Studies on the genetics and molecular pathogenesis of mitochondrial respiratory chain disorders

Teeratorn Pulkes MD

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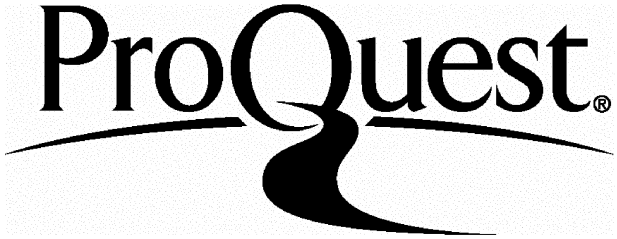

Abstract

This work aimed to identify the molecular genetic basis of disease in thirty patients with classical mitochondrial encephalomyopathy phenotypes without known mutations. The patients exhibited a range of phenotypes including MELAS, MERRF, CPEO, exercise intolerance, isolated myopathy and Kearns-Sayre syndrome. In addition the molecular mechanisms which underlie the clinical diversity associated with the common A3243G and 7472C insertion-mutations were investigated.

Systematic sequencing of the entire muscle mtDNA was undertaken in each of the thirty patients. Seven pathogenic mtDNA mutations were identified in eleven of the patients. Five mutations are previously unpublished. There are the A5874G mutation in $tRNA^{Tyr}$ gene, the G12294A mutation in $tRNA^{Leu(CUN)}$ gene, the G4810A mutation in ND2 gene, the T11232C mutation in ND4 gene and the G15723A in cytochrome *b* gene. The A5874G and G4810A mutations are the first mutations in the $tRNA^{Tyr}$ and ND2 gene, respectively, to be described in association with human diseases. All but the G4810A mutation were associated with sporadic myopathy without involvement of other tissues. For these sporadic cases, mutant mtDNA was present in only skeletal muscle was not detected in other tissues or in the blood mtDNA of the maternal relatives examined. These data suggested that these mutations might be somatic.

Four MELAS cases were found to harbour the G13513A mutation in the ND5 gene previously reported in a single case. Our data indicate that the G13513A is probably the second commonest cause of MELAS in the United Kingdom. Furthermore the phenotypic diversity associated with the G13513A mutation has been extended to include MELAS overlap with Leber's hereditary optic neuropathy.

Despite complete sequencing of the entire muscle mtDNA in the nineteen remaining patients, pathogenic mtDNA mutations were not identified in these patients. These data suggest that these patients might harbour genetic defects in nuclear genes.

The role of mtDNA background in influencing the phenotypic expression of the common A3243G MELAS mutation in forty-eight unrelated cases was studied. The results indicated that

a polymorphism, A12308G, in the $tRNA^{Leu(CUN)}$ gene may increase risk of developing stroke in the A3243G patients (relative risk = 2.17).

The role of mtDNA background on clinical expression of mtDNA mutations was further observed in association with a 7472C-insertion mutation in $tRNA^{Ser(UCN)}$ gene. The author described two new unrelated families harbouring the 7472C-insertion which the first family had myoclonic epilepsy and sensorineural hearing loss similar to previous reports. However, the second case had isolated myopathy, in which she had markedly different muscle histochemical findings from other reported cases. The intragenic polymorphism, T7472G, in the $tRNA^{Ser(UCN)}$ gene was observed in the second case and might be influence in the phenotypic expression of the 7472C-insertion.

In conclusion, this study has defined the molecular genetic basis of mitochondrial diseases in eleven patients. Five novel mtDNA mutations are described and characterised. Evidence is presented that the G13513A mutation is an important cause of the MELAS phenotype. It is suggested that nuclear gene defects probably account for the disease in the remaining nineteen patients. Evidence is presented that the mtDNA haplotype may influence the expression of primary mtDNA defects.

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Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Teeratorn Pulkes (March 2003)

Abbreviations

A	adenine
ADP	adenine diphosphate
Ala	alanine
ANT	adenine nucleotide translocator
Arg	arginine
Asn	asparagine
Asp	aspartic acid
ATP	adenine triphosphate
ATPase	ATP synthase
C	cytosine
CM	cardiomyopathy
CO_2	carbon dioxide
COX	cytochrome *c* oxidase
CPEO	chronic progressive external ophthalmoplegia
Cys	cysteine
Cyt *b*	cytochrome *b*
D loop	displacement loop
DNA	Deoxyribonucleic acid
$FADH_2$	flavin adenine dinucleotide
G	guanine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
H strand	heavy strand
H_2O	water
His	histidine
Ile	isoleucine
kD	kilodaltons
KSS	Kearns-Sayre syndrome
L strand	light strand
Leu	leucine
LHON	Leber's hereditary optic neuropathy
LS	Leigh's syndrome
Lys	lysine
MERRF	mitochondrial encephalomyopathy and ragged red fibres
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
Met	methionine
MNGIE	Myoneurogastrointestinal encephalopathy
MRC	Medical Research Council
MRI	Magnetic resonance imaging
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
NARP	neurogenic muscle weakness, ataxia and retinitis pigmentosa
ND	NADH dehydrogenase
PCR	polymerase chain reaction
Phy	phenylalanine
PMF	proton motive force
Pro	proline
Q	ubiquinone
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RRF	ragged red fibres
rRNA	ribosomal RNA
Ser	serine
SNHL	sensorineural hearing loss
T	thymine
TCA	tricarboxylic acid
Thr	threonine
TP	thymidine phosphorelasez
tRNA	transfer RNA
Trp	tryptophan
Tyr	tyrosine
U	uracil
UK	United Kingdom
Val	valine
WFS	Wolfram syndrome

Chapter 1 Introduction

The mitoc5hondrion is an important intracellular organelle located in the cytoplasm of most eukaryotic cells. Its main function is to produce energy for the cell. Five mitochondrial respiratory chain complexes lying in the inner mitochondrial membrane are responsible for energy production by oxidative phosphorylation [Cooper and Clark 1994]. A primary defect of respiratory chain function may result in multi-organ dysfunction affecting mainly the nervous system and skeletal muscle. These disorders are known as mitochondrial encephalomyopathies. Although the majority of mitochondrial respiratory chain proteins are coded by the nuclear DNA, to date most mitochondrial encephalomyopathies are associated with mutations in the mitochondrial DNA (mtDNA). Mitochondrial encephalomyopathies are clinically and genetically widely heterogeneous. A single mtDNA mutation may associate with several clinical syndromes and a clinical syndrome may associate with several mutations in different genes on the mtDNA and probably nuclear DNA [Morgan-Hughes and Hanna 1999]. Furthermore, mitochondrial respiratory chain dysfunction has been implicated in degenerative diseases, aging and in some form of neoplasia. Such neoplasms include familial pheochromocytoma or paraganglioma and autosomal dominant uterine fibroids, skin leiomyomata and renal cell carcinoma [Astuti *et al.* 2001;Baysal *et al.* 2000;Tomlinson *et al.* 2002].

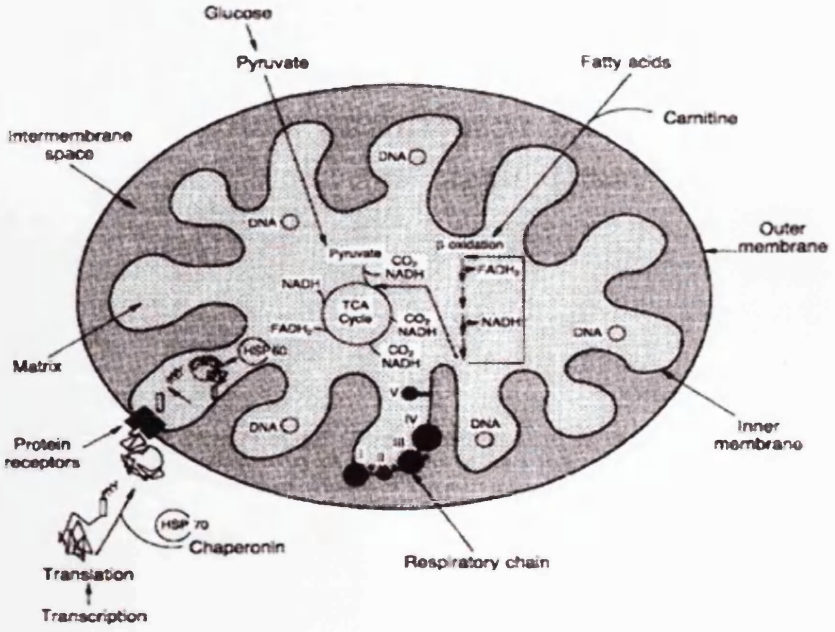
1.1 Mitochondria and the mitochondrial respiratory chain

The mitochondrion is believed to have originated from α-proteobacteria during the evolution of nucleated cells. Eukaryotic cells lacking respiratory organelles imported bacteria which became their energy-producing organelles. This endosymbiotic hypothesis is strongly supported by the similarity of the gene sequences of mitochondria and a subgroup of the α-proteobacteria. Members of the rikettsial subdivision, a group of obligate intracellular parasites including genera such as *Rikettsia*, *Anaplasma* and *Ehrlichia*, are believed to be the closest known eubacterial relatives of mitochondria [Andersson *et al.* 1998].

Most eukaryotic cells contain many mitochondria, varying up to 1,000 mitochondria per cell depending on the cell type [Lodish *et al.* 1995]. Mitochondria are among the largest organelles in the cell. They contain two different membranes: an outer and an inner membrane (Fig. 1.1). The outer membrane is composed of approximately half lipid and half protein. It contains proteins that render the membrane permeable to molecules having molecular weights up to 10 kilodaltons (kD) which is similar to the outer membrane of gram-negative bacteria. The inner membrane is much less permeable, and it is about 20 percent lipid and 80 percent protein. The surface area of the inner membrane is greatly increased by a large number of in-foldings, or cristae, that protrude into the matrix. The matrix and cristae are the sites of the enzymes that catalyze the final oxidation of sugars and lipids and the synthesis of adenine triphosphate (ATP) [Lodish *et al.*1995]. The high-energy bonds in ATP are the usual source of chemical energy for cellular growth and metabolism, and the principal sources of ATP in non-photosynthetic cells are fatty acids and glucose. The complete aerobic degradation of glucose to CO_2 and H_2O is coupled to the synthesis of as many as 32 molecules of ATP:

$$C_6H_{12}O_6 + 6O_2 + 32Pi + 32ADP \rightarrow 6CO_2 + 6H_2O + 32ATP$$

Figure 1.1 The mitochondrion [Cooper and Clark 1994]

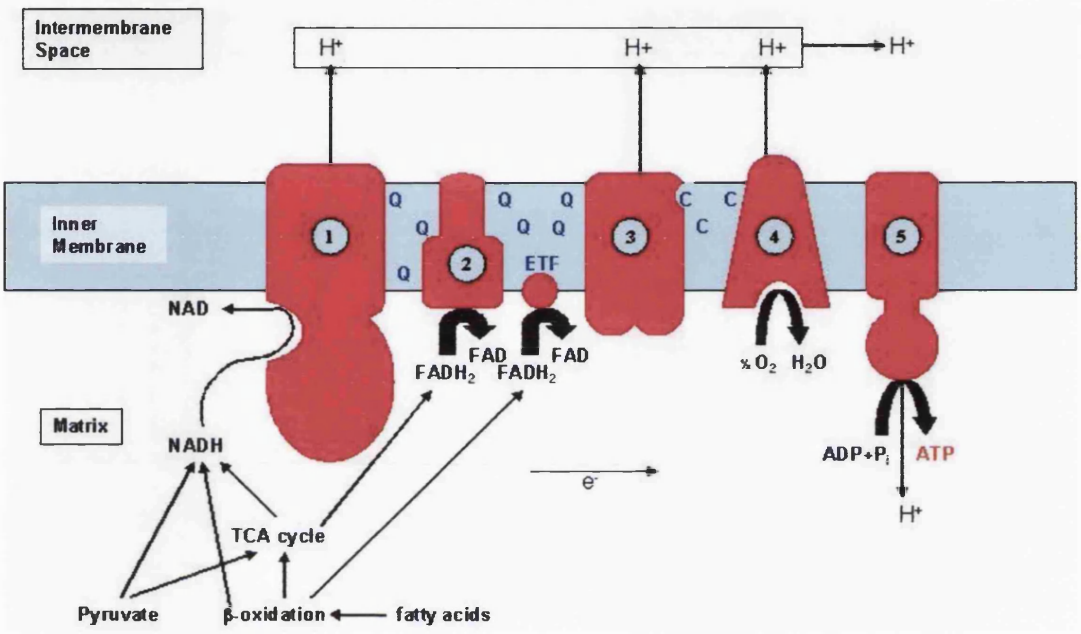


In eukaryotic cells, anaerobic glycolysis in the cytosol generates two ATPs per glucose molecule. In contrast, the mitochondrial ATP synthesis by oxidative phosphorylation is more efficient in ATP production (over 30 mol ATP/mol glucose). The oxidation of fatty acids to CO_2 also generates ATP in the mitochondrion. Therefore, the mitochondrion can be regarded as the "power plant" of the cells [Lodish *et al.* 1995].

1.2 Structural organisation of the mitochondrial respiratory chain

The respiratory chain comprises four multiple polypeptide enzyme complexes: Complex I (NADH-ubiquinone reductase); Complex II (succinate-ubiquinone reductase); Complex III (ubiquinol-cytochrome *c* reductase); and Complex IV (cytochrome *c* oxidase); and two mobile electron carriers: ubiquinone and cytochrome *c*. Together with ATP synthase (Complex V) these complexes and mobile electron carriers comprise the oxidative phosphorylation system (Fig. 1.2). The enzymes for the tricarboxylic acid (TCA) and β-oxidation of fatty acids are in the mitochondrial matrix. Pyruvate and fatty acids are oxidized generating NADH and FADH2. NADH and FADH2 are reoxidized by donating electron to the respiratory electron transport chain. Energy is released and electrons are transported along the respiratory chain finally combing with molecular oxegen to form H_2O [Cooper and Clark 1994]. The released energy is used by complexes I, III and IV to pump proton across the inner membrane into the intermembrane space. An electrochemical proton gradient or proton motive force (PMF) is thus generated across the inner membrane. The PMF is used for the generation of the ATP by ATP synthase and transport of charged molecules i.e. proteins, carboxylic acids and ions [Mitchell 1976].

Figure 1.2 The mitochondrial respiratory chain. 1 = Complex I (NADH-ubiquinone reductase), 2 = Complex II (succinate-ubiquinone reductase), 3 = Complex III (ubiquinol-cytochrome *c* reductase), 4 = Complex IV (cytochrome *c* oxidase), 5 = Complex V (ATP synthase), Q = ubiquinone, C = cytochrome *c*, ETF = electron transferring flavoprotein, TCA = tricarboxylic acid [Cooper and Clark 1994]

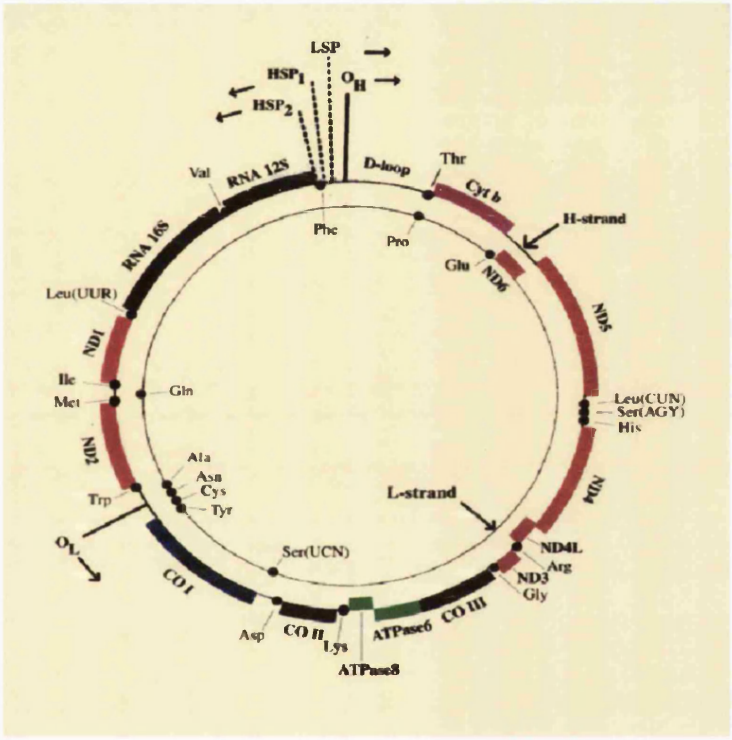


1.3 Mitochondrial DNA and mitochondrial genetics

Mitochondria have their own DNA which is located in the matrix. It is estimated that human mitochondria in a somatic cell typically contain 5-10 copies the mitochondrial DNA (mtDNA) [Thorburn and Dahl 2001]. MtDNA is a circular double-stranded molecule, which consists of 16,568 base pairs (Fig. 1.3) [Andrews *et al.* 1999]. The two DNA strands have significantly different base compositions: the heavy (H) strand is rich in guanines (G) and the light (L) strand is rich in cytosines (C). Although the mtDNA is principally double-stranded, a small section is a triple-stranded DNA structure, which is in the displacement (D) loop [Clayton 2000]. Mitochondria have a separate autonomously replicating DNA system for each strand. Genome replication begins by initiation of the H strand synthesis at a specific origin resulting in the

formation of the D-loop with a newly synthesized H strand known as 7S DNA. Initiation of the L strand synthesis is at a specific origin, two-third of the way around the genome from the origin of the H strand synthesis, and does not occur until this region has been exposed to H strand synthesis [Anderson *et al.* 1981].

Figure 1.3 The human mitochondrial DNA.



Human mtDNA has been entirely sequenced and all the reading frames have been assigned. Unlike the nuclear DNA, the sequence shows extreme economy in that the genes have none or only a few non-coding bases between them. The human mtDNA has no introns and the coding sequences of some genes partially overlap. The genetic code of human mtDNA is slightly different from the universal code, in which UGA codes for tryptophan and not termination, AUA codes for methionine not isoleucine, and AGA and AGG are termination rather than arginine codons (Table 1.1). Furthermore, in many cases the termination codons are not coded in the mtDNA but are post-transcriptionally created by polyadenylation of the mRNAs [Anderson *et al.* 1981]. The mtDNA is not coated by protective histones, and it is tethered to the inner mitochondrial membrane, close to the respiratory chain, which is a potent

source of oxygen free radicals. These factors may contribute to a high rate of mtDNA mutations [Wallace 1999].

Table 1.1 The genetic code of human mtDNA. Four differences from the universal code shown in bold letters, in which UGA codes for tryptophan and not termination, AUA codes for methionine and not isoleucine, and AGA and AGG are termination rather than arginine codons [Anderson *et al.* 1981].

Phe	UUU	Ser	UCU	Tyr	UAU	Cys	UGU
	UUC		UCC		UAC		UGC
Leu	UUA		UCA	Ter	UAA	**Trp**	**UGA**
	UUG		UCG		UAG		UGG
Leu	CUU	Pro	CCU	His	CAU	Arg	CGU
	CUC		CCC		CAC		CGC
	CUA		CCA	Gln	CAA		CGA
	CUG		CCG		CAG		CGG
Ile	AUU	Thr	ACU	Asn	AAU	Ser	AGU
	AUC		ACC		AAC		AGC
Met	**AUA**		ACA	Lys	AAA	**Ter**	**AGA**
	AUG		ACG		AAG		**AGG**
Val	GUU	Ala	GCU	Asp	GAU	Gly	GGU
	GUC		GCC		GAC		GGC
	GUA		GCA	Glu	GAA		GGA
	GUG		GCG		GAG		GGG

The majority of the proteins present in the mitochondrion are encoded by nuclear DNA, synthesized on cytoplasmic ribosomes and imported to the mitochondrion. A few of the mitochondrial protein subunits are encoded by the mtDNA including seven constituting part of complex I (NADH dehydrogenase 1-6 and 4L), one in complex III (cytochrome *b*), three in complex IV (cytochrome oxidase I-III) and two in complex V (ATP synthase 6 and 8) (Table 1.2). They are synthesized by the separate mitochondrial translation system which including twenty-two transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) [Anderson *et al.* 1981]. All remaining mitochondrial respiratory chain proteins, the metabolic enzymes, the DNA and RNA polymerases, the ribosomal proteins, and the mtDNA regulatory factors are encoded by nuclear genes.

Table 1.2 Composition of respiratory chain and phosphorylation system. (Data search from SWISS-PROT/TrEMBL Protein Knowledgebase: http://www.expasy.ch/sprot/)

Respiratory chain complexes	Total no. of Subunits	No. encoded by mtDNA
Complex I	43	7 - ND1-6, 4L
Complex II	4	0
Complex III	11	1 - Cytochrome *b*
Complex IV	13	3 - COX I, II, III
Complex V	14	2 - ATP synthase 6, 8

1.3.1 Maternal inheritance. Human mitochondria and mtDNA are inherited in a maternal line [Giles *et al.* 1980]. This phenomenon is not simply due to a much smaller amount of mtDNA molecules in a sperm than an oocyte (1-15:1000) [Danan *et al.* 1999]. Mammalian sperm mitochondria do enter the oocyte during fertilization [Ankel-Simons and Cummins 1996], but they appear to be eliminated by proteolysis in early embryogenic development [Shalgi *et al.* 1994]. This system probably serves to minimize lethal cytoplasmic gene competition and to prevent the inheritance of sperm mitochondrial DNA that has been subject to degradation by free radicals [Cummins 2000]. This paternal mtDNA elimination appears to occur only when fertilisation is with the same species [Gyllensten *et al.* 1991].

Since mtDNA is maternally inherited, mtDNA mutations have the potential to be inherited in a maternally transmitted fashion. In practice, most mtDNA point mutations are maternally inherited. In contrast, most patients with large-scale rearrangements of the mtDNA, except multiple deletions, are almost invariably sporadic although there is some evidence of the mutant mtDNA in the patients' oocyte [Zeviani *et al.* 1990]. Only one maternal-inherited family has been described to date, in which the mother has progressive external ophthalmoplegia with the common 4977-base-pair deletion, and her son has the Pearson's syndrome harbouring the identical deletion [Bernes *et al.* 1993]. There is also a small group of recently recognised mtDNA point mutations which are not maternally inherited [Andreu *et al.* 1999a]. The precise explanation why certain mutations are inherited maternally and others are not is unclear. For

maternally inherited mutations, a mother harbouring the mtDNA mutation will transmit the mutant mtDNA to all her offspring but this does not imply that all offspring will develop disease. A number of factors seem to be important in determining the penetrance of a mtDNA mutation in a given individual. These include the amount of the mutant mtDNA and its tissue distribution, threshold effect, the mtDNA haplotypes, the mtDNA copy number and the nuclear background. This complexity of factors which influence penetrance present a major problem for accurate genetic counselling.

1.3.2 Heteroplasmy. Individual cells contain many mitochondria up to 1000 mitochondria per cell and each mitochondrion contain 2-10 copies of mtDNA [Satoh and Kuroiwa 1991]. Hence, a large number of the mtDNA molecules, as many as 10,000 molecules, may exist in a healthy individual cell. In general, the DNA sequence in all these copies of the mtDNA molecules is identical. This state is known as homoplasmy. In contrast, when there is a variation of the mtDNA sequence between the different mtDNA molecules within an individual cell or tissue, i.e. there are more than one species of the mtDNA, this is known as heteroplasmy [Solignac *et al.* 1983]. This state is similar to the heterozygous state of the Mendelian genetics. However, the ratio of heteroplasmy of the different mtDNA molecules can vary from one to 99% and the level of the heteroplasmy is often different between cells and tissues. Most pathogenic mtDNA mutations in humans are heteroplasmic [Pulkes and Hanna 2001]. Exceptions include the common mtDNA point mutations associated with Leber's hereditary optic neuropathy (LHON) and non-syndromic sensorineural deafness which are almost invariably virtually homoplasmic [Harding *et al.* 1995;Johns *et al.* 1992;Prezant *et al.* 1993]. It should be noted that the heteroplasmic state is not always pathogenic. For example, heteroplasmy is observed in the mtDNA control region (the D-loop) as non-pathogenic polymorphisms [Tully *et al.* 2000].

1.3.3 Threshold effect. There is a large body of data, mainly from cell culture studies, indicating that mtDNA mutations exhibit a threshold effect [Chomyn *et al.* 1992]. That is a certain proportion of mutant mtDNA is required before there is a reduction in respiratory chain activity. The exact threshold does seem to vary between different mtDNA mutations [Hanna *et*

al. 1995]. For example most studies using cybrid cell lines indicate that the threshold is greater than 90% mutant for tRNA point mutations and greater than 60% for large-scale deletions of mtDNA [Bourgeron *et al.* 1993;Chomyn *et al.* 1992]. However, there is evidence that the precise threshold may be influenced by other factors. For example, the nuclear background of a cell may change the threshold, and differences have been observed when comparing transformed and untransformed cell lines. For some mutations it seems that virtually 100% mutant is necessary but not always sufficient to produce a disease phenotype. For example, the common mutations which associate with leber's hereditary optic neuropathy are generally virtually homoplasmic. However, affected patients generally only develop disease in the optic nerves, furthermore a significant number of patients are homoplasmic for LHON mutations who never develop disease at all [Harding *et al.* 1995]. Hence, whilst the proportion of mutant mtDNA is likely to be an important determinant of threshold other factors must be involved. Importantly, the mechanisms determining threshold for expression are likely to be different for different mtDNA mutations.

1.3.4 Segregation. In addition to the proportion of mutant mtDNA, the tissue distribution is an additional determinant of penetrance, expression and phenotype. Both mitochondrial division and mtDNA replication were believed to be stochastic processes unrelated to the cell cycle or to the timing of nuclear replication [Wallace 1986]. Therefore, at cell division, a heteroplasmic cell may transmit different proportions of mutant mtDNA to daughter cells [Jenuth *et al.* 1996]. A mother may transmit a variable proportion of mutant mtDNA to offspring resulting in a healthy child or an infant with a devastating neurological disorder. It has been documented that a small proportion of the mutant mtDNA in mother can shift to a high proportion in a single generation. This phenomenon may be explained by a mitochondrial genetic bottleneck. During embryonic development and female germline formation there is a dramatic reduction in the number of mtDNA molecules per cell. If a single mtDNA molecule (or a small number) is expanded to populate the next generation this might explain how large shifts in mutant loads might occur in a

single generation. Therefore if the number of effective mtDNA molecules is small, the rate and size of genetypic shift increase dramatically [Chinnery *et al.* 2000a].

However there is also evidence suggesting that this process may in fact not be an entirely random one. For example, analysis of different tissues of a mouse harbouring heteroplasmic polymorphisms suggested a tissue specific distribution. The factors which might control such a process are unknown [Jenuth *et al.* 1997]. Chinnery *et al.* analysed a frequency distribution of the proportion of mutant mtDNA between offsprings and their mothers of the six most common pathogenic mtDNA point mutations. These data suggested that there appear to be some preferential transmission of the mutant genomes for some mutations [Chinnery *et al.* 2000a].

1.4 Clinical aspects of the mitochondrial encephalomyopathies

Mitochondrial encephalomyopathies are a genetically heterogeneous group of disorders associated with impaired oxidative phosphorylation. During the past four decades since the application of histochemistry, and the modified Gomori trichrome stain in particular, the wide range of clinical features of mitochondrial encephalomyopathies have been increasingly recognized [Engel and Cunningham 1963].

In 1962, Luft et al. described the first case of mitochondrial myopathy in a patient with non-thyroidal hypermetabolism with mild weakness (Luft's disease) [Luft *et al.* 1962]. The morphological abnormalities were limited to skeletal muscle with abnormal accumulations of mitochondria in subsarcolemmal and intermyofibrillar spaces [Haydar *et al.* 1971], which a showed mottled red and irregular appearance with the modified Gomori trichrome stain, the so-called ' ragged-red fibre' [Engel 1967]. Luft showed that this patient's mitochondria were uncoupled. Although Luft's disease was the first description of a mitochondrial myopathy, only two such cases have been reported to date [DiMauro *et al.* 1976]. Subsequently, the syndrome of progressive external ophthalmoplegia, sometimes accompanied with mild proximal weakness, cardiac conduction defect, pigmentary retinal degeneration, and/or ataxia, was shown to be commonly associated with ragged-red fibres [Drachman 1968;Olson *et al.* 1972].

