ELECTRONIC LETTER

A novel mutation in the mitochondrial tRNA^{Ser(AGY)} gene associated with mitochondrial myopathy, encephalopathy, and complex I deficiency

L-J C Wong, D Yim, R-K Bai, H Kwon, M M Vacek, J Zane, C L Hoppel, D S Kerr

J Med Genet 2006;43:e46 (<http://www.jmedgenet.com/cgi/content/full/43/9/e46>). doi: 10.1136/jmg.2005.040626

Purpose: To identify molecular defects in a girl with clinical features of MELAS (mitochondrial encephalomyopathy and lactic acidosis) and MERRF (ragged-red fibres) syndromes.

Methods: The enzyme complex activities of the mitochondrial respiratory chain were assayed. Temporal temperature gradient gel electrophoresis was used to scan the entire mitochondrial genome for unknown mitochondrial DNA (mtDNA) alterations, which were then identified by direct DNA sequencing.

Results: A novel heteroplasmic mtDNA mutation, G12207A, in the tRNA^{Ser(AGY)} gene was identified in the patient who had a history of developmental delay, feeding difficulty, lesions within her basal ganglia, cerebral atrophy, proximal muscle weakness, increased blood lactate, liver dysfunction, and fatty infiltration of her muscle. Muscle biopsy revealed ragged red fibres and pleomorphic mitochondria. Study of skeletal muscle mitochondria revealed complex I deficiency associated with mitochondrial proliferation. Real time quantitative PCR analysis showed elevated mtDNA content, 2.5 times higher than normal. The tRNA^{Ser(AGY)} mutation was found in heteroplasmic state (92%) in the patient's skeletal muscle. It was not present in her unaffected mother's blood or in 200 healthy controls. This mutation occurs at the first nucleotide of the 5' end of tRNA, which is involved in the formation of the stem region of the amino acid acceptor arm. Mutation at this position may affect processing of the precursor RNA, the stability and amino acid charging efficiency of the tRNA, and overall efficiency of protein translation.

Conclusion: This case underscores the importance of comprehensive mutational analysis of the entire mitochondrial genome when a mtDNA defect is strongly suggested.

The human mitochondrial genome is a 16 569 bp double stranded circular DNA encoding for two rRNA, 22 tRNA, and 13 mRNA genes essential for the structural and functional maintenance of the mitochondrial respiratory chain.¹ The major function of mitochondria is to generate ATP, the energy currency for cellular activities. Defects in mitochondrial oxidative phosphorylation cause a group of heterogeneous disorders characterised by mitochondrial dysfunction in tissues with a high energy demand and frequently manifest with multisystemic disorders including impairment of the central nervous system, peripheral neuropathy, movement disorders, failure to thrive, and global developmental delay.²⁻³ The most commonly recognised mitochondrial disorders due to mitochondrial DNA (mtDNA) point mutations are MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like

episodes) and MERRF (myoclonic epilepsy and ragged-red fibres).⁴⁻⁵ Diagnostic criteria have been developed to aid in the diagnosis of an electron transport chain (ETC) disorder.⁶⁻⁷ However, since nuclear genes encode most oxidative phosphorylation proteins, the molecular diagnosis of mitochondrial diseases is further complicated by their genetic heterogeneity.

To date, more than 100 pathogenic mtDNA mutations have been reported in association with a wide spectrum of clinical manifestations.⁸ The majority of these mutations are found in tRNA genes that lead to translational defects and subsequent global mitochondrial respiratory chain dysfunction. Screening of 45 recurrent mutations in 2000 patients with suspected mitochondrial disorders revealed that only 6% of the patients had identifiable mtDNA mutations.⁹ Despite ample evidence of clinical, biochemical, neuroradiological, and pathohistological studies to suggest mitochondrial disease, the molecular defects of most of the patients with mitochondrial disease remain unidentified.⁹ Since the number of mtDNA mutations screened are only a small fraction of the entire mitochondrial genome, it is frequently necessary to analyse the whole mtDNA genome to identify mtDNA mutations responsible for the disease.

