNTP, 12.5 pmol of each forward

Table 3: Clinical data of five patients with MERRF features or mitochondrial myopathy

Patients	R1	R2	R3	R4	R5
Age/Sex	8/M	18/F	23/F	25/M	31/M
Age at onset	3	ND	5	2	ND
Race	Chinese	Malay	Chinese	Chinese	Malay
Family history	-	+	+	+	+
Myoclonic epilepsy	+	-	+	+	-
Ataxic gait	-	-	-	+	-
Seizures	+	-	+	+	-
Muscle weakness	+	+	-	+	+
Dementia or mental retardation	-	-	+	+	-
Other manifestations	ptosis	-	-	-	Areflexia
CSF lactate (1.1-2.4 mmol/L)	ND	ND	ND	1.4	ND
Serum lactate (0.6-2.4 mmol/L)	8	0.328	ND	ND	ND
Neuroradiological abnormalities	ND	ND	Cerebral atrophy	ND	ND
RRFs in muscle	-	+	+	+	+
COX activity	Increased	Reduced	ND	ND	Reduced
m.8344A>G	+	+	-	-	+

M, Male; F, Female; +, Present; -, Absent; ND, Not determined

Table 4: Clinical data of six patients with Leigh syndrome **features**

Patient	L1	L2	L3	L4	L5	L6
Age/Sex	8 months/ F	1/F	1/F	2/M	2/M	9/M
Age at onset	Since birth	6mth	6mth	ND	ND	9
Race	Chinese	Chinese	Chinese	Indian	Malay	Chinese
Family history	-	-	-	+	-	-
Parental consanguinity	-	-	-	+	-	-
Global developmental delay	+	+	+	+	+	-
Respiratory failure	+	-	+	+	+	+
Hypotonia	-	+	+	+	+	+
Hyperreflexia	+	+	+	-	-	+
Seizures	+	+	-	-	+	-
Muscle weakness	+	-	-	-	-	-
Optic abnormalities	-	-	-	-	-	-
Failure to thrive	-	-	+	-	-	-
Hypotelorism	-	-	-	+	+	-
Macrocephaly	-	-	-	+	-	-
Microcephaly	-	+	-	-	-	-
Strabismus	-	+	-	-	-	-
Sensorineural deafness	ND	+	-	-	-	+
Other manifestation	Floppyness, hypertonia	Astigmatism	Proximal weakness	-	Divergent squint, dystonic	Retinitis pigmentosa, heart block, short stature, unsteady gait, mild mental retardation, cataract
CSF lactate (1.1-2.4 mmol/L)	ND	4.3	4.7	ND	ND	ND
Serum lactate (0.6-2.4 mmol/L)	ND	6.91	8.64	3.65	Normal	ND
Muscle biopsy	RRFs, reduced COX	Normal	Non specific changes	Non specific changes	Normal	RRFs
Neuroimaging abnormalities	ND	Frontal lobe/ cerebral atrophy with demyelination, white matter changes, hypodense basal ganglia	Hypointense in gray and white matters	Cerebellar atrophy	Brain stem lesion, extensive midbrain, pons, medulla, hyperintensity involving deep nucleus	Hypodense lesion at the thalamus/ basal ganglia
MtDNA mutation	m.8344A>G	m.8993T>GT8993G	-	-	-	m.6843_13583del6739

M, Male; F, Female; +, Present; -, Absent; ND, Not determined

and reverse primers, 1.25 units of *Taq* DNA polymerase (Fermentas, USA) and 100 ng of total DNA template. Restriction enzyme analyses of the amplicons using *Apa*I (m.3243A>G), *Ban*II (m.8344A>G) or *Hpa*II (m.8993A>C/G) were carried out as described previously according to the manufacturer's recommendation (NEB, UK). In the case of *Hpa*II (m.8993A>C/GA8993C/G), primers were designed to contain an A to C mismatch with the template sequence at nucleotide 8347, thus introducing a diagnostic restriction site into the mutant amplicon. Digested products for m.3243A>G and m.8993A>C/G analyses were electrophoresed on a 2% Seakem LE Agarose gel (Cambrex, USA). For the analysis of m.8344A>G mutation, a 4% Metaphor 3:1 Agarose gel was used.