Syndromes exclusively or predominantly involving central nervous system such as MELAS (**M**itochondrial **E**ncephalomyopathy, **L**actic **A**cidosis and **S**troke-like episodes) and MERRF (**M**yoclonic **E**pilepsy with **R**agged **R**ed **F**ibres) were also described in association with ragged red fibres [Fukuhara *et al.* 1980;Pavlakis *et al.* 1984] Although such syndromes are helpful clinically, patients often present with a wide range of clinical features in various combinations not always amounting to a syndromic diagnosis. Mitochondrial respiratory chain has biochemical studies not shown clear correlation between any specific mitochondrial clinical syndromes and particular respiratory chain defects [Petty *et al.* 1986]. Hence, classify mitochondrial encephalomyopathies on biochemical grounds has not been sucessful.

In 1988, Holt et al. described the first mitochondrial DNA mutation, single large deletions, in association with chronic progressive external ophthalmoplegia and Kearns-Sayre syndrome [Holt *et al.* 1988]. Subsequently, Leber's hereditary optic neuropathy, NARP (**N**eurogenic muscle weakness, **A**taxia and **R**etinitis **P**igmentosa), MERRF and MELAS were also shown to be commonly associated with mitochondrial DNA (mtDNA) point mutations.[Goto *et al.* 1990;Holt *et al.* 1990;Shoffner *et al.* 1990;Wallace *et al.* 1988]. Over the last decade, rapid advances in mitochondrial-genetic research revealed some associations between clinical syndromes and the specific mtDNA mutations, although the correlation between mtDNA genotype and phenotype is imperfect. More recently, nuclear DNA mutations have been described in association with various syndromes such as infantile onset mitochondrial encephalomyopathies similar to Leigh syndrome [Schuelke *et al.* 1999;Tiranti *et al.* 1998;Triepels *et al.* 1999]. Patients often presented with rapidly progressive encephalopathy and lactic acidosis sometimes accompanied by cardiomyopathy, hepatic failure or anemia [Allikmets *et al.* 1999;Papadopoulou *et al.* 1999;Valnot *et al.* 2000a;Valnot *et al.* 2000b].

Neurological phenotypes

1.4.1 Chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndrome (KSS).

CPEO is one of the most common clinical manifestations of mitochondrial respiratory chain

diseases. Its onset is often in childhood or adolescence, although onset after age forty can occasionally be seen. Ptosis and ophthalmoplegia are almost invariably the first presentation which may be followed by limb weakness, fatigue and exercise intolerance. The limitation of eye movements is often in all directions, although may be more severe on vertical gaze in some cases. Despite disconjugate gaze diplopia is remarkable uncommon. Opthalmoplegia rarely cause symptoms until it is nearly total, when patients have to turn their heads to look to either side. Ptosis is sometimes initially asymmetrical and may fluctuate. Limb weakness is mainly proximal and mild. Patients often have limitation of daily activities due to exercise intolerance rather than weakness [Petty *et al.* 1986].

KSS is defined as CPEO before the age of 20 years, pigmentary degeneration of the retina, together with one or more of the following: heart block, ataxia and increased protein in cerebrospinal fluid (>1g/L). The other common features variably present included short stature, sensorineural hearing loss, vestibular dysfunction and impaired intellectual function. Ptosis and ophthalmoplegia are similar presentation as CPEO. Pigmentary retinal degeneration is often different from typical retinitis pigmentosa in appearance and has a so-called 'salt and pepper' appearance. It rarely causes severe impairment of visual acuity or visual field defects [Berenberg *et al.* 1977]. Cardiac conduction block can cause syncopal attacks, congestive heart failure or cardiac arrest. Prognosis is poor and most patients die in the third or forth decade often from cardiac problems [DiMauro and Bonilla 1997]. Some patients with CPEO may have some but not all the features of KSS. Single large deletions of the mtDNA are associated with the majority of CPEO and KSS cases. It is suggested that CPEO and KSS are part of a continuous spectrum of the same disease [Hammans and Morgan-Hughes 1994]. Further evidence for this spectrum of severity comes from observation in Pearson's syndrome (sideroblastic anemia and exocrine pancreas dysfunction in infancy) that commonly associates with single large deletions. A few patients with Pearson's syndrome later developed KSS [McShane *et al.* 1991]. In Pearson's syndrome, the mtDNA-deletion molecules were found in blood, bone marrow and pancreas [Cormier *et al.* 1990], in contrast to CPEO and KSS in which blood does not usually harbour detectable mtDNA-deletion molecules by Southern Blot. These

data suggest that single large deletion in the mtDNA are associated with CPEO, KSS and Pearson's syndrome, the clinical presentations are probably related to the proportion of the mutant mtDNA.

About 15% of CPEO harbour the A3243G mutation in the mitochondrial transfer RNA Leucine (UUR) ($tRNA^{Leu(UUR)}$) gene, which is inherited by maternal transmission [Moraes *et al.* 1993a]. Nine other mtDNA point mutations have been reported in association with CPEO as a main presentation, in which each mutation was identified in single case or family (table 1.3). One of the mutations, a G12315A mutation in the $tRNA^{Leu(CUN)}$ gene was identified in a sporadic patient with CPEO, sensorineural hearing loss and a pigmentary retinopathy. The mutation was only present in muscle but not in several other tissues including skeletal myoblasts suggesting this mutation may be new gremline mutation and rapid segregation of mutant mtDNA in particular tissues. The absence of mutant mtDNA in satellite cells suggests that regeneration of skeletal muscle fibres from satellite cells could restore a wild-type mtDNA and possibly normal muscle function [Fu *et al.* 1996].

Multiple deletions of the mtDNA have been identified in a few families with autosomal dominant CPEO. Three linkage loci have been mapped in those families including chromosome 3p14.1-21.2, 4q34-35 and 10q23.3-24.3 [Kaukonen *et al.* 1999;Kaukonen *et al.* 1996; Suomalainen *et al.* 1995]. Recently, Kaukonen *et al.* described mutations in the nuclear gene on chromosome 4q encoding the heart/skeletal muscle isoform of the adenine nucleotide translocator (ANT1) in five families and one sporadic patient with CPEO [Kaukonen *et al.* 2000]. Mutations in two other genes namely the polymerase motif B of the mtDNA polymerase γ (POLG) and the Twinkle genes have subsequently been reported [Spelbrink *et al.* 2001;Van Goethem *et al.* 2001]. Only two autosomal recessive Arabic families with CPEO and cardiomayopathy harbouring multiple deletions of the mtDNA have been described with unknown chromosomal loci [Bohlega *et al.* 1996].

Table 1.3 MtDNA point mutations associated with CPEO.

Gene	MtDNA mutation	References
$tRNA^{Leu(UUR)}$	A3243G	[Moraes *et al.* 1993a]
$tRNA^{Ile}$	T4274C	[Chinnery *et al.* 1997a]
	T4285C	[Silvestri *et al.* 1996]
	G4298A	[Taylor *et al.* 1998]
$tRNA^{Asn}$	G4309A	[Franceschina *et al.* 1998]
	A5692G	[Seibel *et al.* 1994]
	G5703A	[Moraes *et al.* 1993b]
$tRNA^{Tyr}$	T5885del	[Raffelsberger *et al.* 2001]
$tRNA^{Leu(CUN)}$	T12311C	[Hattori *et al.* 1994]
	G12315A	[Fu *et al.* 1996]

1.4.2 Isolated myopathy.

Limb weakness is one of the common accompaniments of mitochondrial encephalomyopathies but myopathy sometimes presents as the sole presentation without other neurological features. The age of onset varies from childhood to adult life, and it is often exhibit a slowly progressive course. It commonly associates with exercise intolerance and exertional fatigue. Objective limb weakness may be mild, proximal weakness with muscle wasting. Exercise intolerance is occasionally the sole presentation without any limb weakness, and it may be difficult to make a diagnosis clinically [Andreu *et al.* 1999a]. Myoglobinuria, rhabdomyolysis, or muscle pain is sometimes the most prominent feature of the syndrome [Chinnery *et al.* 1997b].

Isolated myopathy exhibits genetic heterogeneity. Some families associate with the A3243G MELAS mutation and other families harbour private mtDNA point mutations. Most of the patients are maternally inherited. Except a few mutations in the mitochondrial protein-coding genes, especially cytochrome *b* gene, appear to be association with sporadic cases, and the mutant mtDNA is not identified in family members. In a few cases, mutant mtDNA is only identified in skeletal muscle but other tissues including satellite cells do not harbour the mutant mtDNA suggesting it may somatic mutations [Andreu *et al.* 1999b;Andreu *et al.* 1999a].

1.4.3 Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS).

In 1975, Schapira *et al.* described a syndrome associated with stroke-like episodes, lactic acidemia and ragged red fibres [Shapira *et al.* 1975]. The acronym MELAS was subsequently introduced by Pavlakis to characterise a distinctive group of patients who had young onset stroke-like episodes and lactic acidosis [Pavlakis *et al.* 1984]. Hirano *et al.* reviewed 69 MELAS cases and proposed the following invariant criteria for a diagnosis of MELAS: (1) stroke-like episode before age 40 years; (2) encephalopathy characterised by seizures, dementia, or both and (3) lactic acidosis, ragged-red fibers (RRF), or both. The diagnosis may be considered secure if there are also at least two of the following: normal early development, recurrent headache, or recurrent vomiting [Hirano *et al.* 1992]. Other have noted that MELAS patients with mental retardation or first stroke-like episode after age of 40 years were occasionally observed suggesting the diagnosis of MELAS should be considered at any age in the presence of consistent clinical and imaging features [Damian *et al.* 1995;Hanna *et al.* 1998a].

The stroke-like episodes are frequently not confined to a single vascular territory and commonly affected parieto-occipital areas resulting in hemianopia or cortical blindness. Complete recovery is uncommon. More commonly, patients have some residual deficits and may develop progressive encephalopathy following the stroke-like episodes [Goto *et al.* 1992]. The stroke-like episodes are often accompanied by migrainous-type headache, recurrent vomiting and seizures. Seizures may be focal leading to secondary generalised seizures or myoclonic seizures. Other common additional features include short stature, sensorineural hearing loss and diabetes mellitus. Myopathy, cardiomyopathy, optic neuropathy, cerebellar features, sensorimotor axonal neuropathy, gastrointestinal pseudo-obstruction and nephropathy are less commonly associated features [Ciafaloni *et al.* 1992;Hammans *et al.* 1995;Pavlakis *et al.* 1984].

The primary mtDNA defect associated with MELAS was identified simultaneously by two Japanese groups, Goto *et al.* and Kobayashi *et al.* in 1990 [Goto *et al.* 1990;Kobayashi *et al.*

1990]. The commonest mtDNA point mutation associated with MELAS, A3243G in the $tRNA^{Leu(UUR)}$ gene is identified in over 80% of MELAS cases [Goto *et al.* 1990]. Families harboring the A3243G mutation usually exhibit a maternal inheritance pattern. However, unlike the A8344G MERRF mutation, the A3243G mutation associates with marked clinical heterogeneity even in the same family. Less than half of the patients harboring the A3243G mutation exhibit MELAS [Chinnery *et al.* 2000b;Pulkes *et al.* 2000a]. Other clinical syndromes associated with the A3243G mutation include CPEO, diabetes and deafness, myopathy or MERRF [Hammans *et al.* 1995;Moraes *et al.* 1993a]. Factors underlying such clinical diversity remain unclear. The proportion of the mutant mtDNA, threshold effects, nuclear and mitochondrial DNA backgrounds are thought to influence clinical expression [Morgan-Hughes and Hanna 1999].

Other less common mtDNA mutations associated with MELAS include T3271C mutation in $tRNA^{Leu(UUR)}$ gene [Goto *et al.* 1991]. The majority of MELAS patients harbouring T3271C mutation are in Japan, in which the mutation is identified in 7% of the MELAS cases. Although common in Japan but it has only been identified two Caucasian families [Marie *et al.* 1994;Tarnopolsky *et al.* 1998].

1.4.4 Myoclonic epilepsy with ragged red fibres (MERRF).

The association between progressive myoclonic epilepsy and ragged red fibres was first observed by Tsaris *et al.* in 1973 [Tsaris *et al.* 1973]. Subsequently, Fukuhara *et al.* described two further patients with myoclonic epilepsy associated with ragged red fibres and suggested that this is a distinctive mitochondrial encephalomyopathy syndrome. Hence they proposed the acronym MERRF for this syndrome [Fukuhara *et al.* 1980]. Four other common progressive myoclonic epilepsy syndromes including Unverricht-Lundborg disease, Lafora-body disease, sialidosis and neuronal lipofusinosis, have similar clinical manifestations as MERRF. Unlike other progressive myoclonic epilepsy syndrome, MERRF exhibits maternal inheritance and the age of onset varies widely form childhood up to 50 years old. Early age of onset often correlates with a more severe clinical course leading to death in adulthood [Hammans *et al.* 1993].

MERRF is characterised by myoclonus, seizures and cerebellar ataxia often in association with mitochondrial myopathy. Myoclonus is usually stimulus sensitive i.e. action, noise or photic stimuli. Seizures may be tonic-clonic, focal, absense, or atonic types. Associated features include dementia, optic neuropathy, sensorineural hearing loss, ophthalmoplegia, peripheral neuropathy, foot deformity and lipomas [Hammans and Morgan-Hughes 1994;Rosing *et al.* 1985]. These associated features are clinical clues in the differential diagnosis of progressive myoclonic ataxia. Retinitis pigmentosa, stroke-like episodes, diabetes mellitus, chronic pancreatitis are less common additional features [Austin *et al.* 1998;Byrne *et al.* 1988;Morgan-Hughes *et al.* 1982]. The electroencephalographic features are variable and there are no features to distinguish cases of mitochondrial disease from other causes of progressive myoclonic epilepsy [So *et al.* 1989]. Neuropathology reveals neuronal loss and gliosis affecting dentate nuclei of the cerebellum, globus pallidus, the posterior columns and spinocerebellar tracts of the spinal cord. Abnormal mitochondria were observed in the cells of the cerebellar cortex and of the dentate nuclei [Fukuhara 1991]. The pathological changes of Leigh's syndrome are found in some patients with very severe phenotypes [Berkovic *et al.* 1989;Sweeney *et al.* 1994].

Almost 80% of patients with MERRF are associated with an A8344G mutation in the mitochondrial $tRNA^{Lys}$ gene [Silvestri *et al.* 1993]. Clinical heterogeneity has been described in some large MERRF families harbouring the A8344G mutation. There is a significant correlation between the proportion of mutant mtDNA in blood and both clinical severity, and age of onset. Unlike large-scale rearrangements, all mothers of affected offspring harbour the A8344G mutant mtDNA. Symptomatic mothers are more likely to have affected offspring than asymptomatic mothers [Hammans *et al.* 1993]. MERRF is also associated with other $tRNA^{Lys}$ point mutations including T8356C and G8363A mutations. The 7472C-insertion (7472insC) mutation in the $tRNA^{Ser(UCN)}$ gene has been identified in five families with myoclonus, ataxia, seizures, sensorineural hearing loss and myopathy similar to MERRF phenotype [Jaksch *et al.* 1998;Schuelke *et al.* 1998;Tiranti *et al.* 1995]. In contrast to the A8344G mutations, patients harboring 7472insC mutation do not have ragged red fibres on the skeletal muscle biopsies but

often show that majority of muscle fibres are decrease in cytochrome oxidase (COX) activity.

1.4.5 Neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP).

NARP was originally described in a maternally inherited family with a variable combination of retinitis pigmentosa, ataxia, neurogenic muscle weakness, developmental delay, seizures, dementia, optic atrophy and sensory neuropathy by Holt *et al.* 1990 [Holt *et al.* 1990]. They identified a heteroplasmic T8993G mutation in the mitochondrial ATP synthase subunit 6 (ATPase 6) gene in all studied family members.

In contrast to KSS, retinopathy in NARP patients is often typical of retinitis pigmentosa, with clump of pigment and bone corpuscle formation in retinae leading to constricted visual fields and night blindness. Gait and limb ataxia is cerebellar type and frequently associated with cerebellar atrophy on brain scan. Limb weakness predominantly affects proximal muscles, is often mild, and may be obscured by limb ataxia. Axonal sensory neuropathy may involve any sensory modalities with absent tendon reflexes. Muscle biopsies almost invariably reveal no histochemical evidence of mitochondrial myopathy such as ragged red fibres or COX activity abnormalities [Holt *et al.* 1990]. Since the lack of characteristic muscle histochemistry is generally common in NARP patients, the combination of the described clinical features and maternal transmission are important clues for consideration of molecular analysis.

1.4.6 Leigh's Syndrome (LS).

In 1951 Leigh first described a child who died from subacute severe encephalomyelopathy with strikingly distinct neuropathological abnormalities mainly in the thalamus, basal ganglia and brainstem [Leigh 1951]. Leigh syndrome is the most common mitochondrial encephalomyopathy in infancy and childhood. It is a devastating encephalopathy associated with a range of neurological signs including psychomotor retardation, optic atrophy, ophthalmoplegia, ptosis, nystagmus, ataxia, tremor, dystonia, pyramidal signs and abnormal breathing. Definite diagnosis depends on either characteristic neuroimaging or postmortem neuropathology features of bilateral symmetrical spongiform lesions localised especially in the

thalamus, basal ganglia and brainstem. These findings are microscopically associated with cystic cavitation, demyelination, vascular proliferation and gliosis [DiMauro *et al.* 1994]. Onset of LS is often in the first year of life, however juvenile and adult-onset LS are described [Nagashima *et al.* 1999;Santorelli *et al.* 1996a].

Although the primary molecular pathogenesis of LS was first identified in the ATPase 6 gene of the mtDNA, T8993G mutation [Tatuch *et al.* 1992], LS appears to be more commonly inherited by an autosomal recessive trait than a maternally inherited trait [DiMauro and De Vivo 1996]. Autosomal recessive LS families often have complex IV (cytochrome c oxidase), complex I (NADH-ubiquinone reductase) or less commonly complex II (succinic-ubiquinone reductase) deficiency, and are associated with mutations in the nuclear genes. X-linked recessive families with a defect of pyruvate dehydrogenase complex (PDHC), which does not affect the respiratory chain directly, also exhibit LS [Rahman *et al.* 1996].

1.4.7 Sensory ataxic neuropathy, dysarthria, ophthalmoparesis (SANDO).

Four sporadic patients from North America developed a distinct clinical syndrome presenting with severe sensory ataxic neuropathy, dysarthria and late-onset progressive external ophthalmoparesis. Fadic et al. proposed the acronym SANDO and identified multiple deletions in the mtDNA in all patients. The most prominent feature is sensory ataxic neuropathy and ophthalmoparesis often presenting after 30-years of age. Migraine and depression are also common. Nerve conduction studies often show predominantly sensory axonal neuropathy. Muscle biopsies generally reveal RRF with or without COX-deficient fibres [Fadic *et al.* 1997].

1.4.8 Leber's hereditary optic neuropathy (LHON).

LHON has been recognised as a distinctive syndrome causing subacute bilateral visual loss for over a hundred years [von Graefe 1858]. LHON is characterised by acute or subacute severe bilateral visual loss commonly in young males, which may develop simultaneously or more commonly sequentially. The time interval between affected eyes average eight weeks and the duration of progression of visual loss in each eye usually occur over a period of one to six weeks [Riordan-Eva *et al.* 1995]. Although it commonly occcurs between 11-30 years of age,

patients outside of this age range are not uncommon. In the pedigree analysis of eighty-five LHON families by Harding *et al.*, onset of the first visual symptom ranged from age 6 to 62 years [Harding *et al.* 1995]. Patients often complain of fogging or blurring corresponding to centrocaecal scotoma, enlarged blind spot, or loss of central vision on examination. Loss of vision may be acute or subacute onset, and may occasionally be sudden and complete. Vision generally fails to counting fingers, only 5% of patients experienced the worst visual acuity 6/60 or better [Riordan-Eva *et al.* 1995]. Pain on eye movements, around the affected eye or Uhthoff's phenomenon is an uncommon feature. Pupils usually exhibit a slow symmetrical response to light, although a relative afferent pupillary defect is common at an early stage when one eye is predominantly affected [Harding and Sweeney 1994]. Magnetic resonance imaging (MRI) scans of optic nerves within 4 months of onset of visual loss are often normal. High signal of the affected optic nerves on the MRI scans is commonly shown after 4 months.

Cardiac pre-excitation syndrome has been described in several Finnish LHON families [Nikoskelainen *et al.* 1985]. These observations were not confirmed by studies in Australian, American and British LHON kindreds, although one unaffected mother had ECG evidence of Wolff-Parkinson-White syndrome [Bower *et al.* 1992;Ortiz *et al.* 1992]. It is possible that the high prevalence of pre-excitation syndrome in the Finnish families is due to coincidental genetic predisposition [Riordan-Eva *et al.* 1995].

Almost all of LHON patients are associated with three common mtDNA point mutations in the mitochondrial complex I subunit genes including G3460A in ND1 gene, G11778A in ND4 and T14484C in ND6 gene [Harding *et al.* 1995]. The G11778A mutation is the commonest cause of LHON. The patients harboring T14484C have a better prognosis than patients harboring G3460A or G11778A. In contrast to MELAS and MERRF mutations, LHON mutations are often virtually homoplasmic in blood. Although most families exhibit maternal inheritance pattern, about one third of the patients do not have family history of visual impairment. Males are more commonly affected than females in association with any of the LHON mutations. The excess of affected males suggested a possible X-linked visual loss susceptibility locus (VLSL) initially reported by Bu *et al.* and Vilkki *et al.* in 1991 [Bu and

Rotter 1991;Vilkki *et al.* 1991]. This was not confirmed by other groups [Carvalho *et al.* 1992;Chalmers *et al.* 1996;Juvonen *et al.* 1993;Sweeney *et al.* 1992]. However, pedigree analysis does support the existence of X-linked VLSL gene as an explanation for the propensity for visual loss to occur in the males members of families harboring the LHON mutations [Harding *et al.* 1995].

A multiple sclerosis-like illness has been described in some patients with LHON [Harding *et al.* 1992a]. Patients exhibited clinical, immunological and magnetic resonance imaging (MRI) features indistinguishable from MS. MRI of brain of the affected females with multiple sclerosis-like illness harboring G11778A mutations often show multiple white matter abnormalities predominantly involving the periventricular area. Most patients are females associated with the G11778A mutation. These findings are sometimes observed in females without MS-like illness harboring G11778A mutation. Brain MRI scans of males are generally normal [Riordan-Eva *et al.* 1995] The higher prevalence of MS-like illness associated with LHON than in general populations suggests that these observations do not occur coincidentally [Horvath *et al.* 2000].

1.4.9 Sensorineural hearing loss or deafness.

Sensorineural hearing loss is commonly associated with phenotypes such as MELAS, MERRF or KSS. However mitochondrial sensorineural deafness in isolation had not been recognised until the last decade after the observation of several maternal inheritance families susceptible to antibiotic-induced ototoxicity [Higashi 1989;Hu *et al.* 1991]. Prezant *et al.* proposed that the mitochondrial ribosomal RNA (rRNA) may be a candidate gene due to the similarity of the aminoglycosides binding site of the 16S rRNA of Escherichia coli (E. coli) and a region of the human mitochondrial 12S rRNA [Prezant *et al.* 1993]. They described four families with aminoglycoside-induced and nonsyndromic sensorineural deafness in association with an A1555G mutation in the 12S rRNA [Prezant *et al.* 1993]. Subsequently, the A1555G mutation was shown to be the commonest cause of late-onset familial sensorineural deafness in Spain accounting for 27% of the familial cases [Estivill *et al.* 1998]. This high prevalence of A1555G

mutation in Spain is not caused by a founder effect [Torroni *et al.* 1999]. A study in Japan revealed that the A1555G is accounted for over 3% of all patients with sensorineural hearing loss in out patient clinic and one-third of them had previously aminoglycosides injections [Usami *et al.* 2000]. Patients often have late-onset bilateral gradually progressive sensorineural hearing loss. However age of onset less than 2 years is occasionally occurred. Age of onset vary widely within family however most patients often have symptoms before 40 years old. Hearing loss is both spontaneous or relate to aminoglycosides treatment. It commonly affected high frequency at the beginning. Hearing loss is often mild unless it is aminoglycosides-induced, in which it is generally associated with acute deafness few weeks after received aminoglycosides treatment [Estivill *et al.* 1998;Usami *et al.* 1997]. Family with maternally inherited sensorineural hearing loss are also described in association with A7445G, T7511C and 7472insC mutations in the $tRNA^{Ser(UCN)}$ gene [Reid *et al.* 1994;Sue *et al.* 1999;Verhoeven *et al.* 1999]. The clinical features are similar to the A1555G mutation but the A7445G and T7511C mutations have not been reported in association with aminoglycoside-induced deafness. Families harboring the 7472insC mutation also exhibit other neurological features including ataxia, myoclonus, seizures and polyneuropathy [Ensink *et al.* 1998;Tiranti *et al.* 1995].

Non-neurological phenotypes

Non-neurological features associated with primary mtDNA disorders are less common. They are often part of phenotypes rather than present as a sole manifestation such as short stature and diabetes mellitus in MELAS, cardiomyopathy in KSS or lipomatosis with MERRF. However some conditions may lead to premature death especially cardiomyopathy. Some of the important non-neurological features associated with mitochondrial respiratory chain defects are described here.

1.4.10 Heart.

Cardiac manifestations are relatively common presentations of mitochondrial encephalomyopathies and more importantly, they sometimes are fatal. The common cardiac features are both conduction defects and cardiomyopathy. Studies cardiac manifestations in 17

patients with 6 CPEO, 3 KSS (single large deletions), 5 MELAS (A3243G) and 3 MERRF (A8344G), 3 patients with KSS had cardiac conduction defects. Two-third of MERRF patients had cardiomyopathy and 2 out of 5 patients with MELAS had symmetrical left ventricular hypertrophy [Anan *et al.* 1995]. Hypertrophic cardiomyopathy is occasionally described in Leigh's syndrome (T8993C) [Marin-Garcia *et al.* 1996]. Maternally inherited hypertrophic cardiomyopathy is genetic heterogeneity. It has been identified in association with several mtDNA point mutations including A1555G mutation in the 12S rRNA gene, A3243G mutation in the $tRNA^{Leu(UUR)}$ gene, A4295G and A4300G mutations in the $tRNA^{Ile}$ gene, A8296G and G8363A mutations in the $tRNA^{Lys}$ gene, G15423A mutation in the cytochrome *b* gene and some sporadic cases with mtDNA depletion [Casali *et al.* 1999;Marin-Garcia *et al.* 1997;Merante *et al.* 1994;Merante *et al.* 1996;Santorelli *et al.* 1996b;Santorelli *et al.* 1999;Silvestri *et al.* 1997;Valnot *et al.* 1999].

Dilated cardiomyopathy is less common than hypertrophic type. It has been described in patients with MELAS and MERRF [Anan *et al.* 1995;Grasso *et al.* 2001]. Familial idiopathic dilated cardiomyopathy is described in association with mtDNA multiple deletions and a point mutation, T12297C mutation in the $tRNA^{Leu(CUN)}$ gene [Suomalainen *et al.* 1992].

1.4.11 Endocrine: Diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD) or Wolfram syndrome.

In 1938, Wolfram syndrome (WFS) was first described as familial juvenile-onset diabetes mellitus and optic atrophy [Wolfram and Wagener 1938]. It is characterised by optic atrophy, diabetes mellitus, diabetes insipidus, deafness, urinary tract atony, and other neuropsychological symptoms including ataxia, myoclonus and peripheral neuropathy, also known as DIDMOAD (**D**iabetes **I**nsipidus, **D**iabetes **M**ellitus, **O**ptic **A**trophy, and **D**eafness). Onset is often in the first decade presenting with insulin-dependent diabetes mellitus followed by optic atrophy. Patients often died from respiratory failure associated with brainstem atrophy. The prevalence is estimated 1 in 770,000 in the UK and 1/100,000 in North America, which mode of inheritance is autosomal recessive [Barrett *et al.* 1995;Fraser and Gunn 1977]. Mutations in a gene encoding

a transmembrane protein (WFS1) causing WFS has been recently identified on chromosome 4p16.1 [Hardy *et al.* 1999;Inoue *et al.* 1998]. Pathogenesis of WFS is remained unknown. However WFS1 is thought to maintain normal islet β-cell function since mutations in this gene alone lead to premature death of islet β-cell [Inoue *et al.* 1998].