Recently, we have successfully developed the temporal temperature gradient gel electrophoresis (TTGE) method to detect mtDNA mutations.¹⁰⁻¹² A number of novel mutations have been identified.¹⁰⁻¹⁴ In this study we report the case of a patient who had a history of global developmental delay, muscle weakness, and abnormal basal ganglia MRI results and who was evaluated for biochemical and molecular evidence of mitochondrial disease.

METHODS

Specimens

A skeletal muscle specimen from the proband and a blood specimen from her mother were submitted to the Molecular Genetics Laboratory at the Institute for Molecular and Human Genetics, Georgetown University Medical Center, Washington, DC, for mutational analysis of mtDNA. Total DNA was extracted according to the published procedures.¹⁵⁻¹⁶

Biochemical analysis

Skeletal muscle biopsy and histochemical analyses were performed at Rainbow Babies and Children's Hospital, Cleveland, OH. Mitochondria were isolated and function

Abbreviations: ASO, allele specific oligonucleotide; ETC, electron transport chain; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fibres; mtDNA, mitochondrial DNA; NADH, nicotinamide adenine dinucleotide dehydrogenase; TTGE, temporal temperature gradient gel electrophoresis

analysed through the Center for Inherited Disorders of Energy Metabolism, Louis Stokes VA Medical Center, Cleveland, OH. Oxidative phosphorylation was measured using substrates that enter the mitochondria using different transporters and dehydrogenases, as well as different points of entry of reducing equivalents into the ETC according to the published procedures.^{17, 18}

Mitochondrial DNA mutational analysis

Common mtDNA point mutations, A3243G, T3271C, A8344G, T8356C, T8993G, T8993C, G8363A, G11778A, G3460A, G14459A, and T14484C were screened by multiplex PCR/allele specific oligonucleotide (ASO) dot blot analysis.^{9, 15} mtDNA re-arrangements and deletions were analysed with *EagI* and *HindIII* restriction enzyme digestion followed by Southern blot analysis. TTGE was performed to scan the entire mitochondrial genome for unknown mtDNA alterations using 32 overlapping primer pairs as previously published.^{10, 12} The muscle mtDNA sample from the proband was analysed by TTGE in parallel with the asymptomatic mother's blood mtDNA sample. DNA fragments showing different TTGE banding patterns between the proband's and her mother's mtDNA were sequenced. DNA sequencing was carried out with the BigDye terminator cycle sequencing kit (Perkin-Elmer, Wellesley, MA) and analysed on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Sequencing results were compared with the GenBank mtDNA sequence (accession number NC_001807; <http://www.mitomap.org>).

To confirm the heteroplasmic status of the 12207G>A mutation, the mtDNA region containing the mutation site was amplified using forward primer mt11688F 5'CCGGCGCAGTCATTCTCA3' and reverse primer mt12360R 5'GGTTATAGTAGTGTGCATG3' followed by hybridisation of the PCR products with either the wild type (5'CTTATTTC-CCGAGAAAGCTC3') or the mutant (5'CTTATTACCAAG-AAAGCTC3') ASO probe.^{9, 15} To quantify the percentage of 12207G>A mutant heteroplasmy, PCR/RFLP and real time ARMS quantitative PCR (qPCR)¹⁹ were used. The primers for PCR amplification of the region containing 12207G>A for RFLP were modified forward primer mtF12175-12206-Mod-Xho I: 5'TGACAACAGAGGCTTACGACCCCTTATTtC3' and mtR12238: 5'GGCATGAGTTAGCAGTTCT3'. The forward primers for real time ARMS qPCR assay were ARMS-G12207-1m: 5'GCTTACGACCCCTTATTACaG3' and ARMS-G12207A-1m: GCTTACGACCCCTTATTACaA, and the reverse primer was mtR12277: 5'TCCTTTAAAGTTGAGAAA-GCC3'.