Southern blot hybridization

Southern blot hybridization was carried out to detect mtDNA large-scale deletions. The mtDNA probe was a 16,231-bp PCR product amplified using DNA polymerase having 3'→5' proofreading activity (TaKaRa LA Taq, TaKaRa, US) with primers described previously.[11] Probe labeling and blot hybridization was performed using Amersham Gene Images AlkPhos Direct Labelling and CDP-Star Detection System (GE Healthcare, Buckinghamshire, UK). Briefly, one μg of total muscle DNA was digested with *Pvu*II (NEB, USA), electrophoresed on a 0.8% agarose gel, transferred to a nylon membrane (GE Healthcare, Buckinghamshire, UK), hybridized to the labeled mtDNA probe and the bands were visualized using chemiluminescent detection with CDP-Star.

DNA sequencing analysis

Sequencing analysis of $tRNA^{Leu}$ and $tRNA^{Lys}$ were performed after excluding the common point mutations and large deletions in our patients, as previously reported.[11] Briefly, purified-PCR fragments consisting of np 3130-3404 and np 7804-8380 corresponding to $tRNA^{Leu}$ and $tRNA^{Lys}$, respectively, were subjected to direct sequencing. The sample sequence was compared with reference sequence (NC_012920) to locate any nucleotide variants.

RESULTS

Of the 22 patients, 15 patients (68.2%) were confirmed to have mtDNA mutations. Their mean age was 16.4 years (8-months to 31-years) and 8 were males. Of these, 10 were ethnic Chinese, 3 ethnic Malays, 1 ethnic Indian and 1, Murut, an indigenous ethnic group of Sabah. The clinical features and investigation findings of all 22 patients are summarised in Tables 1- 4.

Molecular analysis showed the presence of the m.3243A>G mutation in 9 of 11 (81.8%) MELAS patients. All patients showed RRFs on muscle biopsies with or without reduced COX activity. For those negative for the m.3243A>G mutation, the $tRNA^{Leu(UUR)}$ gene was screened by direct sequencing but no mutation was found.

One out of 3 MERRF patients (33.3%) had the m.8344A>G mutation. This patient (patient R1, Table 2) was an 8-year-old boy with a Chinese-Malaysian father and Japanese mother, who developed myoclonic jerks since the age of 3 years. He also had bilateral mild ptosis and mental retardation. However, his muscle biopsy did not show typical RRFs although the mitochondria were slightly more prominent. On the other hand, the other 2 patients (R3 and R4, Table 3) were siblings, with history of myoclonic epilepsy and mental impairment since early childhood and RRFs on muscle biopsies. However, no m.8344A>G mutations were detected. The two patients (R2 and R5) with isolated mitochondrial myopathy, also had the m.8344A>G mutation on testing.

Molecular analyses of the 6 patients clinically suspected to have LS revealed 3 different mutations in 3 patients viz. an m.8344A>G mutation, the typical m.8993T>G mutation and a large deletion (patients L1, L2 and L6 respectively, Table 4). All of them had infantile-onset neurodegenerative symptoms while 2 had characteristic neuroimaging changes. Patient L6 first presented with ataxic gait at the age of 8 years. He had intellectual developmental delay with short stature, unsteady gait, bilateral sensorineural deafness, respiratory failure and complete heart block. Neuroimaging showed hypodense lesions in the basal ganglia and thalamus and muscle biopsy had RRFs. DNA sequencing of the PCR fragment flanking the deleted region in L6 revealed a 6739-bp novel deletion ranging from 6843 to 13583. Patient L2 and the 3 cases without the typical m.8993T>G/C mutations showed no specific muscle histopathological changes whereas RRFs are present on muscle biopsies of L1 and L6. Patient L1, an 8-month-old girl was an interesting case in that although she presented with symptoms suggestive of LS, she was found to have the m.8344A>G mutation, the commonest cause of MERRF.