Diabetes mellitus (DM) is one of the common features associated with A3243G (MELAS) mutation. This mutation is also described in families maternally inherited DM and deafness [Hammans *et al.* 1995]. Patients with mitochondrial encephalomyopathies often have short stature, which has sometimes been shown to be associated with deficient growth hormone secretion [Yorifuji *et al.* 1996].

1.4.12 Bone Marrow.

Pearson's syndrome is a fatal infantile disorder characterised by refractory sideroblastic anemia, thrombocytopenia, neutropenia and pancreatic exocrine dysfunction. Their bone marrow pathology is characterised by remarkable vacuolization of erythroid and myeloid precursors, hemosiderosis, and ringed sideroblasts [Pearson *et al.* 1979]. Additional features include renal tubular acidosis and hepatic failure. It has been described in association with mtDNA large-scale rearrangements, single large deletions and less commonly deletion-duplication same as CPEO and KSS [Rotig *et al.* 1989;Superti-Furga *et al.* 1993]. Most patients died at young age however some patients who survived, are subsequently developed KSS [McShane *et al.* 1991;Simonsz *et al.* 1992]. Furthermore, a variant of Pearson's syndrome, an infant with congenital hypoplastic anemia, renal tubulopathy, diabetes mellitus and cerebral atrophy, has been described in association with single large deletion. She subsequently developed progressive external ophthalmoplegia and KSS [Majander *et al.* 1991]. These observations indicate that the two disorders have the same molecular basis, the different phenotypes may be determined by mutation load and threshold effect of various tissues. Mitochondrial related sideroblastic anemia is also described in families harboring A8344G, multiple deletions of the mtDNA and mutation of a putative mitochondrial iron transporter gene (ABC7) suggesting it is genetic heterogeneity [Allikmets *et al.* 1999;Casademont *et al.* 1994;Wang *et al.* 1999].

1.4.13 Gastrointestinal tract: Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE).

MNGIE is a unique autosomal recessive multisytem disorders associated with multiple deletions or partial depletion of mtDNA or both which is ascribed to a defect in communication between nuclear and mitochondrial genomes. It is characterised by gastrointestinal dysmotility, cachexia, ptosis, progressive external ophthalmoparesis, polyneuropathy and leukoencephalopathy on brain MRI [Bardosi *et al.* 1987;Hirano *et al.* 1994]. Onset may vary form 5 months to 43 years of age which majority of patients experienced symptoms before 20 years of age. Patients often died in the forth decade. Almost half of patients developed gastrointestinal symptoms as an initial manifestation. Opthalmoparesis is also a common initial presentation, which may be subtle and insidious and patients may not to recognise at the beginning.

Gastrointestinal features are the most prominent clinical features which are most related to dysmotility including borborygmi, abdominal pain and cramps, diarrhoea, early satiety, nausea/vomiting, diverticulosis, pseudo-obstruction, gastroparesis and dysphagia. Cachexia is another prominent feature but mechanism responsible for such severe wasting is unknown. Despite the apparent of muscle atrophy, muscle strength is well maintained [Nishino *et al.* 2000].

Neurological symptoms are often mild. Peripheral neuropathy affects both motor and sensory nerves leading to glove-stocking sensory loss and mild distal limb weakness. Patients occasionally have debilitating weakness. Hearing loss is observed in almost half of the patients. Pigmentary retinopathy and mental retardation are rare. Nerve conduction studies are invariably consistent with demyelinating neuropathy but it sometimes also shows additional features compatible with axonal neuropathy. Electromyography sometimes shows myogenic pattern in addition to more common neuropathic features. Increased cerebrospinal fluid (CSF) protein is observed in most patients. Brain MRI generally reveals diffuse leukoencephalopathy in all studied cases, which sometimes relatively spare corpus callosum. Despite wide spreading of leukoencephalopathy on MRI brain, it is commonly no clinical manifestation. COX-deficient muscle fibres are invariably observed and almost two-third of patients has RRF on muscle

biopsy [Nishino *et al.* 2000]. In 1999, Nishino *et al.* described mutations in thymidine phosphorylase (TP) gene on chromosome 22q13.32-qter in 12 MNGIE probands. TP activity in leukocytes in MNGIE patients is less than 5% of normal controls indicating that loss-of-function mutation of the TP gene cause the disease [Nishino *et al.* 1999].

MNGIE is a clinically homogeneous disease causing by mutations in the TP gene. All patients developed: (1) gastrointestinal dysmotility, (2) cachexia, (3) ptosis and ophthalmoparesis, (4) peripheral neuropathy, (5) leukoencephalopathy on brain MRI, (6) laboratory evidence of mitochondrial dysfunction including COX-deficient muscle fibres and RRF or multiple deletions of mtDNA. Pseudo-MNGIE has been described in patients with gastrointestinal dysmotility and cachexia but lack of at least one of the other clinical criteria. All pseudo-MNGIE patients had normal TP activity and there are no any mutations in the TP gene. This data suggests that brain MRI is useful to confirm the diagnosis of MNGIE despite the lack of clinical features of the central nervous system involvement is generally observed [Nishino *et al.* 2001].

1.5 Primary defects of mitochondrial DNA and nuclear DNA

1.5.1 MtDNA point mutations involving tRNA and rRNA genes.

The most common and perhaps most complex of all known mtDNA point mutations is the, so-called, MELAS mutation, which is an A3243G transition in the $tRNA^{Leu(UUR)}$ gene. The A3243G mutation has been identified in approximately 80% of patients with MELAS [Goto *et al.* 1992;Hammans *et al.* 1995]. However, it also associates with other phenotypes including deafness and/or diabetes mellitus [Van den Ouweland *et al.* 1992a;Van den Ouweland *et al.* 1999], CPEO [Goto *et al.* 1990;Hammans *et al.* 1995], KSS [Hammans *et al.* 1995], myopathy [Hammans *et al.* 1995], encephalopathy other than stroke [Hammans *et al.* 1995;Moraes *et al.* 1993a] and a MELAS/MERRF overlap syndrome [Ciafaloni *et al.* 1992;Hammans *et al.* 1995]. The precise mechanisms underlying such phenotypic diversity are not understood. There is some evidence that the proportion of mutant mtDNA in muscle is one factor [Hammans *et al.* 1995;Morgan-Hughes *et al.* 1995]. For example patients with the MELAS phenotype have

higher proportions of mutant mtDNA in muscle [Hammans *et al.* 1995]. However, this dramatic phenotypic diversity is not related simply due to differences in proportion and tissue distribution of mutant mtDNA. For example, this laboratory has described a family with virtually 100% mutant mtDNA who exhibited myopathy alone [Hammans *et al.* 1995]. In contrast patients harbouring very low proportion of mutant mtDNA may exhibit disease [Chinnery *et al.* 2000c]. Other factors, which may be involved in determining the genotype-phenotype relationship, include mtDNA haplotype, mtDNA copy number, nuclear background and mtDNA polymorphisms.

All human tRNAs undergo post-transcriptional modification which influence both structural and functional properties including correct folding, aminoacylation and codon-recognition [Degoul *et al.* 1998;Helm *et al.* 1998]. In cell cybrids harbouring A3243G mutation there is evidence of hypomodification at a site in the DHU-stem [Helm *et al.* 1999] and also a deficiency of aminoacylation [Janssen *et al.* 1999]. These data suggest a loss of function of $tRNA^{Leu(UUR)}$ for which the threshold effect in vitro is very high; over 95% of mutant mtDNA is required to cause respiratory chain impairment [El Meziane *et al.* 1998a;Jacobs and Holt 2000]. In contrast, HeLa cell cybrids revealed that mutant mtDNA failed to modify the wobble-base (first anticodon position) which has an important role in restricting decoding to leucine UUR codons and not decoding phenylalanine UUY codons [Yasukawa *et al.* 2000]. This may result in frequent misreading of leucine to phenylalanine. A further study on patient tissue showed a decrease in the level of aminoacylated $tRNA^{Leu(UUR)}$ which may result in stalling of ribosomes at leucine codons, mistranslation of UUR codons or translational frame shifting [Borner *et al.* 2000].

Mutations in the $tRNA^{Leu(UUR)}$ gene appear to be more commonly associated with MELAS than other tRNA gene mutations although MELAS is genetically heterogeneous. Other MELAS-causing mutations include A3260G, T3271C, T3291C mutations [Goto *et al.* 1991;Goto *et al.* 1994;Nishino *et al.* 1996].

Mutations in the $tRNA^{Lys}$ gene are particularly associated with MERRF. An A8344G transition in the $tRNA^{Lys}$ gene is the most common cause of the MERRF syndrome [Hammans

et al. 1993;Shoffner *et al.* 1990]. However, there is a clinical diversity associated with the A8344G mutation. Other phenotypes including CPEO and myopathy [Hammans *et al.* 1993], encephalomyopathy without myoclonus [Hammans *et al.* 1991], myopathy with or without cardiomyopathy [Silvestri *et al.* 1993], Leigh's syndrome [Rahman *et al.* 1996] and multiple symmetrical lipomatosis [Holme *et al.* 1993].

Cultured primary myoblasts harbouring the A8344G mutation revealed a defect of translation process but not transcription and this defect in protein synthesis seemed to preferentially affect COX subunits [Hanna *et al.* 1995]. Further study in cybrid cells showed that the A8344G mutation causes a premature termination of translation at each or near each lysine codon [Enriquez *et al.* 1995]. These data suggested that a deficiency of aminoacylation of $tRNA^{Lys}$ might be the cause of such a phenomenon. However, recent study on A8344G patient tissue revealed that there was no decrease in aminoacylated $tRNA^{Lys}$ residue [Enriquez *et al.* 1995]. Most mutations in other tRNA genes have only been identified in a single individual or family and it is therefore not possible to make genotype-phenotype correlations.

Pathogenic point mutations in the mitochondrial rRNA genes identified in association with human disease appear to be less common than in the tRNA genes. The A1555G mutation in the 12S rRNA gene has been identified in several families with nonsyndromic sensorineural deafness and aminoglycoside-induced deafness [Prezant *et al.* 1993]. The nucleotide 1555 situated near a stem loop in 12S rRNA required for binding of aminoglycosides [Prezant *et al.* 1993]. Increased susceptibility to aminoglycoside toxicity may be explained by action of aminoglycosides on bacterial ribosomes, which have similar structure to a part around nucleotide 1555 of human mitochondrial 12S rRNA. Aminoglycosides may have a similar effect on human mitochondrial 12S rRNA harbouring the A1555G mutation [Estivill *et al.* 1998]. The pathogenic mechanism may involve interference with mitochondrial translation [Inoue *et al.* 1996].

However there is also evidence suggesting nuclear gene factor may influence phenotypic expression in association with the A1555G mutation. Study on lymphoblastoid cell lines derived from members of a large family carrying this mutation revealed that the severity of

mitochondrial dysfunction in the mutant cell lines was correlated with the presence or absence of hearing loss in the donor individuals [Guan *et al.* 1996]. Recently, a locus on chromosome 8 was significantly linked to the A1555G mutation suggesting a nuclear predisposition [Bykhovskaya *et al.* 2000;Bykhovskaya *et al.* 2001].

A pedigree with maternally inherited sensorineural deafness, levodopa-responsive parkinsonism and neuropathy was recently identified a heteroplasmic T1095C mutation in the 12S rRNA gene. Respiratory chain enzyme studies in cultured lymphocytes from the proband revealed a reduction in COX activity [Thyagarajan *et al.* 2000].

1.5.2 MtDNA point mutations involving protein-coding genes.

Until recently, mutations in the mtDNA protein encoding genes were mainly described in three disorders, LHON, Leigh's syndrome and NARP. LHON is associated with three main primary mutations including G3460A mutation in ND1 gene [Huoponen *et al.* 1991], G11778A mutation in ND4 gene [Wallace *et al.* 1988] and T14484C mutation in ND6 gene [Johns *et al.* 1992]. The G11778A mutation is occasionally associated with non-LHON; for example, it has been identified in a family with parkinsonism [Simon *et al.* 1999]. The G14459A [Jun *et al.* 1994] and T14596A mutations in ND6 gene and A11696G mutation in ND4 gene [de Vries *et al.* 1996] have been associated with LHON with or without dystonia and the G13513A mutation in ND5 gene with LHON and MELAS [Pulkes *et al.* 1999]. To date LHON has only been identified in association with mutations in the complex I genes.

NARP and Leigh's syndrome have been associated with the T8993G mutation in the ATPase 6 gene [Holt *et al.* 1990;Santorelli *et al.* 1993]. The proportion of the T8993G mutant DNA a patient harbour has been shown to correlate with clinical severity [Makela-Bengs *et al.* 1995;Tatuch *et al.* 1994]. Individuals with levels of mutant mtDNA less than 70% are usually asymptomatic or mildly affected. NARP often associates with proportions of mutant mtDNA in muscle or blood between 70 and 90%. In contrast patients harboring proportion of mutant mtDNA above 90% generally develop Leigh's syndrome [Santorelli *et al.* 1993;Uziel *et al.* 1997]. Furthermore, the proportions of the T8993G mutant mtDNA in several tissues are

generally homogeneous in contrast to tRNA gene mutations [Ciafaloni *et al.* 1993;Uziel *et al.* 1997]. Therefore the molecular diagnosis of the T8993G is reliable from a easily accessible source of DNA i.e. blood and prenatal diagnosis is a possible approach to the prevention of severe disease caused by this mutation [Dahl *et al.* 2000;Harding *et al.* 1992b]. Other phenotypes associated with the T8993G mutation appear to be uncommon but progressive external ophthalmoplegia, stroke-like episodes and cardiomyopathy have been identified [Pastores *et al.* 1994;Santorelli *et al.* 1997a].

Although mutations in the ND and ATPase 6 genes are mainly associated with the LHON, NARP and Leigh's syndrome, other phenotypes have been observed. Phenotypic heterogeneity has been observed with mutations in these genes (table 1.4). For example mutations in complex I genes have also been described in bilateral striatal necrosis/MELAS [Campos *et al.* 1997a], MELAS [Penisson-Besnier *et al.* 2000;Pulkes *et al.* 1999;Santorelli *et al.* 1997b] and exercise intolerance [Andreu *et al.* 1999b;Musumeci *et al.* 2000].

Several mutations in mitochondrial COX genes have been described recently. Although the first mtDNA COX mutation, T9957C in the COX III gene, was associated with MELAS [Manfredi *et al.* 1995], all subsequent mutations associate with exercise intolerance, fatigue and/or weakness as the main clinical features [Clark *et al.* 1999;Hanna *et al.* 1998b;Keightley *et al.* 1996;Rahman *et al.* 1999]. The observation that mitochondrial protein-encoding mutation may associate with exercise intolerance as the sole clinical feature has recently been confirmed by the discovery of pathogenic cytochrome *b* mutation [Dumoulin *et al.* 1996]. Others and we have recently identified seven point mutations in the cytochrome *b* gene in association with exercise intolerance (table 1.4) [Andreu *et al.* 1999a]. All patients in whom muscle histochemistry was performed revealed COX-positive ragged red fibres (RRF). In contrast to most of the mitochondrial point mutations, these mutations have been identified in sporadic cases without family history of neurological disorders and they all had isolated skeletal muscle involvement and no mutant mtDNA in blood

Table 1.4 MtDNA mutations in protein encoding genes.

Gene	Mutation	Phenotypes	References
ND1	T3308C	Bilateral striatal necrosis/MELAS	[Campos *et al.* 1997a]
	G3460A	LHON	[Huoponen *et al.* 1991]
	3902 (7bp-inv)	Myopathy and exercise intolerance	[Musumeci *et al.* 2000]
ND4	A11696G	LHON/dystonia	[de Vries *et al.* 1996]
	G11778A	LHON	[Singh *et al.* 1989]
	G11778A	Parkinsonism	[Simon *et al.* 1999]
	G11832A	Exercise intolerance	[Andreu *et al.* 1999b]
ND5	G13513A	MELAS	[Santorelli *et al.* 1997b]
	G13513A	LHON/MELAS	[Pulkes *et al.* 1999]
ND6	G14459A	LHON/dystonia	[Jun *et al.* 1994]
		Leigh's syndrome	[Kirby *et al.* 2000]
	T14596A	LHON/dystonia	[de Vries *et al.* 1996]
	T14484C	LHON	[Johns *et al.* 1992]
Cyt b	14787 (4bp-del)	Parkinsonism/MELAS	[De Coo *et al.* 1999]
	G14846A	Exercise intolerance	[Andreu *et al.* 1999a]
	G15059A	Exercise intolerance/myoglobinuria	[Andreu *et al.* 1999c]
	G15084A	Exercise intolerance	[Andreu *et al.* 1999a]
	G15168A	Exercise intolerance	[Andreu *et al.* 1999a]
	T15197C	Exercise intolerance	[Keightley *et al.* 2000]
	G15243A	Hypertrophic cardiomyopathy	[Valnot *et al.* 1999]
	G15498A	Histiocytoid cardiomyopathy	[Andreu *et al.* 1999a]
	G15615A	Exercise intolerance	[Dumoulin *et al.* 1996]
	G15723A	Exercise intolerance	[Andreu *et al.* 1999a]
	G15762A	Exercise intolerance	[Andreu *et al.* 1998]
COX I	T6721C, T6742C	Sideroblastic anemia	[Gattermann *et al.* 1997]
	6015 (5bp-del)	Motor neuron diseasse-like	[Comi *et al.* 1998]
	G6930A	Myoclonic ataxia/deafness	[Bruno *et al.* 1999]
COX II	T7587C	Myopathy/ataxia/deafness	[Clark *et al.* 1999]
	T7671C	Myopathy	[Rahman *et al.* 1999]
COX III	9537insC	Leigh-like syndrome	[Tiranti *et al.* 2000]
	G9952A	Encephalomyopathy	[Hanna *et al.* 1998b]
	T9957C	MELAS	[Manfredi *et al.* 1995]
	9480 (15bp-del)	Exercise intolerance/myoglobinuria	[Keightley *et al.* 1996]
ATP 6	T8993G	Leigh syndrome	[Santorelli *et al.* 1993]
	T8993C	Leigh syndrome	[Santorelli *et al.* 1994]
	T8993G	NARP	[Holt *et al.* 1990]
	T8993C	NARP/MILS	[de Vries *et al.* 1993]
	T9176C	NARP/MILS	[Campos *et al.* 1997b]
	T8851C	Bilateral striatal necrosis	[de Meirleir *et al.* 1995]
	T9176C	Bilateral striatal necrosis	[Thyagarajan *et al.* 1995]

Abreviations as follow: Cyt b = cytochrome b, ATP6 = ATP synthase 6, inv = Inversion, del = deletion, ins = insertion, MELAS = mitochondrial encephalopathy, lactic acidosis and stroke-like episodes, LHON = Leber's hereditary optic neuropathy, NARP = neurogenic weakness, ataxia and retinitis pigmentosa, MILS = matenally inherited Leigh's syndrome.

[Andreu *et al.* 1999a]. Furthermore in some cases, the mutations were not detected in the of myoblast cell lines studied. These data suggest that these mutations may be somatic mutations [Andreu *et al.* 1999a]. There are three other mutations in the cytochrome *b* gene have been described in association with phenotypes other than exercise intolerance. The first mutation was a microdeletion in a 20-year-old man who presented with parkinsonism and MELAS [De Coo *et al.* 1999]. The others, G15243A and G15498A mutations, were described in association with sporadic cardiomyopathy [Andreu *et al.* 2000;Valnot *et al.* 1999].

1.5.3 Large-scale rearrangements of mtDNA.

Large-scale rearrangements of mtDNA were the first defect in the mtDNA to be described in association with human mitochondrial disease [Holt *et al.* 1989a]. The commonest defect is a single large deletion, in which several thousand base pairs are deleted from the 16 kilobase pairs of mtDNA molecule. Following the discovery of single deletion, it has been appeared that patients sometimes harbour other rearrangements form of mtDNA. These include duplication, multiple deletions and deletion-duplication which are commonly associated with CPEO including KSS [Holt *et al.* 1989b] as well as Pearson's syndrome [Rotig *et al.* 1995]. Patient harboured a single large-scale deletion are typically normal at birth and during early development and the amount of deleted mtDNA molecules in skeletal muscle increased over time [Larsson *et al.* 1990]. This may explain why the onset of the CPEO and KSS does not begin in childhood.

Most patients harbouring large-scale rearrangements of the mtDNA are sporadic cases, although the mtDNA deletions are present in low abundance in apparently normal oocytes [Chen *et al.* 1995]. The exception of a few families harbouring multiple deletions of mtDNA which are inherited in a autosomal dominant fashion [Servidei *et al.* 1991]. There is usually no evidence of single large deletions in family members of CPEO and KSS patients [Moraes *et al.* 1989], except in one family, a mother and her son harboured the same single large deletion [Bernes *et al.* 1993]. There was another reported family which a mother and her son harbouring large scale deletions but their deletions were different in sizes and locations suggesting they

were not maternal transmission [Ozawa *et al.* 1988]. Furthermore, heteroplasmy of mitochondrial large-scale rearrangements distributed in wide range of tissues suggests that large-scale rearrangements of the mtDNA may be somatic mutations occurring at a stage earlier than organogenesis [Zeviani *et al.* 1990]. Whereas mtDNA duplications can be maternal transmitted which is possibly less pathogenic [Manfredi *et al.* 1997]. Recently, the first animal model of human mitochondrial disease has been described [Inoue *et al.* 2000]. This mouse harboured a single large deletion of the mtDNA. This will allow studying a pathogenesis of the mutant mtDNA and could lead to a therapeutic implication in the future.

The phenotypic diversity is also observed in association with large-scale rearrangement but in lesser degree compare to mtDNA point mutations. Other phenotypes included KSS/strokes [Brockington *et al.* 1995], Leigh's syndrome [Yamadori *et al.* 1992], Wolfram syndrome [Barrientos *et al.* 1996], ataxia [Nakai *et al.* 1994], enteropathy [Cormier-Daire *et al.* 1994], diabetes mellitus and deafness [Ballinger *et al.* 1992].

1.5.4 Nuclear gene mutations.

Several hundreds of nuclear DNA encoding mitochondrial-related proteins. These proteins are required for mitochondrial functions such as oxidative phosphorylation, mtDNA replication, protein transportation and protein translation. Recently, mutations in these genes caused human diseases have been identified.

1.5.4.1 Complex I disorders

Isolate complex I deficiency appears to be common cause of mitochondrial encephalomyopathies and the most common clinical presentation is Leigh's syndrome (LS) [Morris *et al.* 1996]. LS with complex I deficiency accounts for almost 20% of LS cases [Rahman *et al.* 1996]. It has been described in association with the nuclear-encoded complex I subunit genes including *NDUFS7* and *NDUFS8* genes [Loeffen *et al.* 1998;Triepels *et al.* 1999]. Both families exhibited autosomal recessive LS with typical neuropathological findings. A 5 base-pairs duplication in the nuclear gene encoding the 18-kilodaltons (AQDQ) subunit has been described in a patient with infantile encephalopathy characterised by severe psychomotor

retardation, seizures, bradypnea, cyanosis, hypotonia and symmetrical basal ganglia abnormalities and generalised brain atrophy [van den *et al.* 1998]. Mutations in the *NDUFV1* gene has subsequently been described in families with fatal leukodystrophy with myoclonic epilepsy [Schuelke *et al.* 1999].

1.5.4.2 Complex II disorders

The first description of nuclear genes associated with is LS is flavoprotein subunit gene of complex II (succinate dehydrogenase subunit A or *SDHA*), which is described in two sisters with a family history of consanguineous parents [Bourgeron *et al.* 1995]. Further mutation in the *SDHA* gene has subsequently been identified in a family with late onset optic atrophy, ataxia and myopathy [Birch-Machin *et al.* 2000].

Apart from the above mentioned complex II flavoprotein subunit mutations, mutations in other subunits of the complex II genes namely succinate dehydrogenase subunit B and D (*SDHB* and *SDHD*) have recently been identified in association with neoplasms. These included familial paragangliomas, glomus tumours and pheochromocytoma [Baysal *et al.* 2002;Neumann *et al.* 2002]. It is estimated that sporadic patients who had mutations of *SDHB* and *SDHD* had 20-30% likelihood of subsequent development of a glomus tumours. Therefore periodic physical and ultrasonographic examinations of the neck should be performed to detect the tumour in early stage [Dluhy 2002].

1.5.4.3 Complex III disorders

In 2001, De Lonlay *et al.* identified mutations in *BCS1L* gene, which appears to be involved in the assembly of complex III in four Turkish families with isolated complex III deficiency [de Lonlay *et al.* 2001]. Clinical manifestations were relatively homogeneous including lactic acidosis at birth, neonatal tubulopathy, encephalopathy, psychomotor retardation and liver failure. Mutations in the *BCS1L* gene have been identified in one-third of patients with isolated complex III deficiency [de Lonlay *et al.* 2001].

1.5.4.4 Complex IV disorders

Tiranti et al. described mutations in *SURF1* gene, which account for 75% of LS with COX deficiency [Tiranti *et al.* 1998;Tiranti *et al.* 1999a]. The *SURF1* mutations led to an early blockage of assembly of COX [Tiranti *et al.* 1999b]. Mutations in other COX assembly genes, *SCO1* and *SCO2* genes have subsequently been identified in patients with severe infantile encephalopathy [Papadopoulou *et al.* 1999;Valnot *et al.* 2000a]. In contrast to patients harboring *SURF1* mutations, which generally have LS, patients harboring *SCO2* mutations often exhibit hypertrophic cardiomyopathy and severe encephalomyopathy resembling LS but without the characteristic neuropathological features of LS [Sue *et al.* 2000]. Whereas *SCO1* was described in only two brothers with neonatal-onset hepatic failure, ketoacidotic coma and encephalopathy, which both of them died within two months after birth [Valnot *et al.* 2000a]. Another family with autosomal recessive encephalopathy with isolated COX deficiency has been described in association with a mutation in heme A:farnesyltransferase gene (COX10) [Valnot *et al.* 2000b]. One sister developed LS accompanied with de Toni-Fanconi-Debré syndrome [Ogier *et al.* 1988]. A younger brother and sister had leukodystrophy and renal tubulopathy but no neuroimaging or neuropathological features were described.

1.5.4.5 Nuclear genes affecting mtDNA replication and mtDNA level.

1.5.4.5a Multiple deletions of mtDNA

Mutations in some nuclear genes affect mtDNA replication resulting in multiple deletions of the mtDNA. Affected patients exhibit either autosomal dominant or recessive inheritance implying that nuclear genes are causative. To date patients with the known gene mutations exhibit two major clinical syndromes; CPEO and MNGIE. It appears that almost all CPEO families associated with multiple deletions are autosomal dominant except for some ophthalmoplegia cardiomyopathy families [Bohlega *et al.* 1996]. Although autosomal dominant progressive external ophthalmoplegia (adPEO) has ophthalmoplegia as the clinical hallmark of the syndrome, other manifestations are also observed including proximal limb weakness, severe depression, peripheral neuropathy, sensorineural hearing loss, cataracts, and endocrine

dysfunction. In 2000 Kaukonen et al. described families with adPEO was associated with mutations in the adenine nucleotide translocator gene (*ANT1*) [Kaukonen *et al.* 2000]. The function of *ANT1* is to transport adenosine triphosphate across the inner mitochondrial membrane in exchange for adenosine diphosphate. The *ANT1* mutations disrupt or modify transmembrane alpha helices. However *ANT1* defect causes secondary accumulation of mtDNA mutations in post-mitotic cells by a still unknown mechanism [Kaukonen *et al.* 2000]. Mutations in two other genes namely the polymerase motif B of the mtDNA polymerase γ (*POLG*) and the Twinkle genes have subsequently been reported [Van Goethem *et al.* 2001;Spelbrink *et al.* 2001]. Polymerase gamma is the DNA polymerase responsible for mtDNA replication [Van Goethem *et al.* 2001]. Twinkle is a mitochondrial protein with structural features similar to bacteriophage T7 primase/helicase and may be involved in mtDNA replication [Spelbrink *et al.* 2001]. It is not clear how these diverse protein defects cause multiple deletions of mtDNA. There are two further linkage loci on chromosome 10q [Suomalainen *et al.* 1995] and 3p [Kaukonen *et al.* 1996] but causative genes have not yet been identified.