Real time qPCR analysis of mtDNA content

Multiple deletions and mtDNA depletion undetectable by Southern blot were analysed simultaneously with a real time qPCR method. For mtDNA, two regions, np3212–3319 (tRNA^{Leu(UUR)}) and np12093–12170 (ND4), were amplified for the measurement of mtDNA content. Region np3212–3319 is usually (>97%) present in all mtDNA molecules including the deletion mutants. Therefore this region can be probed for the total mtDNA content. Region np12093–12170 is deleted in 97% of the deletion molecules that have been reported. Thus, a probe in this region was used to measure the amount of non-deletion molecules. The difference in the amount of total and non-deleted mtDNA molecules is the amount of deleted mtDNA molecules. The $\beta 2$ microglobulin ($\beta 2M$) gene is used as the nuclear gene (nDNA) normaliser for the calculation of mtDNA to nDNA ratio. The target sequences were detected by using TaqMan probes: 6FAM-5'TTACCGGGCTCTGCCATCT3'-TAMRA, 6FAM-5'CATCATTA CCGGGTTTCTCTTGTA3'-TAMRA, and VIC-5'TTGCTCCAC-AGGTAGCTCTAGGAGG3'-TAMRA for mtDNA regions

np3212–3319 and np12093–12170, and $\beta 2M$, respectively. The primers were: 5'CACCCAAGAAGAGGGTTTGT3' (forward) and 5'TGGCCATGGGTATGTTGTTA3' (reverse) for the mtDNA np3212–3319 region; 5'TCCTCTATCCCTCAACC CC3' (forward) and 5'CACAATCTGATGTTTGGTTAAAC3' (reverse) for the mtDNA np12093–12170 region; and 5'TGCTGTCTCCATGTTTGATGTATCT3' (forward) and 5'TCT-CTGCTCCCCACCTCTAAGT3' (reverse) for the $\beta 2M$ gene.

The 20 μ l PCR reaction contained 1 $\times$ Platinum qPCR SuperMix-UDG Master Mix (Invitrogen, Carlsbad, CA), 300 nM of each primer, 100 nM of TaqMan probe, 0.4 μ l of Rox dye (supplied by the manufacturer), and approximately 2 ng of total genomic DNA extract. Real time PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s of denaturation at 95°C, and 60 s of annealing/extension at 60°C. Real time qPCR analysis was performed on a Sequence Detector System ABI-Prism 7700.^{20, 21}

Clinical report

The patient was born to a 36 year old G2P1 mother at 38 weeks of gestation with an antenatal course complicated by frequent premature contractions and respiratory infections. Her birth weight was 3 kg (the 50th centile). She had neonatal pneumonia. By age 7 months, weight and head circumference were at the 5th centile and height at the 25th centile, eventually all dropping to less than the 2nd centile. She had developmental delay in motor and cognitive skills, with tremors and poor coordination, balance, and upper trunk stability. Due to trouble chewing, her mother continued breast feeding until age 3 years. The patient also had constipation and a long history of recurrent infections, with fevers, otitis media, and upper respiratory infections. Neurological examination showed proximal weakness, facial diplegia, and mild ptosis. Initial MRI at age 7 years showed increased signal intensity in the basal ganglia, especially the putamen bilaterally, and diffuse atrophy with an enlarged cisterna magna. Chromosome analysis was 46XX. Plasma alanine (907 μ M; normal 200–550) and lactate (9–13 mM; normal 0.3–2.4) were elevated, with a high lactate/pyruvate ratio (30; normal 10–20). Plasma ammonia and carnitine were normal. EKG showed pre-excitation Wolff-Parkinson-White syndrome. Echocardiogram showed a dilated left ventricle, decreased ventricular function, and mild mitral regurgitation. The patient was enrolled in the Dichloroacetate Congenital Lactic Acidosis Clinical Trial at the University of Florida at age 9 years. At age 12 years, repeat brain MRI showed no significant changes with continued mild cerebral atrophy, and increased signal within the lentiform nuclei bilaterally suggestive of demyelination. She could not hop or walk on her heels, had a positive Gower's sign and unsteady gait, and spoke with a slight labial dysarthria; she had mild bilateral alternating exotropia and ptosis, with some weakness of the lower face and neck flexion. She was being treated with coenzyme Q₁₀, carnitine, biotin, thiamine, vitamin C, calcium supplements, and daily multivitamins.