DISCUSSION

In our cohort of patients, the *tRNA^{Leu}* m.3243A>G mutation was the most common mutation found as 81.8% (9 out of 11) of the MELAS patients harbored the mutation. This result coincides with the findings reported previously.[9,12] The m.8344A>G mutation has been reported to be present in over 80% of MERRF[13], while the less common mutations in *tRNA^{Lys}* is seen in about 10% of MERRF cases.[14] We identified the mutation in a child (R1) with MERRF who was born to a Malaysian Chinese father and a Japanese mother. Thus far, we have not found any m.8344A>G-positive MERRF case with a Malaysian maternal ancestry. Interestingly, the same A to G alteration at position 8344 was identified in a patient with Leigh syndrome (L1) and two patients presented with isolated myopathy.

RRFs are thought to be due to subsarcolemmal accumulation of proliferated mitochondria to compensate for impaired energy generation. Alhough RRFs are often evident in mitochondrial dysfunction, they are rarely seen in patients presenting with mitochondrial encephalopathy syndromes with an infantile or early childhood onset such as LHON, neuropathy, ataxia and retinitis pigmentosa (NARP), Leigh syndrome and mitochondrial DNA depletion syndromes.[15] The causative genetic defects are often mutations in mitochondrial or nuclear genes encoding mitochondrial proteins. However, RRFs were evident in 2 suspected Leigh syndrome patients (L1 and L6) harbouring the *tRNA^{Lys}* m.8344A>G mutation and a large deletion, removing part of the tRNA genes. This suggests that mitochondrial proliferation is a compensatory process in response to mitochondrial deficiency but is unlikely a determinant factor of a mitochondrial disorder phenotype.

Although the m.8344A>G mutation is typically found in patients with MERRF and large deletions are often associated with chronic progressive external opthalmoplegia (CPEO) and Kearns-Sayre syndrome (KSS), these mutations have been reported in patients with Leigh syndrome.[16-18] Since a mutation can have different clinical consequences, it has been suggested that the variability of mutant load and tissue distribution are clinically important in addition to the pathogenicity of the mutation.[19,20] However, it has been reported that the severity of the disease may not be proportionally correlated to the mutant load in an indicated tissue.[21] Age-dependent energy requirements in different tissues may also affect the tissue vulnerability to the mutation. In other words, pathogenic significance, mutant load, tissue distribution and the age of patient with the mutation could act together to give rise to a particular phenotype.

A limitation of this study was that the review of the patients' clinical features was retrospective as they were referred from different hospitals. Therefore, some clinical manifestations may have been overlooked and their current status not assessed. Despite this, the general correlation between m.3243A>G mutation and MELAS was seen. Therefore, screening for m.3243A>G mutation is a useful confirmative tool when MELAS is suspected in the Malaysian population. On the other hand, we are unable to make similar conclusions for m.8344A>G mutation and MERRF. In our cohort, the mutation was seen in several clinically diverse patients. More MERRF patients need to be studied to determine the prevalence of m.8344A>G mutation in the Malaysian population. The screening for m.8993T>C/G mutation resulted in a low detection rate, since the mutation was present in only 1 out of 6 patients. This is unsurprising as Leigh syndrome has considerable heterogeneity involving both mitochondrial and nuclear genes.[22] Biochemical tests for the activity of the various respiratory chain complexes may help narrow down the candidate genes if available.[23]

Mitochondrial encephalomyopathies are an overlapping spectrum rather than individual diseases. A distinctive clinical course is indicative of a particular mutation but should not rule out the other mutations. Similarly, a typical mutation does not always predict a specific mitochondrial syndrome. Atypical findings of this study exemplify the importance for the screening of the other mtDNA mutations to have a definite diagnosis of mitochondrial encephalomyopathies.

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