Patients with MNGIE also harbour multiple deletions and sometimes depletion of the mtDNA however they had different mode of transmission and phenotype apart from ptosis ophthalmoplegia was also present in MNGIE suggesting they had different molecular pathogenesis. The causative gene was thymidine phosphorylase (TP) gene on chromosome 22q [Nishino *et al.* 1999]. TP drives the thymidine salvage pathways, which it catalyzes the breakdown of thymidine to be reutilized for dTTP synthesis [Suomalainen and Kaukonen 2001]. TP activity in MNGIE patients were virtually undetectable suggesting mutations were loss-of-function mutations. These patients had increased plasma thymidine level more than 20-fold result in an imbalance of nucleotide pools for DNA synthesis. Since mtDNA constantly replicates even in post-mitotic cells hence the imbalance of nucleotide pools may adversely affects on mtDNA replication more than nuclear DNA. Leading to mtDNA multiple deletion and/or depletion [Nishino *et al.* 2001].

1.5.4.5b MtDNA depletion.

MtDNA depletion syndrome is a rare inherited disease in which patients have a decrease in the amount of mtDNA in tissues often less than 10% of control levels [Moraes *et al.* 1991]. The age at onset is frequently in the first few years of life with rapid deterioration leading to death. However patients with later onset and slowly progressive course were also observed [Barthelemy *et al.* 2001]. Clinical manifestations include myopathy, encephalomyopathy, nephropathy and liver failure depending on which tissue exhibits depletion of the mtDNA [Bakker *et al.* 1996;Moraes *et al.* 1991]. There has been no evidence of maternal transmission. Parents of reported cases have been asymptomatic implying that the mode of transmission may be autosomal recessive trait. Studies on skin fibroblasts of patients with the mtDNA depletion suggested that the molecular pathogenesis might involve the replication or maintainance of the mtDNA [Bodnar *et al.* 1995]. Cybrid cells with the nuclear genome of the rho0 cells and the mtDNA of the mtDNA-depleted cells restored mtDNA levels and mitochondrial respiratory chain function. These data suggested that mtDNA depletion might be caused by the nuclear gene defect [Bodnar *et al.* 1993]. No underlying gene defect has yet been described.

1.6 Aims of the present study

The aims of this project fall into three board areas

1) To define the molecular genetic basis of human mitochondrial neurological disease in thirty patients without any known mitochondrial mutations by mtDNA sequence analysis.

2) To characterise pathogenic status and study molecular mechanism of mutations identified using molecular biology, cell culture and single fibre techniques.

3) To determine the molecular mechanisms which explain the marked phenotypic diversity associated with pathogenic mtDNA mutations causing human neurological diseases. This includes the common A3243G mutation and newly identified mutations in the present work when tissues or cells are available to study.

Chapter 2 Methods

2.1 Overview

This chapter describes materials and experimental methodology employed in this study. The methodological stages of mitochondrial DNA (mtDNA) analysis are described including DNA extraction, measurement of DNA concentration, the polymerase chain reaction (PCR) including all oligonucleotide primers and restriction enzymes, DNA sequencing, quantitation of the proportion of the mutant mtDNA and southern blot analysis. The methods of human muscle cell culture included dissociation of muscle, muscle cell growth conditions and passage, harvesting cell cultures and long-term storage of cell cultures. In addition, the methodological stages of protein electrophoresis including mitochondrial protein extraction, measurements of protein concentration and single-dimension protein electrophoresis are described. Buffers and solutions used are detailed at the end of the chapter.

2.2 Analysis of mitochondrial DNA

All patients studied had common mtDNA mutations excluded. This included large-scale rearrangements and point mutations at the nucleotide position 3243, 8344 and 8993 using standard methods by the service team of this lab, MG Sweeney and V. Stinton. The available clinical, histochemical and biochemical data of the studied patients were reviewed. All patients, who were recruited to sequence the mtDNA, had typically clinical features and at least one of the characteristic histochemical or biochemical features of mitochondrial encephalomyopathic syndromes. The clinical features of the patients studied are given in relevant result chapters.

2.2.1 DNA extraction. All DNA samples used for sequencing the mtDNA were extracted from either fresh or frozen skeletal muscle tissues kept in liquid nitrogen unless otherwise specified.

In brief, DNA extraction from muscle samples and cells was performed by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Firstly, muscle tissue was finely chopped with a razor blade in a petri dish. The tissue was then transfered to a

1.5 ml eppendoff tube and 300 µl of digestion buffer (PE Applied Biosystems) added. Two-hundred µg of proteinase K (Sigma) was added and the tube swirled gently. The tube was placed flat in the shaker (at 30-45 rpm) in the incubator at 50°C and leaved overnight. The tissue would then be fully digested, added a 500 µl of buffered phenol and inverted the tube gently for 5 minute. Spinned the tube at 2000 rpm for 10 minutes. After spinning the mixture would separate into two layers. The upper aqueous layer containing the DNA was removed into a new 1.5 ml eppendoff tube. Added another 500 µl of phenol and repeated the spinning step again. The aliquot aqueous layer was added a 600 µl of chloroform and mix gently for 5 minutes. Spin the mixture again at 2000 rpm for 10 minutes. Transferred the aqueous phase into a clean Sarstedt DNA tube and added ½ of its volume of 7.5M NH_4OAc followed by two volumes of absolute ethanol. Inverted the tube gently, DNA may be seen at this stage (If not place the tube in -70 freezer for 15 minutes). The DNA sample was then centrifuged at 10,000 rpm for 15 minutes. Poured off the supernatant. Washed the DNA again with 70% ethanol. Re-centrifuge at 5000 rpm for 5 minutes and poured off supernatant. Air dried the pellet and resuspended with 1×TE buffer.

2.2.2 Measurement of DNA concentration. DNA concentration was estimated by measurement of optical density (OD) using a spectrophotometer (Cecil, model CE202). OD measurements were performed at a wavelength of 260 nm using quartz cuvettes. Measurements were calibrated using distilled water. DNA dilutions of 5 µl of DNA in 1 ml of distilled water (1 in 200) were used for OD measurements. A 5 µg of the total DNA was used for Southern blot analysis.

2.2.3 Polymerase chain reaction (PCR). This technique carried out to amplify any selective target regions of the DNA by using a pair of primers flanking the region, along with the enzyme *Taq* polymerase (Promega). The amplification reactions were performed on a programmable thermal cycler (PE Applied biosystems model 480, 9600 and 9700). Typical reaction components are listed in table 2.1.

Table 2.1 Typical PCR reaction components (50 µl/reaction)

DNA	100	ng
10×Buffer	5	µl
*dNTP mix	5	µl
Primer (forward)	5-10	pmol
Primer (reverse)	5-10	pmol
Taq DNA polymerase	1-1.5	u

*The deoxynucleotides (dNTP) mixture consisted of 200 µM of each dNTP (Promega).

The above reaction mixtures were overlaid with 40-60 µl of liquid paraffin prior to use. In all PCR reactions, water controls were used to exclude contamination. All reactions consisted of the following three steps repeated thirty times:

1. Denaturation of DNA at 92°C for 30 seconds
2. Annealing of primers to DNA at variable temperature for 30 seconds
3. Primer extension at 72°C for 30 seconds

The annealing temperature varies depending on the base composition of the primers employed (table 2.2). Each reaction is preceded by one denaturation step for 3 minutes at 94°C, followed by adding *Taq* DNA polymerase (Promega) at 80°C for 1 min. The final extension step for 5 minutes at 72°C was followed after 30 cycles of PCR. After amplification 10 µl aliquots of the products were electrophoresed on 1.6% (w/v) agarose gel in lx TBE buffer with the addition of ethidium bromide (50 µg/ml), at 50 volts. Electrophoresed products were identified by illumination on an ultraviolet light box. The PCR reactions are generally performed for 4 major purposes, which were described as follow:

2.2.3.1 To generate template for sequencing analysis. The sequencing techniques employed in this study using two types of sequencing kits: ABI Prism Dye Primer and ABI Prism BigDye Primer. Since the latter kit had produced a better quality of sequencing data and more sensitive in detecting a lower mutant load in the tissues. Therefore we have changed the sequencing kits for mitochondrial works in our lab to detect the mutant mtDNA with a low proportion since early 2000. Both techniques are used the same primers for making the PCR templates and

required the same procedure to clean the PCR templates. Forty-six nucleotide primer pairs spanned through the entire mtDNA (designed by Dr IP Nelson). The size of each PCR product is between 400-600 bp. After preparing the PCR templates in the interested regions of the mtDNA, the PCR products were washed using a QIAquick PCR purification kit (Qiagen, Germany) according to the company's protocol. The purified PCR products were then underwent the second sequencing PCR using the Dye or BigDye primer sequencing kits.

Cycle sequencing using Dye primer sequencing kit. This was carried out on DNA Thermal cycler 480 using a 0.5 ml eppendoff tube. Each PCR fragment was separated into 4 reactions including reagents A and C (2 μl in each tube) and reagents G and T (4 μl) mix with 0.5 μl of the PCR products in A and C tubes and 1 μl of the PCR products in G and T tubes. Then overlaid the reactions with 20 μl of paraffin oil and spinned to layer the oil over the aqueous reactions. The sequencing PCR was performed as follow:

1. Preheat the thermal cycler at 95°C.
2. Fifteen cycles of 95°C for 30 seconds, 55°C for 30 seconds and 70°C for 1 minutes.
3. Then followed by 15 cycles of 95°C for 30 seconds and 70°C for 1 minutes

The samples were then ready to concentrate the pallet by pipette the extension reactions from the bottom of the four (A, C, G and T) tubes into a clean tube with 80 μl of 95% ethanol. Placed the tube in the -70 freezer for 10 minutes and spinned the tubes in the microcentrifuge at maximum speed for 45 minutes. Poured off the supernatant and air dry the pellet. The pellet is then ready for loading into polyacrylamide gel, which is described in section 2.2.4 in this chapter.

Cycle sequencing using BigDye primer sequencing kit. This was carried out on GeneAmp 9600 or 9700 using a 96-well plate. Each PCR fragment was separated into 4 reactions including 2 μl of reagents A, C, G and T mix with 0.5 μl of the purified PCR products. The sequencing PCR was performed as follow:

1. Fifteen cycles of 96°C for 10 seconds, 55°C for 5 seconds and 70°C for 1 minute.

2. Then followed by 15 cycles of 96°C for 10 seconds and 70°C for 1 minute.

The samples were then ready to concentrate the pallet as methods in the Dye primer section.

2.2.3.2 To determine the presence of a particular point mutation. After analysis of the sequencing data all of the previously unreported nucleotide substitutions were analysed. The changes in the protein-coding genes resulted in highly conserved amino acid changes and all changes in other genes were designed PCR and restriction fragment length polymorphisms (PCR-RFLP) or mismatch PCR-RFLP reactions to determine the presence of the particular mutation in the patients and controls and to detect the degree of heteroplasmy if it existed. Restriction enzyme cutting sites were either gained or lost by base changes identified during sequencing used to differentiate between mutant and wild-type DNA. Each digestion was performed in 20 µl reaction, 5-10 units of restriction enzymes and 2 µl of enzyme buffer (provided with enzyme by manufacturer) was added in 10 µl of PCR product and incubated for 2 hours (the optimal assay temperature was shown in table 2.2). The digested products were then separated on agarose gel for electrophoresis. Here is the list of the primers and restriction endonuclease enzymes using in this study.

Table 2.2 PCR and restriction digest protocols for detection of the mt nucleotide substitutions.

Mutations	**Genes**	**Primers** (5′ →3′, forward then reverse) Mismatch nucleotide in bold and underline	**Annealing Temp (°C)**	**Restriction enzymes**	**Digest Temp (°C)**
C3388A	ND1	L3310 (nt 3310-3329) H3512 (nt 3512 -3493)	55	*Bfa* I	37
G3796A	ND1	L3640 (nt 3640-3659) 5'- GTTCTTGTGTTGTGATA**CC**G	55	*Age* I	37
G4810A*	ND2	L4650 (nt 4650-4669) H4860 (nt 4860-4841)	55	*Hinf* I	37
A5874G*	$tRNA^{Tyr}$	5'-TAGATTTACAGTCCAATG**A**T H6020 (nt 6020-6001)	51	*Mbo* I	37
7472C-ins*	$tRNA^{Ser(UCN)}$	5'-AGGAAGGAATCGAACC**A**CCC H7640 (nt 7460-7541)	54	*Xcm* I	37
T7472G	$tRNA^{Ser(UCN)}$	L7442m	55	*Xho* I	37

		H7626 (nt 7426-7607)			
G8616T	ATP6	L8574 (nt 8574-8593) H8726 (nt 8726-8717)	55	*Mbo* I	37
T8594C	ATP6	5'-CCTACCCGCCGCAGTAC**GT**A H8726 (nt 8726-8707)	55	*Rsa* I	
T9083C	ATP6	5'-TAGCAATATCAACCATTA**GG**C H9244 (nt 9244-9225)	55	*Hae* III	37
T9185C	ATP6	L9026 (nt 9026-9045) H9206 (nt 9206-9185)	55	*Bst* OI	60
A10887G	ND4	5'-GCATCATCCCTCTACTATTTGC**T**A H11158 (nt 11158-11139)	55	*Nhe* I	37
T11232C*	ND4	L11091 (nt 11091-11110) H11290 (nt 11290-11271)	52	*Mnl* I	37
A12119C	ND4	5'-TCCTCCTATCCCTCAACCCCG**GG**A H12281 (nt 12281-12262)	55	*Bam*H I	37
T12173A	$tRNA^{His}$	L12016 (nt 12016-12036) 5'-GGTCGTAAGCCTCTGTTG**C**C	55	*Bsr* I	65
G12294A*	$tRNA^{Leu\ (CUN)}$	L12174 (nt 12174-12193) H12363 (nt 12363-12344)	55	*Mbo* I	37
G13513A*	ND5	L13370 (nt 13370–13389) 5'-TGCGGTTTCGATGAGAG**A**T	46	*Bgl* II	37
A13780G	ND5	L13702 (nt 13702-13721) H13841 (nt 13841-13822)	60	*Bsm* FI	65
C13802T	ND5	L13650 5'-GGAAAGTGACAGCGAGGC**A**T	55	*Nde* I	37
A14687G	$tRNA^{Glu}$	L14522 (nt 14522-14541) 5'-CATATCATTGGTCGTGGT**GC**T	52	*Nhe* I	37
G15723A*	Cyt *b*	L15553 (nt 15553-15534) H15996 (nt 15996-15977)	55	*Mse* I	37

* Pathogenic mtDNA mutations identified in this study.

Mismatch nucleotides in the primer sequences are shown in bold and underlined.

2.2.3.3 To quantify proportion of mtDNA point mutations in various tissues (fluorescent-labelled PCR). The author quantified proportions of mutant mtDNA using fluorescently

labelled PCR method followed by particular RFLP reactions. Oligonucleotide primers and conditions of each PCR were the same as in section 2.2.3.2 (table 2.2) but each reaction was performed for only 19 cycles instead of 30 cycles. After 19th cycle added 1 μl of one-tenth dilution of fluorescent dUTP (PE Applied Biosystems) in water. Followed by one cycle of 92°C for 60 seconds, annealing of primers to DNA at variable temperature for 30 seconds and 72°C for 2 minutes. PCR product was then washed using QIAquick PCR purification kit (Qiagen, Germany) according to the company's protocol. RFLP was performed using restriction enzymes and conditions list in table 2.2. The samples were then precipitated by using four times volume of 95% ethanol. Placed the tube in the -70 freezer for 15 minutes and spinned the tubes in the microcentrifuge at maximum speed for 45 minutes. Poured off the supernatant and air dry the pellet. The pellet was loaded into polyacrylamide gel for Genescan analysis, which is described in section 2.2.5.

2.2.3.4 To quantify proportion of mtDNA point mutations in single muscle fibres (single-fibre PCR). Single fibre PCR is a PCR-based system to study heteroplasmic mtDNA mutations in single muscle fibres. Muscle fibres are microdissected under the microscope and subjected to PCR amplification. Molecules originating from mutant and wild-type mtDNAs are distinguished by specifically design of RFLP for each mutation. The presence of higher levels of mutant mtDNAs in cells with an abnormal phenotype is one of the most compelling pieces of evidence of pathogenicity for a mtDNA mutation.

2.2.3.4A Equipment. Microdissection of muscle fibres requires a Pasteur pipette and an inverted microscope. Capillaries are prepared by flaming Pasteur pipettes at the narrow end and pulling the ends apart when the glass starts to melt until they are separate into two pieces. The narrow tip of the Pasteur pipette should be a little bigger than a muscle fibre. Dissection is performed under an inverted microscope.

2.2.3.4B Preparation of muscle sections and isolation of single fibres. The thickness of the section was 30 μm. Slides were pretreated with polylysine to avoid movement during the dissection. Sections are stained for enzyme activity of succinate dehydrogenase, cytochrome *c*

oxidase, or both depending on muscle histochemical abnormalities of each patient. Stained sections provide a visual phenotype that will direct the dissection. Slides are then immersed in a 100-mm cell culture dish containing 50 ml of 50% ethanol. Single fibres are isolated by prepared pastuer pipettes under an inverted microscope. Isolate the chosen fibre by pulling away adjacent fibres surrounding the targeted one, and then pick up the targeted fibre. Fibres are placed directly into 10 µl of purified water in a 0.5 ml Eppendorf tube (Brake the tip of pipettes in the eppendorf tubes).

2.2.3.4C DNA amplification from single fibres. After all fibres are picked, spin tubes in a microcentrifuge at maximum speed for 5 minutes. Remove the supernatant and add 5 µl of an alkaline lysis solution to the fibre and incubating it at 65°C for 30 minutes. The solution is then neutralized by adding 5 µl of a buffered neutralizing solution. For the detection of single nucleotide changes, florescent-incooperated PCR reactions (methods 2.2.3.3 but PCR is performed for 30 cycles instead of 19 cycles) are performed using specific nucleotide primer pairs followed by specific RFLP reaction for each newly identified mutation. Restriction endonuclease-digested fragments are separated by native 5% polyacrylamide gel electrophoresis and analysed by Genescan software as methods 2.2.5. The results of the proportions of the mutant mtDNA in normal and abnormal muscle fibres would then be analysed using student *t*'s test (SPSS version 8.0).

2.2.4 DNA sequencing

Automated sequencing of the entire mtDNA was undertaken with 45 PCRs. Details of the PCR conditions and cycle sequencing is in section 2.3. The oligonucleotide primers are shown in table 2.3 all of which were designed by Dr. IP Nelson. PCR product of each primer pairs has about 100 bp overlapping to other PCR product of primer pairs close to it. Annealing temperature for each PCR is 55°C except for primers, light strand: L4215 and heavy strand: H4643, is 53°C. This section details methods of acrylamide gel electrophoresis and analysis of sequencing data.

Table 2.3 Oligonucleotide primers for sequencing analysis of mtDNA

PCR	Light strand	Nucleotide position	Heavy strand	Nucleotide position
1	L336	336 - 355	H706	706 - 687
2	L660	660 - 679	H1040	1040 - 1021
3	L901	901 - 920	H1430	1430 - 1411
4	L1412	1412 - 1431	H2022	2022 - 2003
5	L1917	1917 - 1936	H2541	2541 - 2522
6	L2466	2466 - 2485	H3023	3023 - 3004
7	L2925	2925 - 2944	H3287	3287 - 3268
8	L3056	3056 - 3075	H3406	3406 - 3387
9	L3285	3285 - 3304	H3745	3745 - 3726
10	L3704	3704 - 3723	H4325	4325 - 4306
11	L4215	4215 - 4234	H4643	4643 - 4624
12	L4393	4393 - 4412	H4899	4899 - 4880
13	L4854	4854 - 4873	H5470	5470 - 5451
14	L5465	5465 - 5484	H5988	5988 - 5969
15	L5866	5866 - 5885	H6485	6485 - 6466
16	L6426	6426 - 6445	H6968	6968 - 6949
17	L6799	6799 - 6818	H7196	7196 - 7177
18	L7115	7115 - 7134	H7650	7650 - 7631
19	L7588	7588 - 7607	H7992	7992 - 7973
20	L7865	7865 - 7884	H8445	8445 - 8426
21	L8196	8196 - 8215	H8726	8726 - 8707
22	L8646	8646 - 8665	H9244	9244 - 9225
23	L9151	9151 - 9170	H9744	9744 - 9725
24	L9638	9638 - 9657	H10107	10107 - 10088
25	L9983	9983 - 10002	H10452	10452 - 10433
26	L10362	10362 - 10381	H10724	10724 - 10705
27	L10651	10651 - 10670	H11158	11158 - 11139
28	L11091	11091 - 11110	H11449	11449 - 11430
29	L11374	11374 - 11393	H11854	11854 - 11835
30	L11758	11758 -11777	H12191	12191 - 12172
31	L12069	12069 - 12088	H12395	12395 - 12376
32	L12309	12309 -12328	H12876	12876 - 12857
33	L12829	12829 - 12848	H13389	13389 - 13370
34	L13301	13301 -13320	H13841	13841 - 13822
35	L13702	13702 - 13721	H14217	14217 - 14198
36	L13926	13926 - 13945	H14446	14446 - 14427
37	L14300	14300 - 14319	H14883	14883 - 14864

38	L14595	14595 -14614	H15038	15038 - 15019
39	L14983	14983 - 15002	H15343	15343 - 15324
40	L15221	15221 - 15240	H15649	15649 - 15630
41	L15553	15553 - 15572	H15996	15996 - 15977
42	L15788	15788 – 15807	H16084	16084 - 16065
43	L15875	15875 – 15894	H16345	16345 - 16326
44	L16240	16240 - 16259	H33	33 - 14
45	L16527	16527 - 16546	H390	390 – 371

2.2.4.1 Polyacrylamide gel electrophoresis. 36-cm-double glass plates were used with the 373A DNA sequencer (PE Applied Biosystems). Plates were cleaned with detergent and rinsed with distilled water. The dry plates were put together and poured the freshly made acrylamide gel mix (6% denature acrylamide gel mixture is in section 2.5) between the plates. Insert spacing strip between the two plates and clamped with small 3 clips on the top of the plates and big 3 clips along each sides of the plates. Left gel to set for 2 hours. Removed all the clips off the plates and assembled the plates to the sequencer. Switched on the sequencer and reset the computer. Filled up Sequence sample sheet correspond to loading samples by selecting 'Sequence sample' icon. Next selected an icon 'Sequence run'. The Sequence run settings are as follow:

Run module: XL scan
Lanes: choose 48 or 64
Collect time: 12 hours
Well-to-read distance: 24 cm
Filter set: A
PMT gain: 840V

Run plate check for background fluorescence for 5 minutes. After plate checked, filled in upper and lower buffer chambers with 1.3 litre of 1 x TBE buffer (section 2.5). Removed the spacing strip and inserted a 48 or 64-well shark's tooth comb in its place. Mixed up the pellet with 6 µl of loading buffer containing 5:1 of formamide:blue dextran. Load 2 µl of odd samples in odd lanes corresponding to the sample sheet. Pre-Run was then run for 15 to 20 minutes and paused

the run. After sequencer stopped running, loaded 2 μl of even samples in each correct even lane. Then stopped the Pre-Run and selected run.

2.2.4.2 Sequencing data analysis. Analysis sequencing data from gel file involves extraction data from the gel file, sequence analysis to get electropherogram of sequence data and analysis using Autoassembler software to compare the results of sequence analysis to control sequences. Extract of data from the gel file is undertaken after the sequencer stopped running. The gel file was selected from 'Run' window. Retracked the lanes on gel file in which the retracked lines should be in the lanes from top to bottom of the lanes. After finished retracking every lane then selected 'Extract lanes'.

A 'Sample Manager' window should be appeared which contained a list of samples. Changed primer set depending on samples was forward or reverse primers in the column headed 'Dye set/primer'. Checked each sample file where the data point start and stop. Then changed the start and stop points depending on the length of the PCR product of each samples. This would cut the fluorescent artefact at the start point result in a better quality of electropherogram. Then selected the 'Start' icon and analysis would be undertaken.

Analysis of the sequencing data compared to normal sequence was carried out using Autoassembler software. The standard mtDNA sequence was downloaded to the computer (Genebank: www.ncbi.nlm.nih.gov/irx/genebank 1997) and created mtDNA sequence text file. Downloaded this control sequence or available previous control sequence data to the Autoassembler new window. Each pair of forward and reverse sample file then downloaded to the Autoassembler and compared to the sequencing data.

2.2.5 Quantitation of the proportion of mtDNA point mutations

Proportions of the mutant mtDNA in this study is quantified by fluorescently labelled PCR - RFLP reactions and visualised the PCR fragments using laser-reading 373A DNA Sequencer (PE Applied Biosystems). Accuracy of this method is confirmed by constructing a standard curve by mixing varying proportions of myoblast cloned wild type and cloned mutant mtDNA ranging from 0-100% by Dr. IP Nelson. This fluorescently labelled PCR method is accurate

while cycles are not more than 20 cycles. The PCR method is described in the section 2.2.3 and buffers and solutions used are detailed in section 2.5.

2.2.5.1 Polyacrylamide gel electrophoresis. 24-cm-double glass plates were used with the 373A DNA sequencer (PE Applied Biosystems). Plates were cleaned as sequencing plates. The dry plates were put together and poured the freshly made acrylamide gel mix (5% native acrylamide gel mixture is in section 2.5) between the plates. Insert spacing strip between the two plates and clamped with small 3 clips on the top of the plates and big 3 clips along each sides of the plates. Left gel for 2 hours to polymerise. Removed all the clips off the plates and assembled the plates to the sequencer. Switched on the sequencer and reset the computer. Filled up Genescan sample sheet correspond to loading samples by selecting 'Genescan sample' icon. Next selected an icon 'Genescan run'. The Sequence run settings are as follow:

Run module: Full scan
Lanes: choose 24 or 36
Collect time: 5 hours
Well-to-read distance: 12 cm
Filter set: B
Voltage: 800V
Power: 40W

Run plate check for background fluorescence for 5 minutes. After plate checked, filled in upper and lower buffer chambers with 1.1 litre of 1 x TBE buffer (section 2.5). Removed the spacing strip and inserted a 24 or 36-well shark's tooth comb in its place. Mixed up the pellet with 1 µl of loading buffer, 0.5 µl of Genescan-500 Tamra and 3 µl of npH_2O. Load 2.5-4 µl of odd samples in odd lanes corresponding to the sample sheet. Pre-Run was then run for 15 minutes and paused the run. After sequencer stopped running, loaded 2.5-4 µl of even samples in each correct even lane. Then stopped the Pre-Run and selected run.

2.2.5.2 Genescan data analysis. Analysis genescan data from gel file involves extract lane from the gel file, analysis to size the PCR fragments and get the genescan data to calculate proportions of wild-type and mutant DNA fragments. To extract data from gel file was

undertaken as extract data from sequencing gel file but choose the genescan gel file instead. After extract lanes, sample. A 'Sample Manager' window would be appeared which contained a list of samples. Click on arrow icon in one of the samples and set size depending on a manufacturer's information for each red peak, which was Tamra standard. Chose the red-colour block icon for Tamra standard and other colour depending on types of dUTP used, then clicked 'analyse' icon. After Genescan analysis, result control window was selected, then selected the colour of the used dUTP, peak of each PCR fragment, size, data point and peak area for each sample would be shown. Calculated proportions of the mutant mtDNA as follow:

$$\frac{\text{(Peak area of mutant peak / data point of its peak)} \times 100\%}{\text{(Peak area of mutant peak/datapoint of its peak)} + \text{(Peak area of wild-type peak /datapoint of its peak)}}.$$

2.2.5.3 Statistic analysis of single fibre PCR results. For single fibre PCR, data of proportions of the mutant mtDNA in each muscle fibres was put in a database created for each mutation using SPSS software version 8.0. The data would then be analysed using independent-sample t test to confirm the differences of the proportions of the mutant mtDNA in normal and abnormal muscle fibres.