The family history is unremarkable. The mother is Chinese and the father is Caucasian, without consanguinity. The patient's 21 year old brother and both parents are without significant medical problems. There are no known similar neurological problems on either side of the family.

RESULTS

Pathology and biochemical analysis

Modified Gomori-trichrome staining of the muscle biopsy showed ragged-red fibres. Histochemical analysis revealed reduced nicotinamide adenine dinucleotide dehydrogenase (NADH) and succinate dehydrogenase stains with evidence of mitochondrial proliferation. Electron microscopy showed pleomorphic mitochondria. The yield of skeletal muscle

Figure 1 Oxidation is reported as the average of the state 3 rates following two additions of 0.1 mM ADP (only one addition with TMPD/ascorbate) for the patient and controls (n = 68). The final concentrations in the incubation media for specific substrates are: 20 mM glutamate; 10 mM pyruvate+5 mM L-malate; 0.04 mM palmitoylcarnitine+5 mM L-malate; 20 mM succinate (+3.75 μ M rotenone); 1 mM duroquinol (+3.75 μ M rotenone); and 1 mM tetramethylethylenediamine/10 mM ascorbate (+3.75 μ M rotenone). OXPHOS, oxidative phosphorylation.

mitochondria was increased at 13.9 mg per gram wet weight muscle compared to controls (mean $\pm$ SD: 5.2 ± 1.3 ; n = 29), suggesting mitochondrial proliferation. Oxidative phosphorylation studies were performed.^{17, 18} Figure 1 shows state 3 rates of oxidation in the presence of 0.1 mM ADP. The data clearly demonstrate decreased oxidation of the three substrates that ultimately utilise complex I for entry into the chain (glutamate, pyruvate plus malate, and palmitoylcarnitine plus malate), whereas the state 3 oxidation rates are in the control range for succinate (entry at complex II), duroquinol (coenzyme Q analog at complex III), and TMPD plus ascorbate (reduces cytochrome c). Furthermore, with glutamate as substrate, the rate was not increased by the addition of 2 mM ADP or the uncoupler DNP, showing that the decreased rate of oxidation is not due to limitations in acceptors such as the adenine nucleotide or phosphate transporter, or ATP synthetase activity (table 1). Additionally, as shown in table 1, state 4 rates of oxidation were normal as were the respiratory control ratio and ADP/O ratio with succinate as substrate, indicating normal control of oxidative phosphorylation (identical results were obtained with duroquinol and TMPD plus ascorbate as substrates; data not shown). These data suggest a complex I defect associated with mitochondrial proliferation and indicate normal oxidative phosphorylation with substrates other than those used by complex I.

The activity of rotenone sensitive NADH-cytochrome c reductase reflecting ETC complexes I and III was not measurable in disrupted freshly isolated mitochondria, whereas the activities of antimycin A sensitive succinate-cytochrome c reductase, reflecting ETC complexes II and III,

and citrate synthase, a Krebs cycle enzyme, were increased compared to prior controls (table 2). Activities of NADH-ferricyanide reductase, the first component of complexes I, III, and IV, are in the normal ranges (table 2). These results indicate lack of activity of the later components of complex I. Analysis of the skeletal muscle tissue revealed similar findings of increased activity of combined complexes II and III (succinate cytochrome c reductase), succinate dehydrogenase, and citrate synthase activity (49.2 U/g wet weight; controls: 19.7 ± 4.9 ; n = 30). The increased yield of mitochondrial protein and the increased activities of skeletal muscle citrate synthase and succinate dehydrogenase, which are not encoded by mtDNA genes, are consistent with mitochondrial proliferation.