2.3 Human muscle cell culture

2.3.1 Dissociation of muscle.[Hanna *et al.* 1995] Human muscle cell cultures employed in this study involved dissociation of muscle fibres in order to release muscle precursor cells namely satellite cells. Fresh skeletal muscle (200-400 mg) was obtained from diagnostic muscle biopsies. The muscle was placed in a sterile 40-ml beaker containing 10-ml of dissociation solution (section 2.5). The muscle fibres were teased apart using sterile needles and after covering with sterile aluminium foil, the beaker was incubated in a shaking water bath at 37°C for 15 minutes. 10 ml of normal growth medium (section 2.5) was then added in order to neutralise the enzymes and the solution was gently pipetted with a 25-ml pipette in order to further dissociate the muscle fragments. The solution was allowed to settle for 10 minutes and the supernatant, containing the dissociated satellite cells, was drawn off and filtered through two layers of nylon mesh (upper layer 50 lower layer 20 pin). This was done through a plastic funnel

into a Sterilin universal container. The mesh and funnel were sterilised prior to filtering using alcohol and then rinsed with a large volume of DMEM. A further 10 ml of dissociation solution was added to the beaker containing the muscle tissue and this cycle of incubation was repeated a further three times. After each cycle, the universal containing the dissociated satellite cells was stored in the incubator at 37°C. The four universals were then centrifuged at 1000rpm for 10 minutes in order to pellet the cells. The supernatants were then removed by gentle aspiration with a sterilised Pasteur pipette and the four pellets resuspended in a final volume of 1.5 ml of growth medium, placed in a non- collagen coated 35-mm tissue culture dish and placed in the incubator for 15 minutes at 37°C. During this time, the majority of fibroblasts will attach to the plastic surface whereas myoblasts will not. Following this the supernatant, which is now fibroblast depleted was drawn off and placed in a collagen coated 35mm plate and incubated overnight at 37°C in a 8% CO_2 atmosphere. The following day the supernatant containing tissue debris and unattached cells, was drawn off and the plate was gently washed with growth medium to remove any further red cells and debris. Inspection of the plate under the light microscope now revealed attached myoblasts.

2.3.2 Muscle cell growth conditions and passage. All muscle cell cultures were grown in growth medium described in section 2.5. All growth medium was supplemented with uridine to aid the growth of respiratory deficient cells. Cultures were incubated at 37°C in a 8% CO_2 atmosphere. Growth medium was changed every three days. Passage of cells was performed by aspirating off the growth medium, washing the cells twice in phosphate buffer saline (PBS) and trypsininized with 1×Trypsin-EDTA in DMEM. The cells were incubated at 37°C for 5 minutes during which time the cells would detach from the culture plate. Growth medium was then immediately added to inhibit the trypsin following which the cells were pelleted by centrifugation for 10 minutes at 10,000 rpm, resuspended in growth medium and plated out. Mass cultures were expanded by passaging to plates of increasing diameter. The order of plate diameters was 35-mm, 60-ml, 100-mm and 70-ml culture flask. Passage was undertaken when cultures were subconfluent before myoblast fusion had commenced.

2.3.3 Harvesting cell cultures. Harvesting cell cultures for mtDNA analysis was undertaken. All growth medium was aspirated off and cultures washed twice with PBS. Cells were then scraped off the dish into ice cold PBS using Teflon coated cell scrapers. The harvested cell solution, taken up into an Eppendorf, was then centrifuged in a microcentrifuge for 30 seconds. The supernatant was aspirated off and the cell pellet was then frozen at -70°C pending further analysis.

2.3.4 Long-term storage of cell cultures. Cell cultures for storage were trypsinized when 80% confluent in 70-ml culture bottle. The cell suspension was then pelleted by centrifugation at 3000 rpm for 10 minutes. The pellet was resuspended in 500µl of mixture between 900µl of FCS and 100µl $DMSO_4$. The cell suspension was placed in a 2-ml cryotube and frozen down immediately. Freezing was carried out in a two-step procedure using a freezing tray. This involved an initial 20 minutes at -70°C followed by 15 minutes at -120°C before transfer to liquid nitrogen for long term storage. When required, frozen cells were allowed to quickly defrost at room temperature. As soon as the solution had defrosted, the cells were suspended in 6-ml growth medium and plated into a 60-mm plate. The following day the supernatant containing unattached cells which had not survived, was removed and fresh growth medium added.

2.4 Blue native protein electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a method for isolation of enzymatically active membrane proteins from biological membranes, which was successfully applied to bovine mitochondria, for the preparation of highly pure oxidative phosphorylation (OX-PHOS) proteins for production of antibodies, N-terminal protein sequencing, and for the analysis of molecular masses and oligomeric states of native complexes. The electrophoretic techniques provide information about the quantity of correctly assembled multiprotein complexes. Methods used in this study were modified from those previously described [Schagger 1996].

2.4.1 Mitochondrial protein extraction. Sample preparation starting from homogenized skeletal muscle. Firstly make stock of buffer consisted of 440 mM sucrose, 20 mM 4-morpholine-propane sulfonic acid (MOPS), 1 mM ethelenediamine tetraacetic acid (EDTA) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). 0.5 mM PMSF freshly prepare before use from 0.5 M stock in dimethyl sulfoxide. Add 250 µl of buffer to 20 mg of muscle and homogenize muscle with a razor blade in a petri dish. Homogenized muscle tissue is then centrifuged at 20,000g for 20 minutes and discarded supernatant. Add 1M aminocaproic acid, 50 mM Bis-Tris-Hcl, pH 7.0 in the homogenized tissue and twirled with a tiny spatula. Then add 10% dodecylmaltoside and centrifuge at 100,000g for 15 minutes and collect supernatant which is ready to load in electrophoretic gel.

2.4.2 Measurement of protein concentration. Protein assays were carried out to estimate total concentration of protein using Bio-Rad protein assay. It is a dye-binding assay based on colour change of a dye in respond to different concentrations of protein. The procedure is performed as follow:

1. Prepare six dilutions of protein standard (bovine serum albumin) from 1 to 25 µg/ml.
2. Place 0.8 ml of standards and appropriately dilutes samples in test tubes. Place 0.8 ml sample buffer in blank test tube.
3. Add 0.2 ml of Dye Reagent Concentrate (filter through Whatman No.1 paper).
4. Mix several times by gentle inversion of the test tubes.
5. After a period of 5 to 60 minutes, measure OD_{595} versus reagent blank.
6. Plot OD_{595} versus concentration of standards and read unknown from standard curve (figure 2.1).

Figure 2.1 Protein standard curve. Unknown concentrations of studied samples are read form this graph.

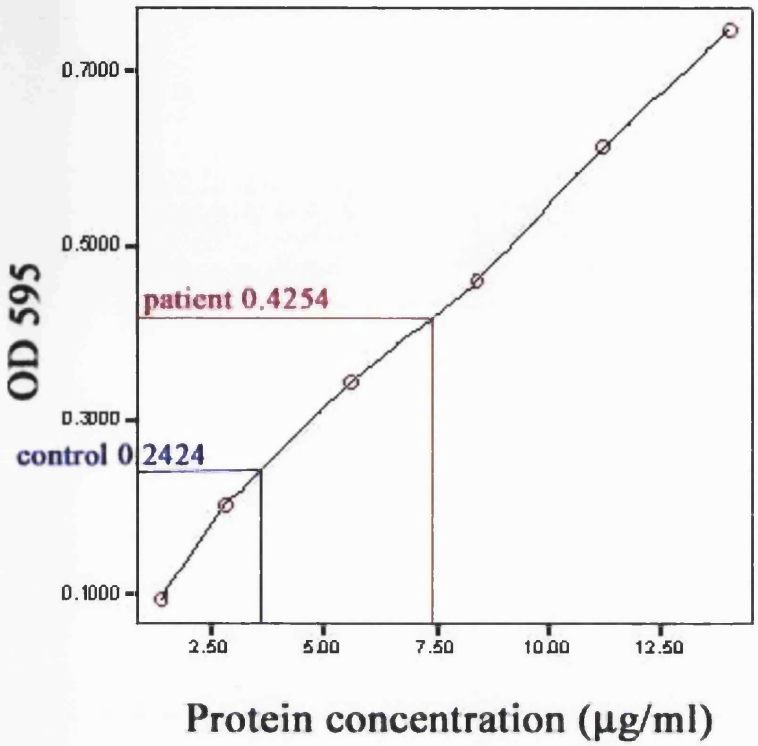
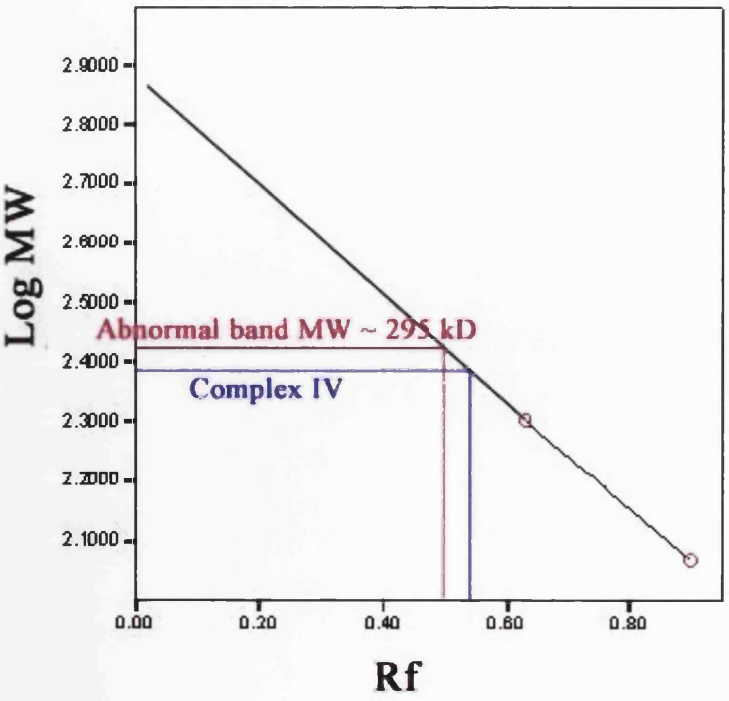


Figure 2.2 Curve generate by plotting the log of molecular weight of the board range standard (red dot: β-galactosidase 116.25 kD and myosin 200 kD) against relative mobility (calculation see section 2.4.3). Unknown MW is calculated by reading value of log MW against its Rf on curve.



2.4.3 Blue native protein electrophoresis. Protein electrophoresis is run using Bio-Rad minigel electrophoresis vertical cell and 4-15% Tris-HCl ready gel with 10-wells (30µl/well). Molecular weight standards, board range 6.5-200 kilo Daltons (kD) was run with the samples at the first experiment in order to calculate the molecular weight of the protein bands of the samples. Molecular weight of the protein bands are calculated by generating a curve of log of the molecular weight of the standards against relative mobility (Rf) which Rf is distance migrate by protein divide by distance migrated by dye (Figure 2.2). Aliquot dilute samples 1:2 with Native sample buffer (Bio-Rad) containing approximately 100 µg of protein into each well. Electrophoresis is performed at 120 V for 6 hours in ice (4°C). After electrophoresis, stain gel with Coomasie blue G-250 for 7-9 minutes and then wash the gel with Coomasie blue G-250 destaining.

2.5 Solutions and buffers

DNA extraction

Digestion buffer	PE Applied Biosystems, California, U.S.A.
Proteinase K	Sigma, MO, U.S.A.
Phenol	Bio-Rad Laboratories, California, U.S.A.
Chloroform	BDH Laboratory supplies, U.K.

Minigel electrophoresis

Agarose gel	Roche, IN, U.S.A.

10×TBE for minigel (1 litre)

121.1g Trisma base	Sigma, MO, U.S.A.
61.8g boric acid (anhydrous)	BDH Laboratory supplies, U.K.
7.4g EDTA	BDH Laboratory supplies, U.K.

Loading dye

4g sucrose	Sigma, MO, U.S.A.

(Make up to 10 ml with purified water)

0.025g Bromophenol blue	Boehringer Mannheim, Mannheim, Germany

Purified PCR kit

QIAquick PCR purification kit	Qiagen, Germany

Single fibre PCR

Alkaline lysis solution

900 µl of 222mM KOH	Boehringer Mannheim, Mannheim, Germany
100 µl of 500mM Dithithreitol (DTT)	Sigma, MO, U.S.A.

Neutralising solution

450 µl of 1M Tris base	Sigma, MO, U.S.A.
8.6 µl HCl (11.6N)	BDH Laboratory supplies, U.K.
40 µl npH_2O	

Automated sequencing and Genescan

Dye primer cycle sequencing kit, -21M13	PE Applied Biosystems, California, U.S.A.
Dye primer cycle sequencing kit, M13 rev	PE Applied Biosystems, California, U.S.A.
BigDye primer cycle sequencing kit, -21M13	PE Applied Biosystems, California, U.S.A.
BigDye primer cycle sequencing kit, M13 rev	PE Applied Biosystems, California, U.S.A.
Fluorescent dUTP	PE Applied Biosystems, California, U.S.A.
Genescan-500 Tamra	PE Applied Biosystems, California, U.S.A.

10×TBE for sequencing and Genescan

108g Trisma base	Sigma, MO, U.S.A.
55g boric acid (anhydrous)	BDH Laboratory supplies, U.K.
8.3g EDTA	BDH Laboratory supplies, U.K.

Sequencing gel mix (80 ml 6% denature polyacrylamide gel)

12ml 40% Acrylamide/Bis solution, 19:1	Bio-Rad Laboratories, California, U.S.A.
40g Urea	Fisher Scientific International Co., U.K.

0.5g Amberlite MB-150	Sigma, MO, U.S.A.
8ml 10×TBE	
30ml npH_2O	
400 µl 10%Ammonium Persulfide	Sigma, MO, U.S.A.
45 µl Tetramethylethylenediamine	BDH Laboratory supplies, U.K.

Genescan gel mix (60 ml 5% native polyacrylamide gel)

7.5ml 40% Acrylamide/Bis solution, 19:1	Bio-Rad Laboratories, California, U.S.A.
6ml 10×TBE	
46.5ml npH_2O	
250 µl 10%Ammonium Persulfide	Sigma, MO, U.S.A.
35 µl Tetramethylethylenediamine	BDH Laboratory supplies, U.K.

Protein electrophoresis

Chemicals using for blue-native protein electrophoresis

Bis-Tris-HCl	Sigma, MO, U.S.A.
MOPS	Boehringer Mannheim, Mannheim, Germany
EDTA	BDH Laboratory supplies, U.K.
PMSF	Boehringer Mannheim, Mannheim, Germany
Dodecylmaltoside	Boehringer Mannheim, Mannheim, Germany
6-aminocarproic acid	Fluka,
DTT	Sigma, MO, U.S.A.

Protein electrophoretic system

Minigel electrophoresis vertical cell	Bio-Rad Laboratories, California, U.S.A.
4-15% Tris-HCl ready gel	Bio-Rad Laboratories, California, U.S.A.
10×Tris-Glycine buffer	Bio-Rad Laboratories, California, U.S.A.
Coomasie blue G-250 staining	Bio-Rad Laboratories, California, U.S.A.
Coomasie blue G-250 destaining	Bio-Rad Laboratories, California, U.S.A.

Blue loading buffer	Bio-Rad Laboratories, California, U.S.A.

Protein assay

Biorad protein assay – BSA	Bio-Rad Laboratories, California, U.S.A.

Cell cultures

1×Dulbecco's phosphate buffer saline (PBS)	ICN Biomedicals Inc., Ohio, U.S.A.
1-β-D-ribofuranosyluracil	Sigma, MO, U.S.A.
DMEM with pyruvate, glucose, pyridoxine	GibcoBRL, Scotland
Gentamicin	GibcoBRL, Scotland
Foetal bovine serum albumin	GibcoBRL, Scotland
1×Trypsin-EDTA	GibcoBRL, Scotland

Dissociation Solutions (40 ml)

40mg Bovine serum albumin (0.1% w/v)

60mg collagenase type II (0.15% w/v)

2.4ml trypsin (0.15% w/v)

Made upto a final volume of 40ml with Dulbecco's Modified Eagle's Medium (DMEM)

Growth medium

500ml DMEM with glucose 4.5g/litre, sodium pyruvate, pyridoxine .

100ml 20% Fetal calf serum (FCS)

30 mg Uridine in 2ml DMEM filtered before mix

5mg Gentamicin.

Chapter 3 MELAS

3.1 Identification of the G13513A mutation in the mitochondrial ND5 gene

3.1.1 Patients

A group of patients with typical stroke-like episodes, ragged red fibres on muscle biopsy, and lactic academia who did not harbour any of the known defects of mitochondrial DNA.

Patient 1. This right-handed man was well until the age of 17 years, when he developed painless loss of central vision in both eyes simultaneously over the course of a few days. An examination showed visual acuity to be hand movement in left eye and 1/60 in the right eye. The optic discs appeared normal, and there was no peripapillary microangiopathy. There was no recovery, and he was registered blind. Optic disc pallor developed after a few months. A diagnosis of Leber's hereditary optic neuropathy (LHON) was made. He remained otherwise well until 2 years later, when he had an episode of left-sided throbbing headache with nausea, photophobia, and fortification spectra that was most prominent in his right visual hemifield. A computed tomography (CT) brain scan showed a low-density area in the left occipital lobe consistent with infarction. He had further stroke-like episodes accompanied by headache, some of which led to focal motor and generalized epileptic seizures. Interictal lactic acidemia was documented on several occasions. From the age of 20 years, there was progressive cognitive decline, and he died following an epileptic seizure at the age of 25 years. There was no family history of neurological disease. His mother is alive and well. A muscle biopsy at the age of 20 years showed 5% ragged red fibres (RRFs) (modified Gomori trichrome method), which were all strongly cytochrome *c* oxidase–positive. No succinate dehydrogenase–positive blood vessels were seen.

Patient 2. This left-handed man developed sensorineural deafness in childhood but was otherwise well until the age of 52 when over the course of a few days he developed headaches, nausea, vomiting, and an unsteady gait with a tendency to veer to the left. This culminated in

left focal motor seizures affecting his arm and leg. Examination showed a mild left hemiparesis. Brain CT showed a low density area in the right parietal lobe consistent with an area of infarction. He made an initial recovery then represented three weeks later with a sudden increase in weakness of his left side followed by left focal motor seizures. He became confused and drowsy and an EEG was consistent with non-convulsive epileptic status. Plasma lactate was raised at 7.49 mmol/l (normal < 1.8 mmol/l). T2 weighted MRI of his brain showed high signal bilaterally in the occipital regions and in the right tempero-parietal region (Figure 3.1). Although he improved after this second stroke-like episode, he had a fixed neurological deficit comprising fluent dysphasia, cortical blindness, and a dense left hemiparesis. Muscle biopsy showed 10% ragged red fibres with the SDH stain, which were all COX positive. He died 6 months after the initial presentation from pneumonia. There was no family history of neurological disease.

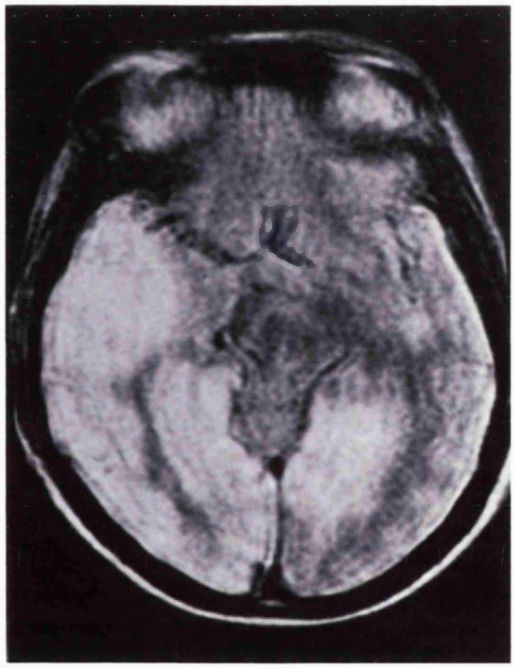


Figure3.1 T2 weighted MRI of patient 2's brain showed high signal intensity bilaterally in the occipital regions and in the right tempero-parietal region. ⇒

Patient 3. This right-handed man was well until the age of 7 years when he developed episodic headaches with nausea, vomiting, and photophobia, which were diagnosed to be common migraine. At the age of 9 years, in addition to the headaches, he was having difficulty reading small print and was found to have bilateral optic atrophy. His headaches settled and he was otherwise well until the age of 34. At this age, he became confused over the course of a day and was found to have a right homonymous hemianopia, nonfluent dysphasia, and a mild right hemiparesis. He made a complete recovery but at the age of 46 developed sudden onset bilateral visual loss with visual acuities reduced to 6/60 in both eyes, and confusion. CT scan of brain

showed areas of decreased attenuation in both occipital lobes consistent with areas of infarction. He subsequently developed generalised seizures and progressive cognitive decline. Examination at the age of 50 years showed marked cognitive impairment with disorientation in time and place. He had bilateral optic atrophy and a salt and pepper type retinopathy. He had a broad based gait with limb incoordination and he exhibited generalised dystonic posturing. There was no clinical evidence of a myopathy. He had bilateral grasp reflexes and generalised hyperreflexia. Plasma lactate was raised at 4.4 mmol/l. Muscle biopsy showed 30% ragged red fibres with the SDH stain, which were all COX positive. Polarography of freshly isolated muscle mitochondria showed a defect of complex I. There was no family history of neurological disease.

Patient 4. This right-handed man had normal motor and cognitive milestones but developed sensorineural deafness in his late teens. He developed cataracts in his 30s requiring surgery. At the age of 45, he experienced headaches, vomiting, and increasing confusion over a period of three days culminating in a generalised seizure. Examination disclosed a fluent dysphasia, dyslexia, dysgraphia, and a right homonymous hemianopia. Brain CT disclosed a low density area in the left occipito-parietal region. He recovered fully but continued to have generalised seizures. At the age of 53 years, he had a respiratory arrest after a generalised seizure and was ventilated. His conscious level remained impaired despite the absence of sedation. Examination showed roving eye movements and generalised stimulus sensitive myoclonus. His best motor response was flexion to pain. He had frequent focal motor seizures affecting his face and arms. Plasma and CSF lactate were raised at 8 and 7 mmol/l respectively. Brain MRI showed generalised atrophy and high signal areas in the paramedian regions extending from the medulla up to the medial thalamus, the appearance being consistent with Leigh's disease. Muscle biopsy showed 10% ragged red fibres with the SDH stain. Most of the ragged red fibres had increased COX staining and there were no COX negative fibres. He was successfully removed from ventilation but his conscious level remained unchanged. He died 2 months later after a further respiratory arrest. There was no family history of neurological disease.

Table 3.1 Clinical details and investigations in four patients with MELAS.

Patient	Age at 1st stroke*	Age last examined	Lactic acidosis	Additional features	Muscle Histochemistry
1	17 y (3)	22 y	Yes	Optic neuropathy Seizures Dementia	5% RRF No COX-ve
2	52 y (2)	52 y	Yes	Deafness	10% RRF No COX-ve
3	34 y (2)	50 y	Yes	Optic atrophy Retinopathy Cerebellar ataxia Dystonia Dementia	30% RRF No COX-ve
4	45 y (1)	53 y	Yes	Cataracts Deafness Seizures	20% RRF No COX-ve

LA=lactic acidaemia (in plasma); RRF=ragged red fibre; COX=cytochrome c oxidase.

*Number in parentheses indicates the total number of stroke-like episodes.

3.1.2 Results

Southern blot analysis showed no evidence of a large-scale rearrangement. Systematic sequence analysis of all of the mitochondrial tRNA genes was undertaken in all 4 patients. This work was performed by Dr. MG Hanna [Hanna *et al.* 1998a]. There were a number of identified nucleotide substitutions compared to the Cambridge sequence [Anderson *et al.* 1981], all which were previously reported as polymorphisms. Therefore, we (Dr. L Eunson and the author) subsequently analysed the entire mitochondrial complex I subunit genes in these patients. We identified a heteroplasmic G to A transition at the nucleotide position 13513 in the NADH dehydrogenase (ND) subunit 5 gene (Figure 3.2A). This was confirmed by a mismatch PCR-RFLP reaction (see methods table 2.2), in which the restriction endonuclease *Bgl* II recognised the presence of the wild-type nucleotide. The G13513A mutation was not found in 100 healthy control samples, 52 patients with other mitochondrial encephalomyopathy phenotypes, which did not include strokes, and 11 patients with LHON who lacked any of the known pathogenic mtDNA mutations. The G13513A mutation was absent from the blood sample obtained from the sister of patient 2. No samples were available from relatives of patient 1, 3 and 4. The results of quantitation of the G13513A mutation in the tissues available on patients 1, 2, 3 and 4 are

shown in table 3.2 and figure 3.2B. The proportion of the G13513A mutation in 14 RRFs and 16 non-RRFs was determined (Figure 3.2C, D). The mean proportion in RRFs (42.2%) was significantly higher than in non-RRFs (14.3%) ($p = 0.003$ by Student's t test).

Figure 3.2 **(A)** Electropherogram showed a G13513A in the ND5 gene (arrow). Upper panel = normal, lower panel = patient 3. **(B)** The proportions of mutant DNA in various tissues of Patients 1 through 4. **Patient 1** (lanes A–G): cerebral cortex (A), optic nerve (B), skeletal muscle (C), heart (D), liver (E), kidney (F), and leukocytes (G). **Patient 3** (lane H): muscle (H). **Patient 2** (lanes I and J): muscle (I) and blood (J). **Patient 4** (lanes K and L): muscle (K) and blood (L). 164 = mutant band, 149 = wild-type band. **(C)** Quantitation of the proportion of the mutant mtDNA in single-muscle fibres compare between ragged red fibres (RRF) and normal fibres by Genescan analysis. The smaller-sized peak is a wild-type mtDNA and the bigger-sized peak is the mutant mtDNA. Each graph represented superimposition of ten different fibres. **(D)** A box-plot graph illustrates the proportion of mutant mtDNA in RRF and non-RRF. Red boxes represented 50% of the samples and a horizontal line in each box indicates median.

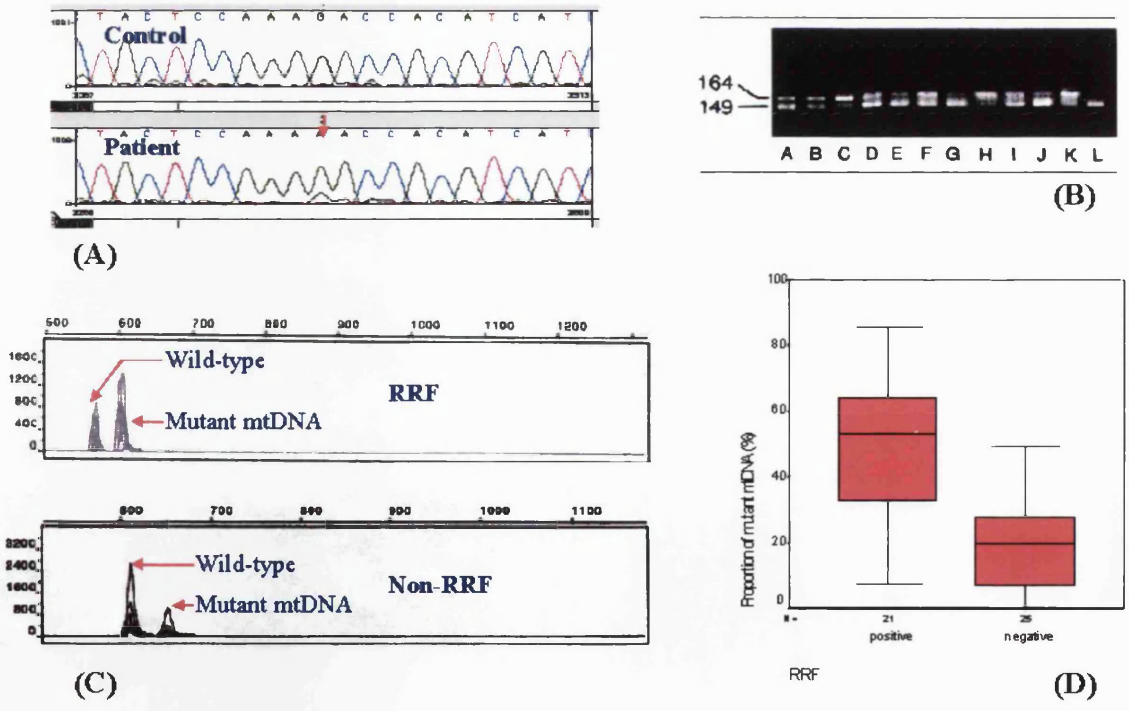


Table 3.2 The proportions of mutant mtDNA in various tissues of reported cases.

Patients	Tissue	Percent of mutant mtDNA
1	Brain	31
	Optic nerve	32
	Muscle	64
	Heart	24
	Liver	25
	Kidney	28
	blood	10
2	Muscle	29
	blood	11
3	Muscle	70
4	Muscle	33
	Blood	0

3.1.3 Discussion

Extensive sequence analysis of mtDNA in patient 1 identified only one base change, G13513A, which had features to suggest that it was of pathogenic importance. First, it was heteroplasmic, second, it resulted in an amino acid substitution at a highly conserved position in the ND5 subunit (Table 3.2) and third, it was not present in 100 healthy controls. Only patient 3 in this group had respiratory chain biochemical analysis performed and this showed an isolated complex I defect (performed by Dr. JM Cooper), as one might expect in association with an ND5 subunit mutation. Further support for the pathogenicity of this mutation is provided by its discovery in a patient with MELAS from North America [Santorelli *et al.* 1997b], and its presence in the three further patients studied here. Moreover, mtDNA sequence analysis has previously ruled out other pathogenic base changes in these three patients [Hanna *et al.* 1998a].

Table 3.3 Alignment of amino acid sequences in the ND5 subunit, showing high degree of conservation (bold letters). An amino acid substitution at codon 393 is pointed by arrow.

	↓
Patient	**L**AG**M**P**FLTGFYSKN**HI**I**E**T**ANM**S**YT**N**AWALSIT
Human	**L**AG**M**P**FLTGFYSKD**HI**I**E**T**ANM**S**YT**N**AWALSIT
Gorilla	**L**MG**M**P**FLTGFYSKD**LI**I**E**T**ANM**S**HT**N**AWALSII
Orangutan	**L**AG**M**P**FLS**GFYSKD**LI**I**E**T**ANM**S**YT**N**TWALSIT
Bovine	**L**TG**M**P**FLTGFYSKD**LI**I**EAANK**S**YT**N**AWALLMT
Horse	**L**TGI**P**FLTGFYSKD**LI**I**E**T**ANT**S**YT**N**AWALLMT
Chicken	**L**MGT**P**FLAGFYSKD**LI**I**E**NLNT**S**YI**N**TWALSLT
Platypus	**L**TG**M**P**FLAGFYSKD**LI**I**E**SLNT**S**NT**N**AWALSLT
Opossum	**L**MGT**P**FLAGFYSKD**SI**I**E**AMNT**S**YT**N**SWALTIT
Wallaroo	**L**MG**M**P**FLTGFYSKD**SI**I**E**AMNT**S**HT**N**TWALIIT
Fruit fly	**L**CG**M**P**FLAGFYSKD**MI**L**E**IVSI**S**NI**N**MFSFFLY

These observations indicate that the G13513A mutation may be a more frequent cause of the MELAS phenotype than previously recognised. To our knowledge, only the A3243G mutation has been reported in more than one MELAS case in the UK. Other MELAS-associated mtDNA mutations have been identified in single cases only [Hanna *et al.* 1998a;Manfredi *et al.* 1995;Manfredi *et al.* 1996;Morten *et al.* 1993;Nishino 1996;Sato *et al.* 1994;Taylor *et al.* 1996]. Therefore we suggest that other laboratories should screen for this mutation in the genetic workup of patients with MELAS [Pulkes *et al.* 1999]. Subsequently other studies in Europe have supported this hypothesis, in which further MELAS patients have harboured the G13513A mutation [Corona *et al.* 2001;Penisson-Besnier *et al.* 2000].

All five cases now reported [four in this study [Pulkes *et al.* 1999] and one from Santorelli et al [Santorelli *et al.* 1997b] had stroke-like episodes characteristic of MELAS, but the phenotype of patient 1, a LHON/MELAS overlap syndrome, appears to be unique. The commonest LHON-associated mtDNA mutations all occur in subunits of complex I, but are generally present in much higher proportions in both blood and optic nerve tissue than we observed in patient 1. Therefore the G13513A mutation was screened in a panel of eleven LHON patients without any known mtDNA mutations. None of the LHON patients had the G13513A mutation in their leukocyte mtDNA as assessed using PCR-RFLP reactions and

visualised under UV light. This method should detect the proportion of the mutant mtDNA down to at least 10%. However, as shown in this study this mutation is very low in proportion or may even be absent in blood (patient 4). It remains a possibility that some LHON patients, negative for known LHON mutations, may habor higher amount of the G13513A mutant mtDNA in optic nerve. As a result of this work our DNA service lab currently screens the G13513A mutation in both MELAS and LHON phenotypes in our routine service protocol.

Whilst the available data indicate that the G13513A change is a pathogenic mutation, the relatively low proportions of this mutation in the affected tissues of patient 1 is notable. For example, cerebral cortex and optic nerve contained only 31% and 32% mutant mtDNA respectively. Interestingly, Santorelli and co-workers also reported relatively low proportions of this mutation in autopsy tissues. According to the RFLP data they presented, the highest proportion in their single case was 57% in the cerebellum [Santorelli *et al.* 1997b]. Although it is possible that the distribution of this mutant may differ between different cell types within brain and optic nerve tissue, and the low proportions of mutant we observed may reflect a sampling phenomenon, such low proportions have not been observed for other pathogenic mtDNA mutations in post-mortem tissue analyses reported previously [Macmillan *et al.* 1993]. It suggests that the G13513A mutation may have a lower threshold for expression than many other mtDNA mutations. Some other mitochondrial protein coding subunit mutations have recently been shown to have lower thresholds for expression compared to common mtDNA tRNA gene point mutations [Hanna *et al.* 1998b].

The function and precise stoichemitry of the ND5 subunit in human complex I is unknown. In bovine complex I, the ND5 subunit is located in a hydrophobic protein fraction and may be involved in proton translocation [Smeitink *et al.* 1998]. The experimental model, *Rhodobacter capsulatus*, showed that disruption of the ND5 gene lead to the suppression of the NADH dehydrogenase activity at the level of the bacterial membrane and the disappearance of the complex I-associated iron-sulfer clusters [Dupuis *et al.* 1998]. It is possible that low levels of mutant mtDNA are sufficient to disturb this critical function of complex I.

In conclusion, these data indicate that the G13513A point mutation is pathogenic and it

may be the second commonest cause of the MELAS phenotype, at least in the UK population. In addition, this is the first report of a LHON/MELAS overlap phenotype associated with a complex I subunit gene mutation.

3.2 The A12308G polymorphism in the $tRNA^{Leu(CUN)}$ gene influences phenotypic expression of the A3243G (MELAS) mutation

The commonest mtDNA point mutation associated with mitochondrial encephalomyopathies is in the transfer RNA Leucine (UUR) ($tRNA^{Leu(UUR)}$) gene (A3243G) and is identified in over 80% of MELAS patients [Hammans *et al.* 1995]. However, half of the patients who harbour the A3243G mutation exhibit non-MELAS phenotypes including myopathy, chronic progressive external ophthalmoplegia (CPEO), diabetes mellitus and deafness. The mechanisms underling this diversity remain unclear and it is not entirely explained by mutation load. In 1998 El Meziane *et al* identified a G12300A mutation in the mitochondrial $tRNA^{Leu(CUN)}$ gene in lung carcinoma cybrid cells containing the A3243G mutation [El Meziane *et al.* 1998a]. The G12300A mutation was shown to ameliorate the impaired respiratory chain function induced by the A3243G mutation raising the possibility that the $tRNA^{Leu(CUN)}$ polymorphisms may be relevant to the phenotypic diversity observed in humans [El Meziane 1998a;El Meziane *et al.* 1998b].

3.2.1 Patients and methods

In order to investigate the role of the G12300A or other mutations in the $tRNA^{Leu(CUN)}$ gene in influencing the phenotypic expression of the A3243G mutation, forty-eight unrelated A3243G cases were studied. These patients previously had the A3243G mutation identified in their skeletal muscle or leukocyte mtDNA. Their medical records were reviewed in details. The G12300A mutation was analysed by fluorescent-incorporated PCR-RFLP reactions (see details in methods). Automated sequencing of the $tRNA^{Leu(CUN)}$ gene was also performed in all patients. The association between A12308G polymorphism and clinical phenotypes were statistically analysed by a Fisher's exact test (SPSS version 8.0 software).

Table 3.4 Clinical features of 48-unrelated patients with A3243G mutation.

Patients	Clinical features	Patients	Clinical features
M1	MELAS, deaf, enteropathy	M25	Myopathy
M2**	DM, deaf, encephalopathy	M26	DM, deaf, cardiomyopathy
M3	DM, deaf, myopathy, neuropathy,	M27	Myopathy
	encephalopathy, retinopathy	M28	Myopathy
M4	MELAS, deaf	M29**	MELAS
M5**	MELAS, deaf, PEO	M30	MELAS, deaf, cardiomyopathy
M6	Myopathy	M31	MELAS, PEO, DM
M7	MELAS	M32	Encephalopathy
M8	MELAS	M33**	MELAS, DM, deaf
M9	MELAS, deaf	M34	MELAS
M10**	MELAS	M35	Encephalopathy, deaf
M11**	MELAS, MERRF	M36	MELAS, deaf
M12	DM	M37	PEO, deaf
M13**	Myopathy, PEO	M38	MELAS
M14**	MELAS	M39	Encephalopathy
M15	DM, deaf	M40	DM, retinopathy
M16	PEO	M41	DM, retinopathy
M17**	MELAS	M42	Myopathy
M18	Myopathy, deaf	M43	DM, deaf, retinopathy
M19	MELAS, enteropathy	M44	Encephalopathy, deaf, neuropathy,
M20	DM, deaf		PEO
M21	MELAS	M45	DM, deaf
M22	MELAS	M46	Myopathy
M23	Myopathy	M47	Myopathy
M24	MELAS, DM, deaf	M48	Myopathy

** Patients harboured a A12308G polymorphism at the $tRNA^{Leu(CUN)}$ gene.

Abbreviations as follow: MELAS = mitochondrial encephalopathy, lactic acidosis and stroke-like episodes, MERRF = myoclonic epilepsy and ragged red fibres, DM = diabetes mellitus, PEO = progressive external ophthalmoplegia, deaf = sensorineural hearing loss.

3.2.2 Results

Phenotypes of all 48 patients are listed in table 3.4 and 3.5. The most common clinical feature associated with the A3243G mutation was stroke-like episodes, which was identified in 44% of the patients. Other clinical manifestations included sensorineural hearing loss, diabetes mellitus, proximal myopathy, encephalopathy, ophthalmoplegia, pigmentary retinopathy, neuropathy,

myoclonic epilepsy, cardiomyopathy and gastrointestinal dysmotility. Automated sequencing of the mitochondrial $tRNA^{Leu(CUN)}$ gene failed to identify the G12300A mutation in this group of patients. However el Meziane *et al.* reported that as little as 11% of the G12300A could correct the biochemical abnormalities in their cell system, and our sequencing method (ABI dye primers) in this lab is only able to reliably detect the proportion of heteroplasmy between the ranges of 30-70% (test by Dr. MG Hanna). Therefore fluorescent-based PCR and RFLP analysis was performed to allow detection of very low proportions of this mutation down to at least 5%. Even using this assay none of the 48 cases had detectable levels of G12300A.

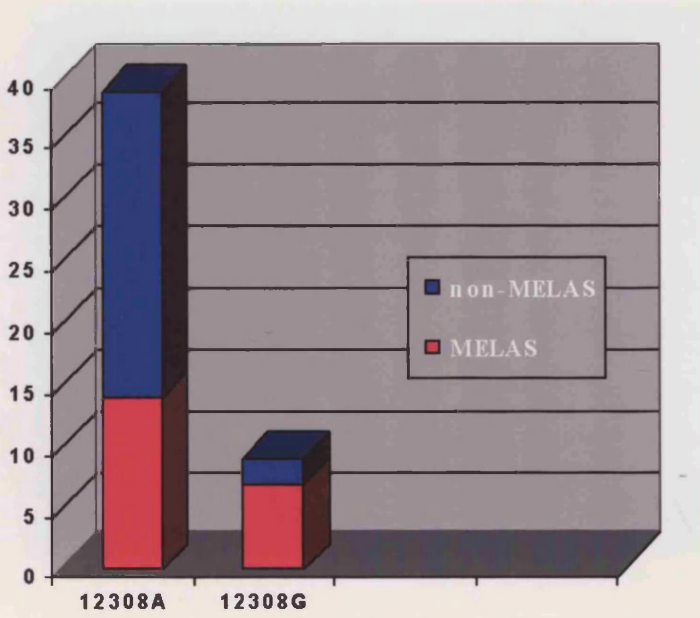
However, one homoplasmic change, A12308G, in the $tRNA^{Leu(CUN)}$ gene compared to the Cambridge mitochondrial sequence were identified in 9 patients (18.8%) (Table 3.4 and figure 3.3). The relationships between clinical features and the A12308G change were analysed. The statistic analysis was by 2×2 table and Chi-square test . Since the sample size was small the analysis selected was Fisher's exact test. Patients who harboured the A12308G transition had a relative risk of 2.167 (95% confidence interval 1.255-3.739; p = 0.031) of developing strokes. There were no statistically significant differences in the expression of the other clinical phenotypes between patients who harboured A12308G and patients who did not. Cardiomyopathy was not analysed due to a lack of echocardiogram or electrocardiographic data in several patients.

Table 3.5 Frequency of the clinical features in 48 patients with A3243G. The cases that also had A12308G are indicated.

Clinical features	Cases (%)	12308-cases
Strokes	21 (44)	7
Deafness	19 (40)	3
Diabetes Mellitus	13 (27)	2
Myopathy	12 (25)	1
Encephalopathy	6 (13)	1
Ophthalmoplegia	5 (10)	2
Retinitis pigmentosa	4 (8)	0
Neuropathy	2 (4)	0
MERRF	1 (2)	1

Abbreviations as follow: MERRF = myoclonic epilepsy and ragged red fibres.

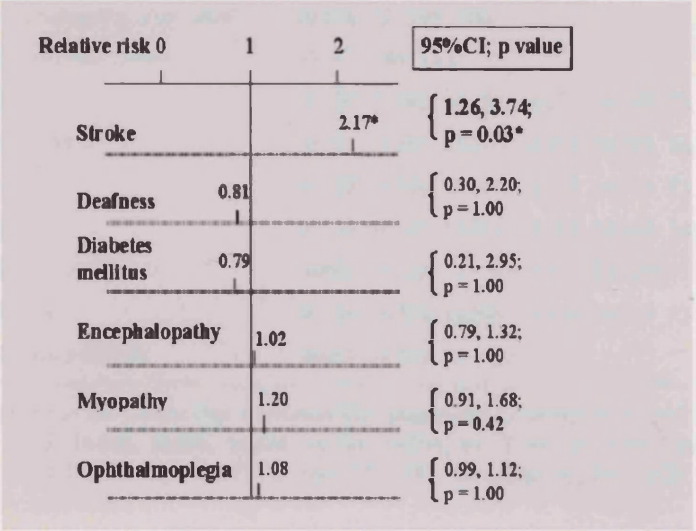
Figure 3.3 Bar chart illustrating 3243-patients are divided into 12308A and 12308G groups. The 12308G group have a greater proportion of strokes.



3.2.3 Discussion

The proportion of heteroplasmic A3243G and its tissue distribution may be important determinants of phenotype [Zeviani and Klopstock 2001]. These parameters alone are not enough to explain the clinical diversity observed. For example some families with A3243G mutation had homogeneous phenotypes regardless how different of the proportion of the mtDNA such as diabetes and deafness family or isolated myopathy family [Hammans *et al.* 1995]. On the other hand, a very low proportion of mutant mtDNA even less than 6% can also exhibit organ dysfunction [Chinnery *et al.* 2000c]. Therefore there could be other genetic factors responsible for the differences observed between families. Chinnery *et al.* found no correlation between the A3243G mutation load and the maximum rate of ATP production as measured by phosphorus magnetic resonance spectroscopy in muscle. They suggested that factors besides mutation load, such as nuclear genes, influence the expression of the A3243G mutation in vivo [Chinnery *et al.* 2001a]. This study showed that the mtDNA background, specifically A12308G, might influence the phenotypic expression of the pathogenic A3243G mutation [Pulkes *et al.* 2000a]. All data suggest that multiple factors might be involved in clinical diversity of the A3243 mutation.

Figure 3.4 illustrates the relative risks of developing different clinical features associated with A3243G mutation in patients harboring A12308G polymorphism.



There is a good evidence that the A3243G mutation in patients with the MELAS phenotype has occurred independently, and is not associated with specific mtDNA haplotypes [Morten *et al.* 1995]. Despite this hypervariable regions I and II in the D-loop were sequenced in order to rule out the possibility that this finding was simply a haplogroup effect. Nine A3243G patients who also harboured A12308G and a list of their polymorphisms in the d-loop are shown in table 3.6. In the seven MELAS patients harbouring the A12308G change six different haplogroups was observed. Only two of these patients shared a similar haplogroup (patient M14 and M17 in table 3.6). These data indicate the observation was not a haplogroup effect and suggest that A12308G may directly influence the risk of stroke in the A3243G patients.

The A12308G change is at a highly conserved nucleotide and was originally suggested to cause CPEO but it has subsequently been reported in 16% of the normal population indicating it is likely to be a neutral polymorphism [Van den Ouweland *et al.* 1992b]. Our data suggest that the A12308G may have a role in influencing the expression of the pathogenic A3243G mutation. The role of common polymorphisms in influencing clinical phenotypes is well documented in other diseases e.g. codon 129 in Creutzfeldt-Jakob disease [Windl *et al.* 1996]. However, the precise mechanism requires further study. One possible mechanism may be

Talle 3.6 MtDNA sequence haplogroups in patients with A3243G and A12308G.

Patient	Phenotype	MtDNA haplogroup
M2	Encephalopathy, Dm, deaf	16298 72 195 200
M5	MELAS, deaf, PEO	57 61 146 183
M 0	MELAS	16129 16192 16256 16270 16399 73
M 3	Myopathy, PEO	16192 16256 16270 16362 16399 16428
M 4	MELAS	16129 16244 16298 16318 16519 73 195
M 7	MELAS	16129 16140 16224 16298 16362 16519 73 152
M13	MELAS, Dm, deaf	16069 16126 73 185 195 228 295
M19	MELAS	16126 16294 16296 16304 16519 73
M 1	MELAS, MERRF	16093 16224 73 152

Hapotypes are given as the Cambridge mitochondrial sequence [Anderson *et al.* 1981]. The base changes were C→T at 16069, 16192, 16256, 16270, 16294, 16296, 61; T→C at 16093, 16126, 16140, 16224, 16298, 16304, 16311,16362, 16519, 57, 72, 146, 152, 195; G→A at 16129, 16428, 185, 228; A→G at 16338, 16399, 73, 183, 200.

Abbreviations as follow: MELAS = mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes, deafness = sensorineural hearing loss, Dm = diabetes mellitus, MERRF = myoclonic epilepsy and ragged red fibres, PEO = progressive external ophthalmoplegia.

that the A12308G may cause a mild defect of the transcription or the translation process of leucine (coded by CUN) but such defect is not sufficient to result in a defect of respiratory chain in the absence of a pathogenic mutation such as the A3243G mutation. Therefore the A12308G polymorphism alone is not pathogenic. However with the coexistance of the A3243G mutation, the defects of the transcription or the translation process caused by A12308G polymorphism may worsen the biochemical defect caused by the A3243G mutation. A defect of translation of leucine codon by both changes (UUR and CUN) may result in a lower threshold effect to the level of the A3243G mutant mtDNA or expression of more severe phenotype. However, this does not explain why the effect should be tissue specific involving brain more than other tissues or why other tissue do not appear to have more severe diseases in the presence of the A12308G.

In conclusions, the proportion of heteroplasmic A3243G and its tissue distribution may be important determinants of phenotype but these parameters alone are not enough to explain the diversity observed. This study indicates that, in some patients at least, the coinheritance of the A12308G polymorphism is an additional determinant of phenotypic expression. In particular, A12308G, seems to be a predictive factor for strokes in A3243G patients. Although

this is the largest collection of unrelated A3243G patients yet reported, confirmation by others in a larger patient group is necessary. If confirmed these observations may have important genetic counseling implications for patients with the commonest pathogenic tRNA gene mutation in human mtDNA.

Chapter 4 Mitochondrial myopathy

4.1 Identification of three novel mutations in association with mitochondrial myopathy characterised by severe exercise intolerance

4.1.1 Patients

Patient 5. This previously reported Greek male developed exercise intolerance due to severe fatigue and myalgia from the age of 7 years [Morgan-Hughes *et al.* 1977]. He was unable to run after the age of 10 years. There was a slow progression of these symptoms over the years. Investigations revealed intermittent lactic acidosis and ragged-red fibres with cytochrome *c* oxidase (COX)-positive fibres in his muscle histopathology. EMG was consistent with a myopathic process. He was lost to follow up at the age of 38 years. No family history of neurological disease. Examination at the age of 38 revealed mild bilateral facial weakness without ptosis or ophthalmoplegia. There was mild proximal weakness (MRC grade 4/5) with striking fatigue after sustained contraction or repetitive contraction.

Patient 6. This Chilean woman with exercise intolerance and complex III deficiency was originally reported in 1984 [Hayes *et al.* 1984]. She developed normally until the age of 5 years when she began to experience exercise intolerance. She tired quickly on minimal exercise and complained of weakness and stiffness in her limbs. Her exercise intolerance fluctuated in severity although became progressively worse. There was no history of muscle pain or pigmenturia. There was no family history of neurological disease. On examination, she had mild bilateral ptosis and mild weakness of her eye closure. Ocular movements were normal. She had fatigable weakness of sternomastoid muscles. There was mild diffuse limb weakness (MRC 5-/5) which became more marked after sustained muscle contraction. There was an abnormal increase in blood lactate during and after exercise (4.5 mmol/l; normal value < 1.8 mmol/l). All muscle sections contained ragged red fibres (Gomori Trichrome stain). The proportions varied in different sections ranging between 24 to 73 percent. All ragged red fibres were cytochrome *c*

oxidase (COX) negative.

Polarographic studies of freshly isolated skeletal muscle mitochondria were performed as previous reported. This showed markedly decreased oxygen consumption with the natural substrates (pyruvate + malate, glutamate + malate and succinate). Oxygen consumption with the artificial substrates (ascorbate + tetramethyl-p-phenylenediamine dihydrochloride (TMPD)), which bypasses electron complex III by shuttling reducing equivalents directly to cytochrome *c*, was increased threefold. The cytochrome content measurements showed a marked decrease in the level of cytochrome *b* (31% of control value). The conclusion from the polarographic data was a decrease in complex III activity. The histochemistry also suggested a reduction in complex IV activity [Hayes *et al.* 1984].

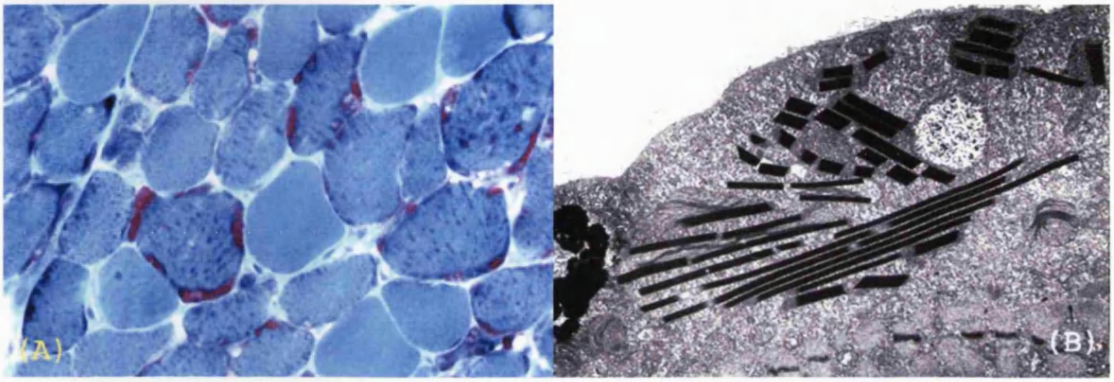
Patient 7. A 49-year-old woman has been suffered from exercise intolerance since the first decade of life. She could only walk about 100 yards before experiencing aches and pains in her legs and breathless. She had 2 seizures at the age of 33 years, which took the form of generalized tonic clonic seizures without warning. She has since been seizure free. There was a history of mild degree of kyphoscoliosis and asthma. On examination, she had bilateral mild ptosis and a mild reduction gaze in all directions. There was mild weakness (MRC grade 4+/5) of her neck flexion. She had some wasting around the shoulder muscle-girdle musculature and the quadriceps muscle. She had symmetrical proximal limbs weakness (MRC grade 4/5).

Her mother became deaf in her 30's but there was no other neurological disease on either side of her family. She had 2 healthy children, son aged 28 and daughter aged 23.

Her serum lactate level was elevated at 4.7 mmol/L (normal < 1.65 mmol/L) with increased lactate/pyruvate ratio of 50 (normal 10-20). Her serum creatine kinase was mildly elevated at 318 IU/L (normal <160 IU/L). EMG was consistent with myopathic process. Muscle biopsy was undertaken on the right triceps. The histochemical studies showed ragged red fibres in 86% of the muscle fibres and these were all COX positive fibres (figure 4.1). Biochemical studies were performed on freshly muscle tissue by I. Hargreaves and Dr. S Heales [Bolanos *et al.* 1994]. Mitochondrial complex I activity ratio = 0.010 [normal = 0.014 – 0.268 (ratio to

citrate synthase activity)]. Spectrophotometry of muscle complex II/III and IV activities were normal.

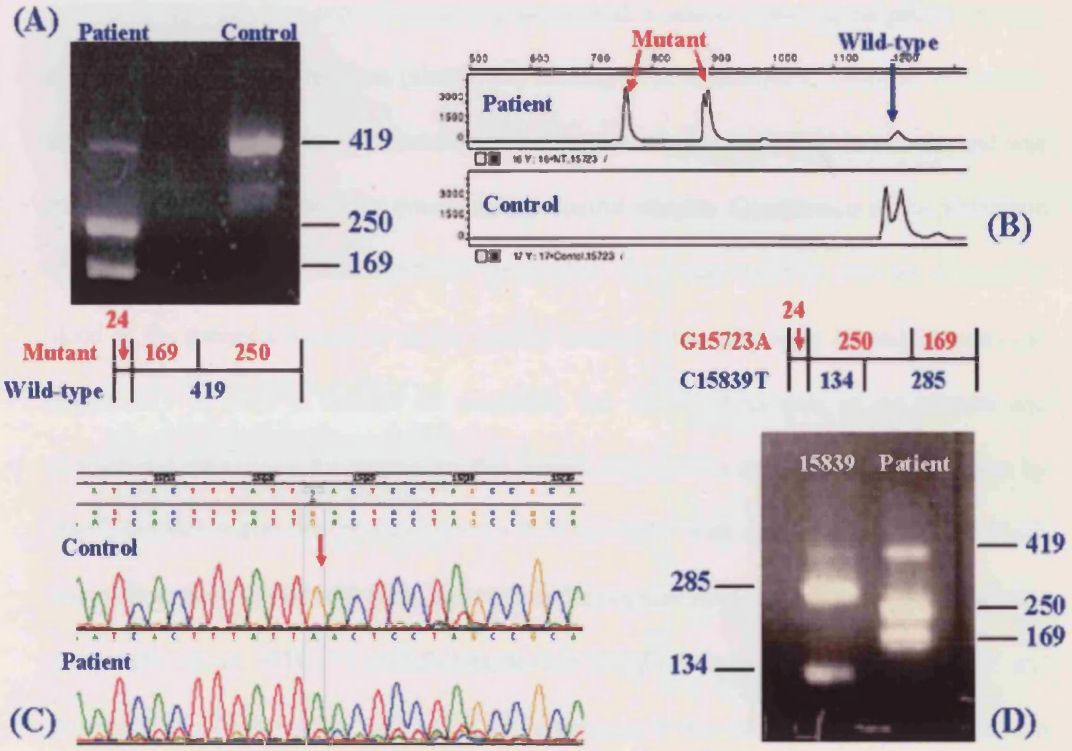
Figure 4.1 (A) Skeletal muscle histochenistry of patient 7 shows an abundance of ragged red fibres. (B) Electron microscope reveals abnormal elongated mitochondria contained with paracrystalline inclusion bodies. (Histochemistry and electron microscopic study were performed by Prof. DN Landon)



4.1.2 Results

Patient 5. Automated sequencing of the entire mt tRNA and cytochrome *b* gene revealed a G to A transition at the mtDNA nucleotide position 15723 in the cytochrome *b* gene resulting in a change of an amino acid tryptophan to stop codon (figure 4.2C). This nonsense mutation predicts to loss of 55 amino acids at the C terminus of the cytochrome *b* protein. The mutation was absent in a panel of 100 normal control samples and 40 patients with mitochondrial encephalomyopathies without any known mutations. Quantitation of the proportion of the mutant mtDNA showed 86.87% in skeletal muscle. There were no other tissue samples or collected blood to study since the patient has been lost to follow up for over twenty years. There was also no collected frozen muscle to analyse by single fibre PCR. PCR product of the primers: L15553 and H15996 (the primers for testing the G15723A mutation) had another restriction site for endonuclease *Mse* I at a nucleotide position 15839 (C to T) in one control sample. This was confirmed by direct sequencing in this region. The PCR product after digestion had 3 fragments which may resemble the fragments of G15723A mutation (figure 4.2D). This may be confusion between the G15723A mutation and the C15839T polymorphism.