Analysis of mtDNA

A direct mutational screen of common mtDNA point mutations in skeletal muscle mtDNA did not detect any previously identified pathogenic mtDNA mutations. Southern blot analysis also did not reveal any large deletions or mtDNA rearrangements. Since the clinical features of the patient and the biochemical and histopathological findings indicated a mitochondrial disorder, more extensive mutational analysis of the entire mitochondrial genome was performed using TTGE.^{10, 12} TTGE analysis revealed a different banding pattern between the affected child and the asymptomatic mother in the mtDNA region (nucleotide position 11688 to np12360) containing tRNA genes for histidine, serine, and leucine. The affected child showed a heteroplasmic banding pattern, whereas her mother showed a homoplasmic banding pattern on TTGE analysis (fig 2A). Direct sequencing of the tri-tRNA region revealed a G to A transition at np12207 (fig 2B) in tRNA^{Ser(AGY)}. To confirm the status of homo- or heteroplasmic state of the 12207G>A novel mutation, the ASO probes for the wild type and the mutant 12207G>A were synthesised, labelled with ³²P, and hybridised with the PCR product containing this mutant site. As shown in fig 2C, the mutation in the patient was heteroplasmic. Her mother's blood mtDNA is homoplasmic for wild type nucleotide G at this position.

To further determine the proportion of mutant heteroplasmy, RFLP and ARMS real time qPCR were performed (table 3).¹⁹ The proportion of mutant heteroplasmy in the patient was found to be 92% and 0.3% in the patient's muscle and hair follicles, respectively. Her mother's blood and hair follicles did not contain any detectable 12207G>A mutant mtDNA (table 3).

The novel 12207G>A mutation occurs at the first base of the 5' end of the tRNA^{Ser(AGY)} gene, which forms the amino acid acceptor stem (fig 3). Mutation at this nucleotide position is expected to cause disruption of the acceptor stem structure leading to unstable tRNA, inefficient amino acid charging, and overall reduction in protein translation. Since np12207 is at the first base of the 5' position of tRNA^{Ser(AGY)}, it may also affect the proper processing of the polycistronic RNA precursor. In addition, this nucleotide position is engaged in base pairing at the highly conserved structural

Substrates	State 3	State 4	RCR†	ADP/O	2 mM ADP	0.3 mM DNP
20 mM glutamate						
Patient	18.3	18.3			15.5	15.5
Prior control‡, mean $\pm$ SD	150 $\pm$ 44	16.1 $\pm$ 6.6	13.2 $\pm$ 8.5	2.84 $\pm$ 0.23	176 $\pm$ 47	201 $\pm$ 70
20 mM succinate+3.75 μ M rotenone						
Patient	220.7	57.8	3.8	1.71	227.5	362.2
Prior control‡, mean $\pm$ SD	295 $\pm$ 40	68 $\pm$ 13	4.5 $\pm$ 1.0	1.60 $\pm$ 0.16	300 $\pm$ 44	299 $\pm$ 47

*nAtomO/min/mg protein; †RCR, respiratory control ratio; ‡number of controls = 68.

Table 2 Electron transport chain enzyme complex activities in freshly isolated skeletal muscle mitochondria*

Enzymes	ETC complexes	Patient	Prior controls‡, mean ± SD	Range
NADH-cytochrome c reductase - rotenone sensitive	I-III	0.0	481 ± 37	441–528
NADH-ferricyanide reductase	"I"	1833	1621 ± 364	1284–2131
Succinate-cytochrome c reductase	II-III	350	146 ± 42	82–187
Succinate dehydrogenase	"II"	131	75 ± 30	53–96
Decylubiquinol-cytochrome c reductase	III	2380	4328 ± 415	4034–4621
Cytochrome c oxidase†	IV	57.4	73.3 ± 22.7	38.4–96.8
Citrate synthase	TCA cycle	3613	1820 ± 348	1338–2555

*μmol/min/mg protein (except for cytochrome oxidase); †first order rate constant k/mg protein; ‡number of controls = 12.

region throughout evolution and is not present in the patient's unaffected mother or 200 healthy controls. Thus, this 12207G>A mutation is most likely a pathogenic mtDNA mutation that is responsible for the disease state of the patient.