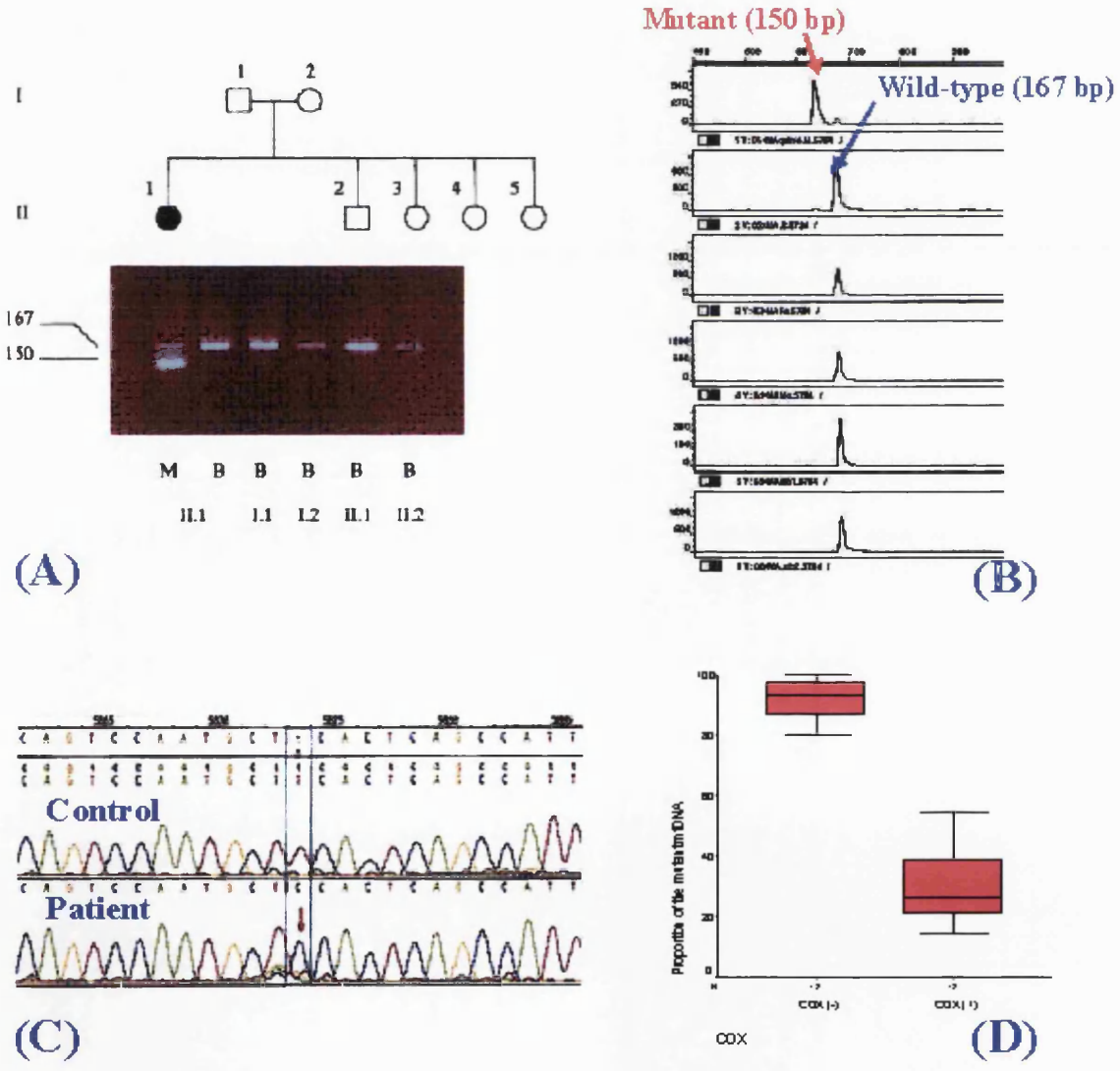
Figure 4.2 Analysis of the G15723A mutation. (A) Agarose gel containing products following digestion of the original 433 bp product with *Mse* I. In the presence of the G15723A mutation the 443 bp product is cleaved into three fragments 24, 169 and 250 bp (the 24-bp fragment is not shown). (B) Quantitation of the proportion of the mutant (G15723A) mtDNA using Genescan, the data shows that the G15723A was heteroplasmic (86.87%) in skeletal muscle. (C) Electropherogram shows the G to A transition (arrow) at position 15723 of the cytochrome *b* gene. (D) Comparison of results of restriction digest PCR products containing G15723A mutation and C15839T polymorphism by *Mse* I, both PCR products were digested into 3 fragments but had different-sized band.



Patient 6. Large-scale mtDNA rearrangements and common mtDNA point mutations were excluded. DNA sequence analysis of all of the mt tRNA and cytochrome *b* gene revealed seven changes compared with the published mtDNA sequence [Anderson *et al* 1981]. These changes were T414G, C447T and T16126C in the mtDNA control region (D-loop), A1832G in the 16S rRNA, T12173A in the $tRNA^{His}$ gene, A15326G in the cytochrome *b* gene, and A5874G in the $tRNA^{Tyr}$ gene. The changes at nucleotide position 447, 1832, 12173, 15326 and 16126 have been reported as polymorphisms (Mitomap website). The change at position 414 has not been previously reported. However, it was homoplasmic in all available tissues in the patient (muscle and blood) and maternal relatives (blood), it is unlikely to be a pathogenic mutation. In contrast, the A5874G change in the mitochondrial $tRNA^{Tyr}$ gene was heteroplasmic in muscle, and was not detected in blood. It was not present in 110 control samples. Quantitation of the proportion of the mutant mtDNA revealed 89% in the muscle. The mutant mtDNA was not detected in blood of the patient's parents or in two healthy siblings when they were 23 and 27 years old (figure 4.3). In order to exclude the possibility that the blood samples of the patients and relatives did not contain the mutant mtDNA missed detection by partially digested reaction by *Mbo* I, the author also designed a mismatch PCR and RFLP with endonuclease *Hind* III which would allow detection of wild-type nucleotide at the position 5874. The oligonucleotide primers were, light strand: GTCTTTAGATTTACAGTCCAA**A**G (mismatch nucleotide is bold and underlined) and heavy strand: 6001-6020. In the presence of the wild-type nucleotide at position 5874, the 172 bp fragment is cleaved into two fragments of 151 bp and 21 bp. This confirmed that only wild-type mtDNA are present in blood in all family members [Pulkes *et al.* 2000b].

Single-fibre PCR studies were performed to investigate the relationship between the mutation and COX deficiency fibres at the level of single muscle fibre (figure 4.3D). The mean proportion of the mutant mtDNA in the COX-negative fibres was 92.3 ± 6.7% (n = 12), and that in the COX-positive fibres was 29.9 ± 11.8% (n =12). This was statistically significant ($P < 0.000001$, by *t*-test).

Figure 4.3 Analysis of the A5874G mutation. (A) *Top*, Pedigree of family (blackened symbol denote affected individual). *Bottom*, gel containing products following digestion of the original 167 bp product with *Mbo* I (see methods). In the presence of the 5874 mutation the 167 bp product is cleaved into two fragments 150 and 17 bp (17 bp was not shown). (M = muscle, B = blood). (B) Quantitation of the proportion of the mutant mtDNA in the studied family revealed that the mutant mtDNA was present only in muscle of the patient but not in her blood or blood of her maternal relatives. *Top panel* is patient's muscle, *the lower panels* are blood samples of the patient, father, mother brother and sister of the patient, respectively. (C) Electropherogram from the automatic sequencer shows the T to C transition (heavy strand) at position 5874 of the $tRNA^{Tyr}$ gene. (D) Box plot graph of single-fibre PCR results showed a significantly higher proportion of mutant DNA in COX (-) fibres than normal COX (+) fibres.

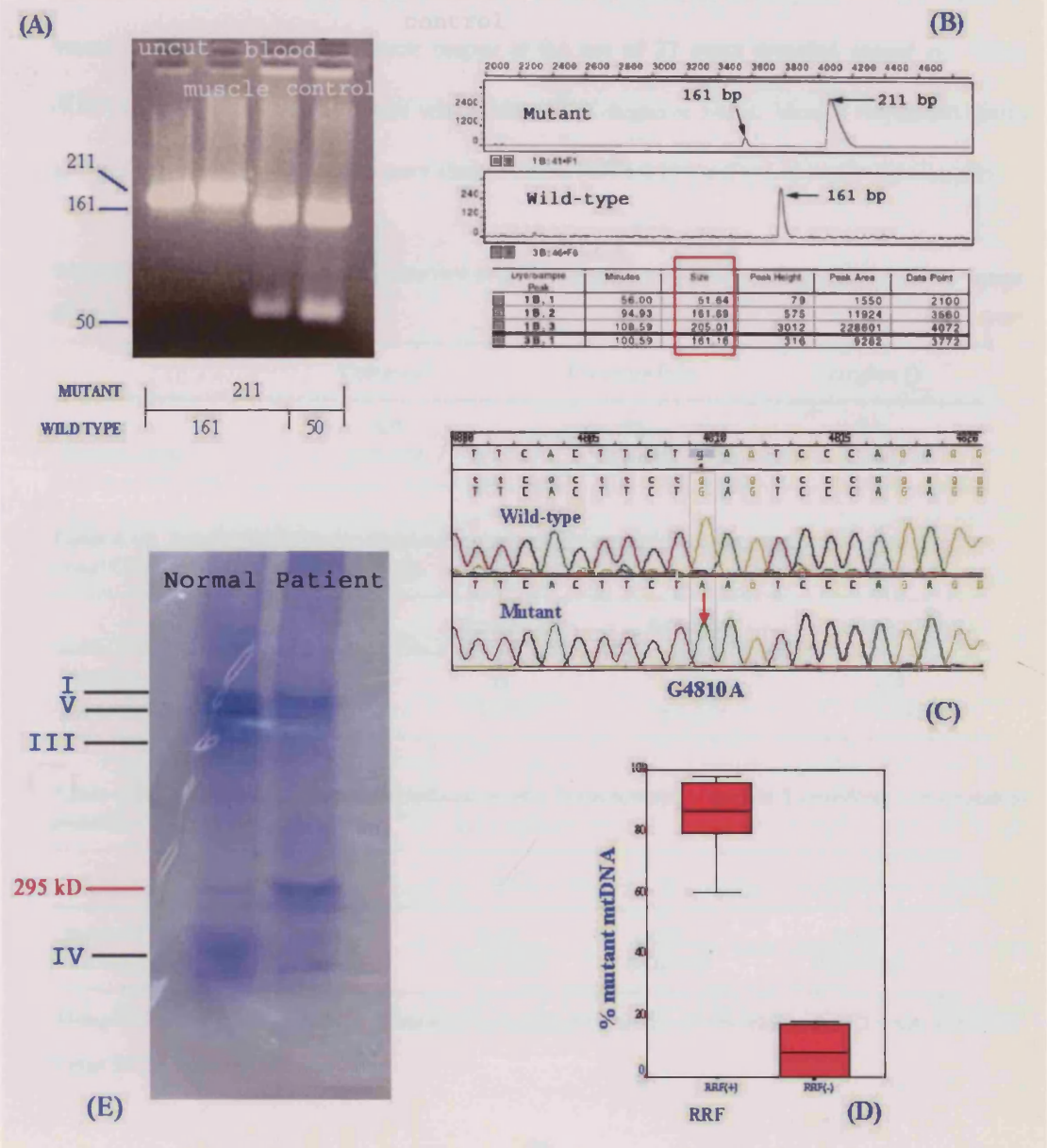


Patient 7. DNA sequence analysis of all the mt tRNA and complex I subunit genes revealed twenty-one changes compared with the Cambridge mtDNA sequence [Anderson *et al* 1981]. There were four previously unpublished changes: the first change was a G4810A nonsense mutation in the ND2 gene; two other changes were missense mutation, A12634G and A13630G in the ND5 gene; and the last change was silent mutation, A12634G in the ND5 gene. Both missense mutations in the ND5 genes were homoplasmic in muscle and blood of the patient and they were in non-conserved regions of the ND5 polypeptide. However, the G4810A transition in the ND2 gene results in a change of tryptophan to the termination codon at the codon 114 out of total 347 codons. It was absent in 105 control samples. The G4810A mutation was heteroplasmic in muscle (94% mutant mtDNA) and absent from blood (figure 4.4A). These data were confirmed by fluorescent-labelled PCR, which is more sensitive in the detection of very low level of the proportion of the mutant mtDNA (figure 4.4). In order to exclude the possibility of a partial digestion another RFLP by the endonuclease *Hinf* I was designed to detect the presence of the wild-type base at the position 4810. In leukocytes mtDNA the *Hinf* I was completely digested, the 211-bp PCR product generating 161 and 50 bp fragments confirmed by fluorescently labelled PCR. These data suggest that the patients did not harbour the mutant mtDNA in her blood.

Single-fibre PCR studies were performed to investigate the relationship between the mutant G4810A mtDNA load and ragged red fibres at the level of single muscle fibre (figure 4.4D). The mean proportion of the mutant mtDNA in the ragged red fibres was 84.27% [standard errors of mean (SEM) = 3.66, n = 15], and that in the non- ragged red fibres was 7.15% (SEM = 1.91, n =14) (figure 1b). This was statistically significant ($P < 0.0001$).

Native protein gel electrophoresis of the mitochondrial protein extracted from skeletal muscle of the patient 7 showed that all the protein complex bands were less strong compared to normal control (figure 4.4E). In addition an abnormal dense approximately 295-kilodatltons (kD) band was observed in the patient's lane. Similar-sized band was seen in the control lane but it was much weaker in intensity.

Figure 4.4 Analysis of the G4810A mutation. (A) Agarose gel containing products following digestion of the original 211 bp product with *Hinf I*. In the presence of the wild-type nucleotide at the position 4810 the 211 bp product is cleaved into two fragments 161 and 50 bp. (B) Quantitation of the proportion of the mutant mtDNA in patient's muscle and blood by Genescan analysis shows that leukocyte mtDNA contains only wild-type, 161 bp mtDNA compare to a heteroplasmic stage in muscle ragged red fibres. Due to the electrophoresis of the two samples started running at different time therefore the peak of the bands in the muscle and blood are in the different data points. (Sizes of the bands are shown in the table.) (C) Electropherogram from the automatic sequencer shows the G to A transition (arrow) at position 4810 of the ND2 gene. (D) Comparison of the proportion of the mutant (G4810A) mtDNA in single muscle fibres classified in two groups: RRF = ragged red fibres and non-RRF = normal fibres. (E) Blue native protein electrophoresis shows decrease in amount of all respiratory chain complexes and dense abnormal 295-kD unknown band (see graph in figure 2.2, chapter 2).



4.2 Identification of two novel mutations in association with chronic progressive external ophthalmoplegia

4.2.1 Patients

Patient 8. A 45-year-old woman has complained of bilateral ptosis without diplopia since 17 years old. She had normal motor milestones however her photographs from age 14 years showed mild bilateral ptosis. From approximately 35 years of age, she developed progressive exercise intolerance leading to a limitation of walking to only short distances. She did not experience muscle pain or pigmenturia. There was no family history of neurological diseases. On examination at the age of 44 years, she had bilateral ptosis and complete ophthalmoplegia. She also had mild proximal limb weakness (MRC grade 4/5). There was normal serum creatine kinase and lactate (resting). Muscle biopsy at the age of 27 years revealed ragged red fibres (RRF) in 20% of the muscle fibres which were COX-negative fibres. Muscle respiratory chain activities showed multiple respiratory chain defects (table 4.1) (performed by Dr JM Cooper).

Table 4.1A Muscle respiratory chain enzyme activities of patient 8 (nmol/min/mg mitochondrial protein). (Cooper JM)

	Complex I	Complex II/III	Complex IV
Patient 1	ND	**72**	**7.5**
Normal range	117-224	113-430	28-65

Table 4.1B Polarographic study of isolated muscle mitochondrial O_2 consumption of patient 8 (nmol/O/min/mg mitochondrial protein).

	Pyruvate	Glutamate	Succinate	Ascorbate+TMPD
Patient 1	**60**	**33**	100	**110**
Normal range	77-129	72-148	97-230	167-485

Table 4.1C Cytochrome contents of isolated muscle mitochondria of patient 8 (nmol/mg mitochondrial protein).

	B	c_1	*c*	aa_3
Patient 1	**0.23**	**0.18**	0.56	**0.10**
Normal range	0.29-0.51	0.48-0.62	0.56-0.83	0.33-0.59

Abbreviations as follow: TMPD = tetramethyl-p-phenylenediamine dihydrochloride, ND = not determine.
Value below normal value in bold

Patient 9. A 48-year-old man developed chronic progressive external ophthalmoplegia which onset of 42 years. He had normal motor milestones. He was able to play sport normally at school. At the age of 42 years, he developed progressive drooping eyelids and intermittent diplopia with a horizontal separation. He had non-insulin dependent diabetes mellitus. There was no family history of neurological diseases. On examination at the age of 47 years, he had bilateral ptosis, which was more severe on the left side. Ocular movements were mildly restricted in horizontal and upgazes. He had bilateral facial weakness and no limb weakness or wasting. There was an abnormal increase in serum lactate, 2.67 mmol/L (normal value < 1.65 mmol/L). Muscle biopsy revealed a scattered RRF and COX negative fibres. Muscle respiratory chain enzyme activities were shown in table 4.2 (Performed by I. Hargreaves and Dr. SJR Heales). All respiratory chain enzyme activities were within normal ranges.

Table 4.2 Muscle respiratory chain enzyme activities of patient 9 (ratio to citrate synthase activity)

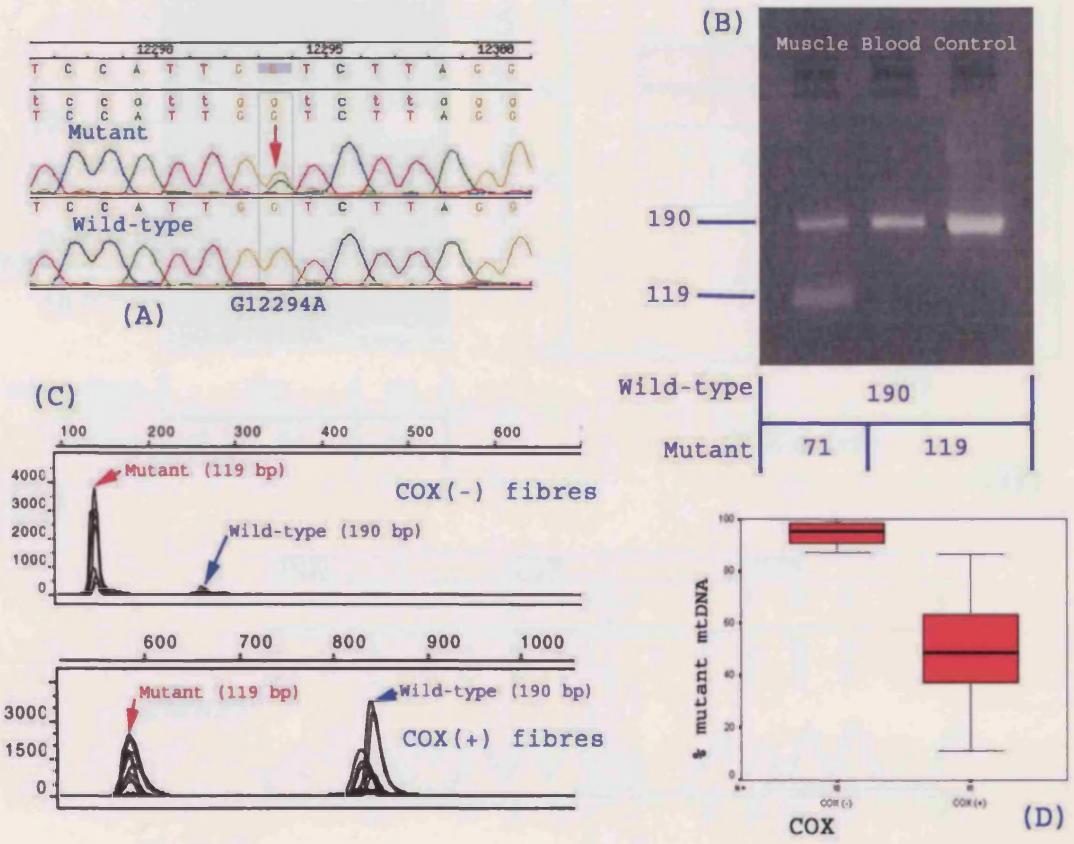
	Complex I	Complex II/III	Complex IV
Patient 9	0.223	0.158	0.015
Normal range	0.104-0.268	0.040-0.204	0.014-0.034

4.2.2 Results

MtDNA sequence analysis of the entire mtDNA genome was undertaken on muscle mtDNA. This revealed three previously unpublished changes compared with the published mtDNA sequence [Anderson *et al* 1981]. One change was in the $tRNA^{Leu(CUN)}$ gene, a G12294A mutation (patient 8) and two changes were in the mtDNA protein-coding genes, a T6911C transition in the COX I gene and a T11232C mutation in the ND4 gene (patient 9). In patient 8, the G12294A mutation in the $tRNA^{Leu(CUN)}$ gene was heteroplasmic.. The proportion of the mutant, G12294A, mtDNA was 59.81% in muscle but it was absent in blood (Figure 4.5). The author had carried out a primary myoblast culture in patient 8 in order to study a pathogenesis of the mutation in the cybrids model. The mutant A12294G was also absent in the myoblast cultures at 4 weeks. The mutation was absent in 112 control samples. Single fibre PCR analysis

was undertaken to investigate the relationship between the mutation and the histochemical abnormality at the level of the single fibre. The mean proportion of the mutant mtDNA in the COX-negative fibres was 92.92 ± 7.37% (n = 10) and that in the COX-positive fibres was 48.31 ± 22.09% (n = 18) ($p < 0.00001$).

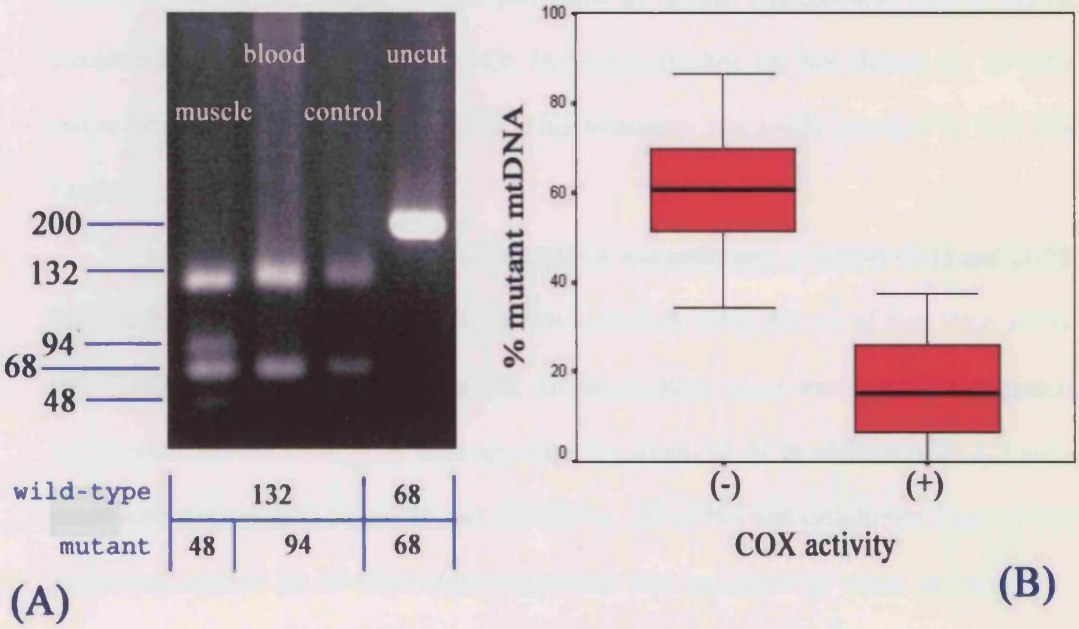
Figure 4.5 Analysis of the G12294A mutation (A) Electropherogram shows the G to A transition (arrow) at position 12294 of the $tRNA^{Leu(CUN)}$ gene. *Top panel,* patient 8's muscle. *Bottom panel,* control. (B) Agarose gel containing products following digestion of the original 190 bp product with *Mbo* I. In the presence of the G12294A mutation the 190 bp product is cleaved into two fragments 119 and 71 bp. (C) Genescan analysis of the G12294A mutation in COX (-) fibres and COX (+) fibres. Each panel shows an overlap of 10 muscle fibres' results) (D) Comparison of the proportion of the mutant (G12294A) mtDNA in single muscle fibres. COX (-) = fibres with cytochrome *c* oxidase (COX) deficiency and COX (+) = fibres with normal COX activity.

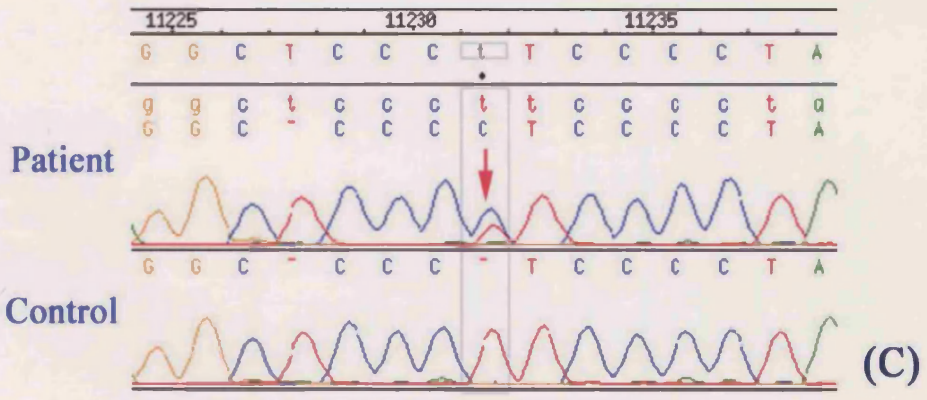


In patient 9 the T6911C transition in the COX I gene was homoplasmic and it was silent mutation. Therefore it is likely to be polymorphism. In contrast the 11232C in the ND4 gene was heteroplasmic (Figure 4.6). It results in an amino acid substitution, leucine to proline, at the highly conserved amino acid codon 158 in the ND4 gene. Patient 9 harboured 39.63% mutant

mtDNA in muscle and again it was absent in blood. It was also absent in 101 control samples. The single fibre PCR study revealed the mean proportion of the mutant mtDNA in the COX (-) fibres was 60.37 ± 14.57% (n = 14) and that in the COX (+) fibres was 16.15 ± 12.51% (n = 16) ($p < 0.00001$, *t*-test).