Sequencing of the entire mitochondrial genome

To further investigate the pathogenic role of the 12207G>A mutation, the entire 16.6 kb of mitochondrial genome was sequenced. Six novel mutations were identified, including the 12207G>A mutation (table 4). Three of them were silent mutations, and the other two were 5331C>A in ND2 (L288I) and 15090T>C in cytochrome *b* (I115T). In addition, two reported missense polymorphisms, T8A in ND5 and T7I in cytochrome *b*, were also detected. Except for the novel mutation 12207G>A in tRNA^{Ser(AGY)}, all were homoplasmic and all were present in the asymptomatic mother. Thus, a pathogenic mtDNA mutation other than 12207G>A has been ruled out. Also, no multiple mtDNA deletions were detected by either PCR or Southern analyses.

Elevated mtDNA content in muscle

Mitochondrial proliferation is a cellular compensatory mechanism in response to mitochondrial dysfunction.²⁴

Mitochondrial respiratory chain enzyme complex assays revealed increased citrate synthase and succinate dehydrogenase (complex II) activities, suggesting mitochondrial proliferation. To test if the mitochondrial proliferation was accompanied by mtDNA amplification, we evaluated the mtDNA content in the muscle specimen of the patient using a real time qPCR method.²⁵ The results showed that the mtDNA content in the skeletal muscle of the patient was 250% of the age matched mean, consistent with the observation of ragged red fibres.

DISCUSSION

Identification of molecular defects in either the nuclear or the mitochondrial genes facilitates the definitive diagnosis of mitochondrial disorders. The patient described in this report presented with a MELAS-like phenotype, consistent with a mitochondrial disorder. Despite biochemical and pathological evidence of a mitochondrial disorder with mitochondrial proliferation in this child, screening of common mtDNA mutations failed to identify a molecular defect. To confirm the genetic basis of mitochondrial disease and to provide accurate genetic counselling, more extensive mutational analysis of the entire mitochondrial genome was carried out. We analysed mtDNA from the proband's skeletal muscle

Figure 2 Identification of the 12207G>A mutation. (A) TTGE analysis of the mtDNA region containing tRNA genes for his, ser, and leu. Lane 1: patient, lane 2: mother, lane 3: mixture of patient's and mother's DNA. (B) Identification of the 12207G>A mutation by direct DNA sequencing. (C) ASO analysis to show the heteroplasmic mutation in the proband and homoplasmic mutation in her mother. Lane 1: proband, lane 2: mother, lanes 3 and 4: normal controls, lane 5: no template control, and lane 6: synthetic positive control.²²

Table 3 Analysis of G12207A mutant heteroplasmy

Method	Patient			Mother		
	RFLP	ARMS-qPCR	TTGE	RFLP	ARMS-qPCR	TTGE
Blood	NA	NA	NA	0	0	0
Muscle	~100	~92	~94	NA	NA	NA
Hair follicles	ND	0.3	ND	ND	0	ND

Numbers are % G12207A mutant heteroplasmy. NA, not available; ND not done.

and her mother's blood side by side. Since each individual's mtDNA contained 10–20 polymorphisms and the mother is asymptomatic, we sequenced only those DNA fragments that displayed differences in TTGE banding patterns between the proband and her mother.¹⁰ This approach avoided sequencing benign mtDNA polymorphisms in this family. In addition, a mixture of mtDNA from the proband and her mother was analysed, to increase the sensitivity of detecting near homoplasmic mutations that might not separate well from homoduplexed wild type DNA on TTGE. Following mixing, the DNA forms heteroduplex mismatched DNA species that usually move much slower than the homoduplex bands (for example, the upper two faint bands in p lane of fig 2A).

One challenge in the molecular diagnosis of mtDNA disorders is establishment of the homoplasmic and heteroplasmic status and determination of the proportion of mutant load. Although DNA sequencing is the gold standard for the identification of mutations, it does not detect heteroplasmy present at a low percentage. Our previous studies have demonstrated that TTGE is sensitive enough to detect heteroplasmic mutations at as low as 4%.¹⁰ Figure 2A clearly showed the presence of a heteroplasmic mutation; however, sequencing results showed apparent homoplasmy (fig 2B). As a general practice, mutations identified from sequencing should always be confirmed by a second, direct mutation detection method, such as PCR/RFLP or PCR/ASO.^{12–14} ASO probes were designed and the results (fig 2C) showed a clear heteroplasmic mutation of 12207G>A, consistent with the TTGE results.

Figure 3 Structure of tRNA^{Ser}. Arrow indicates the location of the G12207A mutation in the mitochondrial tRNA^{Ser(AGY)}. Cloverleaf structure of human mitochondrial tRNA^{Ser(AGY)} is derived from Florentz *et al.*²³

Identification of a deleterious mtDNA mutation not only confirms the diagnosis of mitochondrial disease but also provides information for more accurate genetic counselling. With knowledge of the pathogenic mutation, at risk asymptomatic family members can be screened for carrier status. The percentage of mutant heteroplasmy can also be determined.¹⁹ In this case, the mother does not carry the mutant mtDNA. The mutation appears to be a sporadic de novo mutation in the affected child. Compared to the mutant load in the patient's muscle specimen, extremely little of the mutant mtDNA is present in the patient's hair follicle. This observation emphasises that the mutant load in one tissue does not necessarily represent the mutant load in other tissues. Thus, caution is required in predicting disease outcome based on measurement of mutant load in a single tissue.

Most (approximately 60%) characterised pathogenic mtDNA mutations occur in the tRNA genes, which represent only about 9% of the entire mitochondrial genome.^{23–26} Numerous mutations in mitochondrial tRNA genes have been described in association with diverse clinical phenotypes. Some tRNA genes appear to be more frequently affected, such as the tRNA^{Leu(UUR)} gene. Eighty percent of MELAS cases are associated with an adenine to guanine base substitution in the gene encoding tRNA^{Leu(UUR)} at nucleotide position 3243 in the mtDNA.^{27–28} More than 21 pathogenic mutations in this 74 nucleotides long tRNA have been reported to be responsible for MELAS disease. To date, only two pathogenic mutations have been identified in tRNA^{Ser(AGY)}. A C12246A point mutation occurring in the highly conserved T arm of this tRNA is associated with chronic intestinal pseudo-obstruction with myopathy and ophthalmoplegia.²⁹ Another heteroplasmic C12258A point mutation that alters a highly conserved base pair in the acceptor stem of the tRNA was found in a patient with diabetes mellitus and deafness.³⁰ Our finding of the novel mutation 12207G>A adds to the few reported tRNA^{Ser(AGY)} mutations and underscores the importance of comprehensive mutational analysis of mtDNA when a patient's clinical features and laboratory findings strongly support the diagnosis of a mtDNA disorder.

Pathogenic mtDNA mutations in tRNA genes often cause mitochondrial proliferation as a mechanism to compensate for deficiency in mitochondrial function.^{24–33} Our results of real time qPCR analysis revealed a 2.5-fold over-replication of total mtDNA, consistent with the observation of mitochondrial proliferation. This was further supported by the findings of increased yield of isolated mitochondria and increased activity in skeletal muscle of mitochondrial enzymes not encoded by mtDNA (citrate synthase and succinate dehydrogenase). The proportion of mutant mtDNA alone is not sufficient to predict the severity of disease course. Patients with a high percentage of mutant loads may express relatively mild clinical phenotypes due to the compensatory amplification of the total mtDNA.³⁴ Therefore, it is important to always investigate total mtDNA content whenever possible. This patient has 92% of the mutant mtDNA in her muscle specimen. A 2.5-fold amplification would give

Table 4 Sequencing results of entire mitochondrial genome of the proband

Nucleotide position	Gene/region	Base change	Amino acid change	Novel or reported	Heteroplasmy
73	HV2	A>G			No
263	H-strand origin	A>G			No
310	CSB2	T>C			No
311–315	CSB2	insC			No
750	12S rRNA	A>G			No
1438	12S rRNA	A>G			No
2706	16S rRNA	A>G			No
5231	ND2	C>A	Silent		No
5331	ND2	C>A	L288I	Novel	No
5417	ND2	G>A	Silent		No
10275	ND3	T>C	Silent	Novel	No
11368	ND4	T>C	Silent	Novel	No
11465	ND4	T>C	Silent	Novel	No
12207	tRNA ^{ser(AGY)}	G>A		Novel	Yes
12358	ND5	A>G	T8A		No
12372	ND5	G>A	Silent		No
14766	Cytochrome <i>b</i>	C>T	T7I		No
15090	Cytochrome <i>b</i>	T>C	I115T	Novel	No
16223	HV1	C>T			No
16257	HV1	C>A			No
16261	HV1	C>T			No
16295	HV1	C>T			No

CSB, conserved sequence block; HV, hypervariable segment; ND, NADH dehydrogenase.

approximately 20% of wild type mtDNA content of age matched mean.

Several lines of evidence support the pathogenic role of the 12207G>A mutation as follows: (a) it is not present in 200 normal controls; (b) it is a heteroplasmic mutation in the affected patient but is not detectable in the asymptomatic mother; (c) it locates at a structurally/functionally important amino acid acceptor stem region; (d) it is the first nucleotide of the tRNA, important for proper processing of the precursor RNA; (e) the presence of this mutation in a tRNA gene is consistent with mitochondrial proliferation and mtDNA amplification; and (f) sequencing of the entire mitochondrial genome did not identify any other pathogenic mutation. More importantly, the 12207G>A mutation at the region where ND4, trtRNA (tRNA^{his}, ser, and leu) genes, and ND5 are adjacent, may not only affect the processing of tRNA^{ser(AGY)} but also the tRNA^{His}, tRNA^{Leu} (CUN), ND4, and ND5.^{35–36} Furthermore, when the ser(AGY) content in each ETC complex subunit encoded by mtDNA was examined, it was found that complex I has the highest average percentage of ser(AGY) content compared to complexes III, IV, and V (table 5). This may account for complex I deficiency in this patient.

In conclusion, we utilised TTGE analysis to identify a novel mutation in the tRNA^{ser(AGY)} gene of mtDNA in a patient with a mixed phenotype, including some features of the MELAS/MERRF syndromes, and complex I deficiency. Our

results support the importance of the first nucleotide at the 5' end of tRNA, and its mutation leads to a mixed spectrum of MELAS/MERRF syndromes. This study extends the molecular aetiology of MELAS/MERRF syndromes to include mutations in tRNA^{ser(AGY)}.

ELECTRONIC-DATABASE INFORMATION

The MITOMAP site is at <http://www.mitomap.org>

Authors' affiliations

L-J C Wong, R-K Bai, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA

H Kwon, Institute for Molecular and Human Genetics, Georgetown University Medical Center, Washington, DC, USA

D Yim, Pediatric Genetics, Kaiser Permanente Medical Group, Honolulu, HI, USA

M M Vacek, Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

J Zane, Kaiser Permanente Medical Group, Honolulu, HI, USA

C L Hoppel, D S Kerr, The Center for Inherited Disorders of Energy Metabolism, Case Western Reserve University School of Medicine, Cleveland, OH, USA

This study is supported in part by a grant from Muscular Dystrophy Foundation to LJCW

Competing interests: none declared

Correspondence to: Dr Lee-Jun C Wong, Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, NAB 2015, Houston, TX 77030, USA; ljwong@bcm.edu

Revised version received 5 March 2006

Accepted for publication 8 March 2006

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Table 5 Content of ser(AGY) in mtDNA encoded respiratory enzyme subunits

Subunit	Percentage ser(AGY)	Subunit	Percentage ser(AGY)
ND1	0.9	Cytochrome <i>b</i> (CIII)	1.1
ND2	1.4	COXI	0.8
ND3	0.9	COXII	0.4
ND4	2.2	COXIII	1.5
ND4L	0.0	Average CIV	0.96
ND5	2.2	ATP 6	1.3
ND6	2.9	ATP 8	0.0
Average CI	1.5	Average CV	0.65

ATP 6, ATPase subunit 6; ATP 8, ATPase subunit 8; CI, CII, CIII, CIV, and CV complexes I, II, III, IV, and V; COX, cytochrome c oxidase; ND, NADH dehydrogenase complex (I).

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