Figure 4.6 Analysis of the T11232C mutation (A) Agarose gel containing products following digestion of the original 200 bp product with *Mnl I*. In the presence of the T11232C mutation the 200 bp product is cleaved into three fragments 48, 68 and 94 bp. (B) Comparison of the proportion of the mutant mtDNA in single muscle fibres classified in two groups: COX (-) = fibres with cytochrome *c* oxidase (COX) deficiency and COX(+) = normal COX activity fibres. (C) Electropherogram shows the T to C transition (red arrow) at position 11232.





4.3 Mitochondrial myopathy without any mutations in the mitochondrial DNA

4.3.1 Patients

Patients with isolated mitochondrial myopathy in the Neurogenetic database at the Institute of Neurology, Queen Square were reviewed. The author identified sixteen patients who already had the common mtDNA mutations excluded. Then patients have been assigned number 10 to 25. They exhibited typical clinical, histochemical and/or biochemical features of mitochondrial myopathy (not including patients in the two previous sections in this chapter). The clinical features included progressive external ophthalmoplegia, ptosis, proximal myopathy, muscle pain, exercise intolerance, fatigue, cardiomyopathy and lactic acidosis (table 4.3). Prof. DN Landon and/or Dr. JA Morgan-Hughes performed all muscle histochemical studies. Muscle biochemical studies were performed by Dr. JA Morgan-Hughes, Dr. SJR Heales, Dr. JB Clark and/or Prof. JM Land. The result of muscle histochemistry was kindly provided by Prof. DN Landon.

Automated sequencing of the entire mtDNA was performed in patient 10-15 and 21-25. Patients 16-20 have not been sequenced the whole mtDNA due to run out of their DNA and no collected frozen tissue for DNA extraction. All the mt tRNA genes were sequenced in patient 16. All mt tRNA and COX genes were sequenced in patients 17-20. In addition ND1, 2, 3 and 4 genes were sequenced in patient 17 and 12S rRNA , 16S rRNA and cytochrome *b* genes were sequenced in patient 18. All of the remaining patients were sequenced the whole mtDNA.

Table 4.3A Clinical details of the patients (patients 10-17) with mitochondrial myopathy from section 4.3.1.

Patient	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Sex	F	M	F	M	F	F	M	M
Age of onset	26	20	28	Early 20	39	childhood	22	6
Age when examined	43	35	58	72	51	31	38	50
Ophthalmoplegia and ptosis	+	+	+	+	+	+	+	+
Limb weakness	-	-	+	-	-	+	+	+
Exercise intolerance or fatigue	-	-	-	-	-	+	-	-
Muscle pain	-	-	-	-	-	+	-	-
Family history of similar features	-	-	-	-	-	-	-	-
Cardiac abnormalities	NA	NA	NA	NA	NA	Dilated CM	NA	NA
Serum CPK (normal = 10-70 iu/L)	NA	NA	NA	NA	NA	331	NA	NA
Resting lactic acidosis (<1.8mmol/L)	NA	NA	NA	NA	NA	NA	NA	NA
Muscle histochemistry	cox-, no RRF*	RRF, cox- *	9%RRF	RRF*	RRF*	RRF, cox(-)*	13%RRF*	5% RRF
Muscle respiratory chain activity	NA	NA	NA	NA	NA	NA	NA	NA

Abbreviations as follow: M= male, F = female, NA = not available, CM = cardiomyopathy, RRF = ragged red fibres, cox = cytochrome oxidase, + = present, - = absent.

* RRF or COX (-) fibres present but proportion not quantified.

Table 4.3B Clinical details of the patients (Patients 18-25) with mitochondrial myopathy from section 4.3.1.

Patient	18	19	20	21**	22	23	24	25
Sex	F	F	M	F	F	F	M	M
Age of onset	38	45	20	Childhood	19	Childhood	12	49
Age when examined	40	58	66	27	34	44	24	52
Ophthalmoplegia and ptosis	+	+	+	+	-	-	-	-
Limb weakness	-	+	-	+	+	+	+	+
Exercise intolerance or fatigue	-	+	-	+	+	+	+	+
Muscle pain	-	-	-	+	-	+	+	+
Family history of similar features	-	-	+	Younger sister	-	Mother	Twin brother	-
Cardiac abnormalities	NA	NA	NA	-	CD	-	-	-
Serum CPK (normal = 10-70 iu/L)	NA	Normal	NA	NA	153	NA	Normal	NA
Resting lactic acidosis (<1.8mmol/L)	NA	Normal	NA	2.6-7.5	NA	Normal	Normal	Normal
Muscle histochemistry	RRF*	10%RRF	RRF*	70%RRF	60%RRF, cox+	RRF*	70%RRF, cox+	RRF*
Muscle respiratory chain defects	NA	NA	NA	Complex I	Complex I	Complex I	Complex I	Complex I

Abbreviations as follow: M= male, F = female, NA = not available, CD = conduction defect, RRF = ragged red fibres, cox = cytochrome oxidase, + = present, - = absent.

* RRF or COX (-) fibres were not quantify the proportion.

** Patient 21 and her sister were previously reported clinical, muscle histochemical and respiratory chain activity [Morgan-Hughes *et al.* 1979].

4.3.2 Results

All of the studied patients had isolated myopathy with ragged red fibres or COX-deficiency fibres. The age at onset ranged from childhood to 49 years old. Six of these patients (37.5%) had onset before 20 years of age. Although progressive external ophthalmoplegia was identified in 12 patients (75%), diplopia was present in only one patient (8.33%). Ten patients (62.5%) had limb weakness, which was mainly in the proximal muscle muscles. All of these patients complained of exercise intolerance or they had fatigable muscles on examinations. Muscle aching after exercise was observed in five patients (31.25%). This was significant leading to a limitation of some functional abilities in all patients. Two patients had history of episodes of worsen weakness and severe metabolic acidosis after taking a small amount of alcohol (one episode each). The patients improved after supportive treatment and rest. No patients had rhabdomyolysis or myoglobinuria. Four patients had family history of muscle problems. No patients had family history of other neurological diseases, blindness or deafness. Patient 20 had several family members with similar muscle symptom including his mother, three of his brothers and two of his sisters. One of three of his brother's sons also had ptosis. Each of three other patients had only one other affected relative.

EMG was performed in eight out of sixteen patients and were consistent with myopathic process. Medical records of full cardiac evaluations were identified in two patients. Dilated cardiomyopathy was observed in patient 15, and patient 22 had an intraventricular conduction defect. Only one patient had documented resting lactic acidosis. Two other patients had abnormal raised serum lactate after exercise. Electron microscopy often identified abnormal accumulations of the abnormal shape mitochondria with paracrystalline inclusion bodies. Muscle respiratory chain activity data was available in only five patients which all showed a complex I defect.

Automated sequencing of the mtDNA in these patients identified many changes from standard sequence, thirty three of these changes were not previously published (table 4.4).

Table 4.4 The unpublished nucleotide changes from standard mtDNA sequence [Anderson *et al.* 1981].

Patient	Nucleotide change	Gene	Amino acid change	Heteroplasmy	% in controls
10	T9128C	ATP6	Ile→Thr	-m, -b	ND
	T10388C	ND3	Gly (silent)	-m	ND
	T13965C	ND5	Leu (silent)	-m	ND
	A14687G*	$tRNA^{Glu}$	-	-m, -b	2.4 (3/126)
11	961insC	12S rRNA	-	-m, -b	1.25 (1/80)
12	C3990T	ND1	Tyr (silent)	-m	ND
	T4023C	ND1	Thr (silent)	-m	ND
	G8616T*	ATP6	Leu→Phe	-m	2.7 (1/37)
	T13488C	ND5	Pro (silent)	-m	ND
13	A9052G	ATP6	Ser→Gly	-m	ND
14	C4052T	ND1	Ala→Val	-m	ND
	T8594C*	ATP6	Ile→Thr	-m, -b	3.7 (2/54)
	T8987C	ATP6	Met→Thr	-m, -b	ND
	T9708C	COX III	Leu (silent)	-m	ND
	A10754G	ND4L	Leu (silent)	-m	ND
	C14544T	ND6	Leu (silent)	-m	ND
15	C3388A*	ND1	Leu→Met	-m, -b	0.87 (1/115)
	T3847C	ND1	Leu (silent)	-m	ND
17	A7385G	COX I	Trp (silent)	-m	ND
	A7768G	COX II	Met (silent)	-m	ND
	C8625T	ATP6	Thr (silent)	-m	ND
	T10927C	ND4	Phe (silent)	-m	ND
18	A7768G	COX II	Met (silent)	-m	ND
	G7912A	COX II	Glu (silent)	-m	ND
	T15721C	Cyt b	Tyr (silent)	-m	ND
20	T6524C	COX I	Thr (silent)	-m	ND
21	T4592C	ND2	Ile (silent)	-m	ND

	C11761T	ND4	Tyr (silent)	-m	ND
	C13188T	ND5	Thr (silent)	-m	ND
	A3796G*	ND1	Thr → Ala	-m, -b	0.43 (1/230)
22	C3450T	ND1	Pro (silent)	-m	ND
23	A3796G*	ND1	Thr → Ala	-m, -b	0.43 (1/230)
	A13413G	ND5	Met (silent)	-m	ND

Abbreviations as follow: 961insC = C insertion at nucleotide position 961, ND = NADH dehydrogenase, Cyt = cytochrome, COX = cytochrome oxidase, ATP = ATP synthase, tRNA = transfer RNA, Ala = alanine, Glu = glutamic acid, Gly = glycine, Ile = isoleucine, Leu = leucine, Lys = lysine, Met = methionine, Phe = phenylalanine, Pro = proline, Ser = serline, Thr = threonine, Trp = tryptophan, Tyr = tyrosine, Val = valine, +m = heteroplasmic in muscle, +b = heteroplasmic in blood, -m = homoplasmic in muscle, -b = homoplasmic in blood, ND = not determine.

* The nucleotide transition in the position that evolutionarily conserved.

Of these thirty-three changes twenty-three were silent, eight were missense and two were RNA gene mutations. All of these changes were homoplasmic in skeletal muscle mtDNA. Four of the missense mutations resulted in changes in evolutionarily conserved regions between a few species. Therefore the author screened these four changes and tRNA and rRNA mutations in control samples. All of the primers, PCR conditions and restriction endonuclease enzymes are listed in chapter 2, table 2.2 except for the 961insC mutation in 12S rRNA. Due to the sequence of mtDNA in the region of 961insC mutation had long run of C it was was not possible to design a PCR-RFLP reaction to detect this change. The author designed a PCR reaction using oligonucleotide primers: L901 (nt901-920) and H1070 (nt1070-1051) to measure a size of the PCR fragment by fluorescently labelled PCR and Genescan. In normal control sample, the size of fragment was 170 bp but the 961insC mutant fragment had one more base pair, 171 bp. All these changes were identified in normal control population in various frequencies (table 4.4). The remaining changes were either silent or not evolutionarily conservation.

4.4 Discussion

This chapter describes an extensive mtDNA analysis in twenty-one patients with mitochondrial myopathy. The author identified five new heteroplasmic mtDNA mutations two of which were in the tRNA genes and three were in the protein-coding genes. MtDNA mutations were not detected in the remaining patients.

4.4.1 Evidence supporting the pathogenicity of five newly identified mutations.

Patient 5. Evidence that support a role of pathogenicity of the G15723A mutation in the cytochrome *b* gene are as follow: (1) It is a nonsense mutation predicting the loss of 55 amino acids at the C terminus of the cytochrome *b* protein. Some parts of this 55-amino acids region are highly conserved between different species (Swiss-prot/TrEMBL Protein Knowledgebase website: http://www.expasy.ch/sprot/sprot-top.html); (2) The mutation was absent in a large panel of normal and disease-controlled samples; (3) It is heteroplasmic in muscle which is a characteristic feature of the mtDNA point mutation; (4) The mutation is in a cytochrome *b* gene which is consistent with the abnormal complex III activity detected in the patient. Although there was no analysis of single-fibre PCR or correlation of mutation loads and biochemical defects to confirm the pathogenicity, this mutation is nonsense mutation leading to a loss of 15% of the cytochrome *b* molecule. This should be sufficient to cause a respiratory chain defect in the patient.

Patient 6, 8 and 9. The A5874G mutation in the $tRNA^{Tyr}$ gene, the G12294A mutation in the $tRNA^{Leu(CUN)}$ gene and the T11232C mutation in the ND4 gene are fulfilled criteria for pathogenic status as follow: (1) the changes are heteroplasmic within and between different tissues; (2) they change highly evolutionarily conserved nucleotides and an amino acid (table 4.5A,B and C); (3) they were absent in over 100 controls; (4) single fibre PCR demonstrated greater proportions of mutant mtDNA in COX-negative fibres compared with COX-positive fibres suggesting an association between the proportion of the mutant mtDNA and muscle respiratory chain defects.

Table 4.5A demonstrates that nucleotide position 5874 (arrow) is highly conserved.

```
                                        ↓
PATIENT          GGTAAAATGGCTGAGT....G G AGCATTGGACTGTAAATCT
HUMAN            GGTAAAATGGCTGAGT....G A AGCATTGGACTGTAAATCT
BOVINE           GGTAAAATGGCTGAGC....A A AGCATTAGACTGTAAATCT
SEAL             GGTAAAATGGCTGATA....G A AGCATTAGACTGTAAATCT
CHICKEN          GGTAAGATGGCTGAGTGT TG A AGCGTTAGGCTGTAGTCCT
FROG             GGTAAGGTGGCCGAGTAA..T A GGCGGCGGATTGTAGCTCC
FRUIT FLY        GATTAAGTGGCTGAA.GTT.T A GGCGATAGATTGTAAATCT
```

Table 4.5B demonstrates that nucleotide position 12294 (arrow) is highly conserved.

```
                                      ↓
PATIENT          GGATAACAGC..TATCCATTG G TCTTAGGCCCCAAAA
HUMAN            GGATAACAGC..TATCCATTG G TCTTAGGCCCCAAAA
BOVINE           GGATAGTAGTT.TATCCGTTG G TCTTAGGAACCAAAA
MOUSE            GGATAATAGT..AATCCATTG G TCTTAGGAACCAAAA
CHICKEN          GGATAACAGCT.CATCCATTG G TCTTAGGAACCAAAA
FROG             GGAAAACAGTC.TATCCGCTG G TCTTAGGAACCAGAA
CRAP             GGATAACAGTT.CATCCGTTG G TCTTAGGAACCAAAA
BUDWORM          GCAGAT....TATATGTAATG G ATTTAAACCCCATTT
```

Table 4.5C demonstrates that amino acid codon 158 (arrow) is highly conserved.

```
                                  ↓
PATIENT          ERLNAGTYFLFYTLVGS P PLLIALIHTHNTLGSLNIL
HUMAN            ERLNAGTYFLFYTLVGS L PLLIALIHTHNTLGSLNIL
PIG              ERLNAGLYFLFYTLVGS L .LLVALLYLQNSTGSLNFL
RHINOSORUS       ERLNAGFYFLFYTLTGS L .LLVALIHIQNLTGSLNFL
OSTRICH          ERLSAGIYLLFYTLISS L .LLITILHLHTQTGTLHLP
LANCELET         ERYQAGLYFMFYTLFGS L .LLASLLFLSNTLSTLYIP
SHRIMP           ERLPASYYFLFYTLLSS L .LPLLLIMMLFKNQTLNYI
FRUIT FLY        ERLQAGIYLLFYTLLAS L .MLIGIFYIMNKMGTMNFY
YEAST            ERIQASYYMFFYTLFGS L FMLLSMGTYSYIMGNTDYD
```

Patient 7. The G4810A mutation in the ND2 gene exhibited support pathogenic features: 1) it is a nonsense mutation resulting in a change from tryptophan to stop codon at codon 114, which causes a truncated protein with a loss of 233 amino acids from the C terminus of the ND2 polypeptide; 2) The mutation was heteroplasmic within and between various tissues, which is a recognised feature of the pathogenic mtDNA mutation; 3) It was absent in a large group of normal control samples; 4) Single-fibre PCR analysis revealed the relationship between the high

proportion of mutant mtDNA and abnormal muscle morphology; 5) The mutation is consistent with the biochemical finding of the complex I defect in muscle.

4.4.2 Exercise intolerance

This chapter describes twenty-one patients with isolated mitochondrial myopathy with no known mutations. All patients had some degree of exercise intolerance and fatigable muscle weakness. These observations emphasis that exercise intolerance and fatigue are important features in this group of patients [Hayes *et al.* 1984]. Muscle weakness can sometimes be absent or very mild especially in the early stages, hence muscle strength testing after a brief period of exercise should routinely be performed. Lactic acidosis at rest or after exercise is also an important feature, which may help to diagnose patients especially in whom exercise intolerance is difficult to document objectively.

Exercise intolerance is increasingly recognised as a sole or a main feature of mitochondrial myopathy in association with mtDNA point mutations especially in the protein coding genes. In 1996 Dumoulin *et al.* identified a mt cytochrome *b* mutation in a 29-year-old man with progressive exercise intolerance associated with a marked deficiency of complex III activity and a decreased amount of mitochondrial-encoded cytochrome *b* [Dumoulin *et al.* 1996]. Subsequently Andreu *et al.* identified two further patients with severe exercise intolerance in association with mutations in the cytochrome *b* gene and a defect of complex III activity [Andreu *et al.*1998;Andreu *et al.* 1999c]. These data led us and mitochondrial study groups in the U.S.A., Spain and Germany to collaborate work on a group of five patients with severe exercise intolerance and complex III deficiency. All five patients had cytochrome *b* mutations and our patient is patient 5 in the publication and also **patient 5** in this chapter [Andreu *et al.* 1999a]. The combined data indicated that mutations in the cytochrome *b* gene might be important causes of exercise intolerance. However these data also suggest that mitochondrial myopathy characterised by severe exercise intolerance is genetically heterogeneous. There is no significant common mtDNA mutation despite patients has exhibitly similar clinical features, muscle histochemistry and respiratory chain defect.

In this chapter, section 4.1 describes two further patients (patients 6 and 7) who had severe exercise intolerance. Although both patients had mild ptosis the main features was exercise intolerance leading to functional disabilities. They had only mild proximal limb weakness but significant fatigue after a brief exercise was observed. **Patient 6** had severe complex III deficiency on skeletal muscle biochemical analysis [Hayes *et al.* 1984]. However muscle histochemistry showed that almost all ragged red fibres had no COX activity which was different from all patients with cytochrome *b* mutations whom ragged red fibres were all COX-positive fibres. Combined histochemical and biochemical data suggest that there may be a combined complex III+IV defect. Therefore the author analysed the mtDNA commenced on the cytochrome *b* and all of the tRNA genes, which might cause a predominant complex III, or a possibility of combined biochemical defects. The mtDNA analysis revealed a novel heteroplasmic, A5874G, mutation in the $tRNA^{Tyr}$ gene, which is the first mutation in the $tRNA^{Tyr}$ gene to be described in association with human disease.

It is notably that the clinical features and respiratory chain abnormality is similar to that associated with cytochrome *b* mutations. In order to determine why this $tRNA^{Tyr}$ gene mutation might preferentially affect complex III+IV the author analysed the proportion of tyrosine in all 13 mitochondrial proteins, which are encoded by mtDNA (table 4.6). The analysed data shows that the proportion of tyrosine in highly conserved positions is highest in the cytochrome *b* and COX III subunits. This could be a factor relevant to the observed combined complex III and IV deficiency.

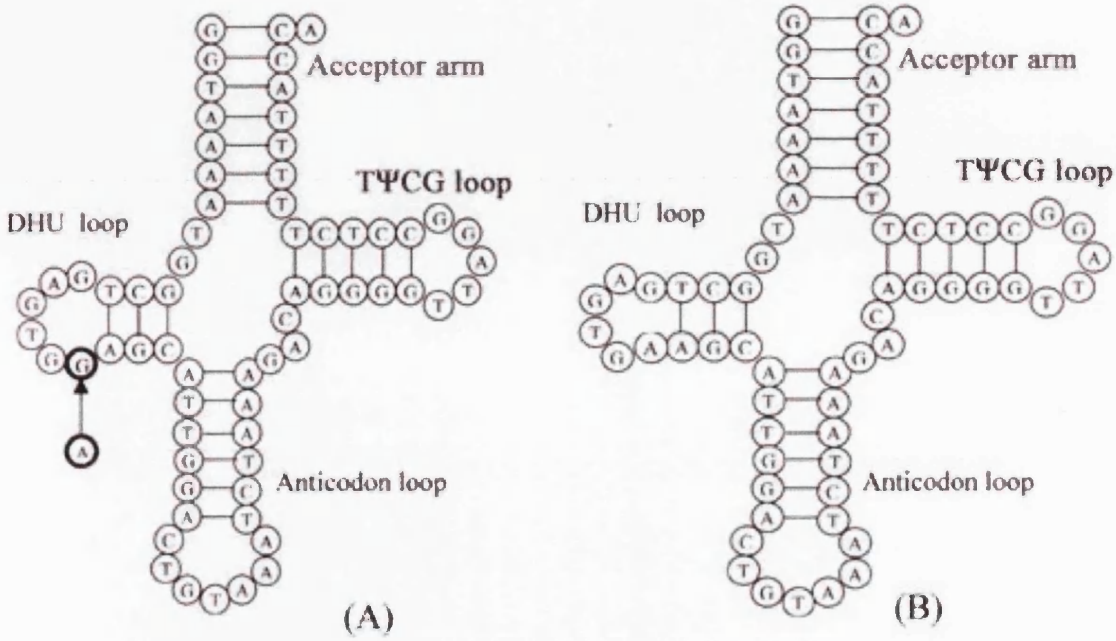
Table 4.6 The total number of tyrosine residue and the number of tyrosine residues in highly conserved positions in mitochondrial proteins encoded by mtDNA. Cytochrome *b* and COX III contain the highest proportions of conserved tyrosine residues.

Protein	Total number of amino acids	Number of tyrosine	% of tyrosine	Number of conserved tyrosine	% of conserved tyrosine
Cytochrome b	**380**	**17**	**4.47**	**12**	**3.16**
ND1	318	13	4.09	6	1.89
ND2	347	8	2.31	4	1.15
ND3	115	3	2.61	2	1.74
ND4	459	13	2.83	5	1.09
ND4L	98	4	4.08	2	2.04
ND5	603	16	2.65	4	0.66
ND6	174	11	6.32	5	2.87
COX I	513	22	4.29	11	2.14
COX II	227	9	3.96	6	2.64
COX III	**261**	**11**	**4.21**	**10**	**3.83**
ATP 6	226	3	1.33	0	0
ATP 8	68	2	2.94	0	0

The abbreviations are as follow: ND = the subunit of NADH-coenzyme Q oxidoreductase, COX = the subunit of cytochrome c oxidase, ATP = the subunit of ATP synthase

The A5874G mutation is in the dihydrouridine (DHU) stem of the $tRNA^{Tyr}$ molecule and would not cause any structural changes of the predicted conformational secondary structure of the $tRNA^{Tyr}$ molecule (figure 4.7). The underlying pathogenesis of the A5874G mutation causing respiratory chain defects should explain by other mechanism. Since this mutation is in a highly conserved region during evolution this nucleotide position may need to be post-transcriptional modification. This observation has been described in association with the A3243G mutation which was also in the dihydrouridine loop [Helm *et al.* 1999]. The modification of nucleotide base in the tRNA influences both structural and functional properties including correct folding, aminoacylation and codon-recognition. A second mutation in the $tRNA^{Tyr}$ gene, T deletion at the np 5885, has recently been described in a patient with CPEO and exercise intolerance [Raffelsberger *et al.* 2001]. This patient was also sporadic and no mutant mtDNA was detected in the patient's maternal relatives.

Figure 4.7 The predicted secondary structure of the mitochondrial $tRNA^{tyr}$ molecule in mutant (A) and normal molecule (B).



This study emphasizes the importance of careful biochemical and histological analysis in detecting the molecular genetic basis of exercise intolerance. These data showed that the syndrome of exercise intolerance with predominant complex III deficiency is genetically heterogeneous and may associate with this newly identified A5874G tRNA tyrosine somatic mutation, as well as with cytochrome *b* gene mutations.

Patient 7 had mitochondrial myopathy and isolated complex I deficiency presenting with severe exercise intolerance as a major presentation. Other clinical features were proximal myopathy, external ophthalmoparesis and 2 seizures. Although she had features of ptosis and external ophthalmoplegia the author describes her as a syndrome of exercise intolerance. Her clinical presentations was marked predominant exercise intolerance since childhood while ptosis and external ophthalmoparesis were only minimal and observed on examination at the age of 49 years. The author analysed all of the mtDNA tRNA and ND genes because she had isolated complex I deficiency. The sequencing analysis revealed a novel nonsense mutation, G4810A, in the mitochondrial ND2 gene which is the first mutation in the ND2 gene to be described in

human disease.

This patient had a questionable family history as her mother had deafness. It is possible that the G4810A may account for maternal deafness but this is uncertain. However the mother's tissues were not available for investigations, and we are not able to prove that this mutation is indeed inherited maternally.

Mutations in the mtDNA ND genes have been previously mainly identified in LHON, although others phenotypes have been described during the last few years (table 4.7). The diseases affect various tissues including optic nerves, skeletal muscle and brain similar to mutations in tRNA and other protein-encoding genes. The mechanisms to explain such phenotypic diversity remain unclear.

Table 4.7 Mutations in the mitochondrial complex I subunit genes associated with human disease.

Gene	Mutation	Disease	References
ND1	T3308C	Bilateral striatal necrosis/MELAS	[Campos *et al.* 1997a]
	G3460A	LHON	[Huoponen *et al.* 1991]
ND2	**G4810A**	**Myopathy/Exercise intolerance**	**This study**
ND3	T10191C	MELAS/optic atrophy	[Armstrong *et al.* 1996]
ND4	A11696G	LHON/dystonia	[de Vries *et al.* 1996]
	G11778A	LHON	[Wallace *et al.* 1988]
	G11832A	Exercise intolerance	[Andreu *et al.* 1999b]
ND5	G13513A	MELAS	[Santorelli *et al.* 1997b]
	G13513A	LHON/MELAS	[Pulkes *et al.* 1999]
	A13514G	MELAS	[Corona *et al.* 2001]
ND6	G14459A	LHON/dystonia	[Shoffner *et al.* 1995]
	T14596A	LHON/dystonia	[de Vries *et al.* 1996]
	T14484C	LHON	[Johns *et al.* 1992]
	A14495G	LHON	[Chinnery *et al.* 2001b]

Abbreviations as follow: MELAS = Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; LHON = Leber's hereditary optic neuropathy; ND = NADH dehydrogenase.

The NADH-ubiquinone oxidoreductase (complex I) is the first complex of the mitochondrial respiratory chain responsible for oxidative phosphorylation. It comprises at least 43 protein subunits, which mainly encoded by nuclear DNA. All of the seven mtDNA-encoded ND subunits are situated in the membrane domain of the complex I protein which may have a

role in anchoring the complex I in the mitochondrial membrane. The ND2 subunit is also predicted to be involved in proton pumping [Dupuis *et al.* 1998]. In bacterial models disruption in the gene equivalent to the ND2 gene results in loss of assembly of the membrane domain and disappearance of complex I-associated iron-sulfur clusters [Dupuis *et al.* 1998;Alves and Videira 1998]. The G4810A mutation appears to have low threshold of expression in the muscle as shown in single fibre PCR analysis. Moreover the mutant mtDNA result in loss of two-thirds of the ND2 polypeptide which should severely affect the structural and functional properties of the polypeptide. Such a major structural change of the ND2 subunit may result in a partial defect of the assembly of the complex I protein. As shown in blue native protein gel electrophoresis the mitochondrial protein concentrations of the patient were clearly less than of the normal control. Although the author is unable to explain why all complexes were reduce compare to controls, rather than only complex I this result is unlikely to be technical errors since these results were consistent in three repeated experiments. We also observed an approxiamtely 295–kD band which was increase in the patient. One possibility is that it may be a partially assembled of complex I. The components of the complex I band and the 295-kD band between complex III and IV are currently been studied by Prof. Winchester's group at the Institute of Child Health.

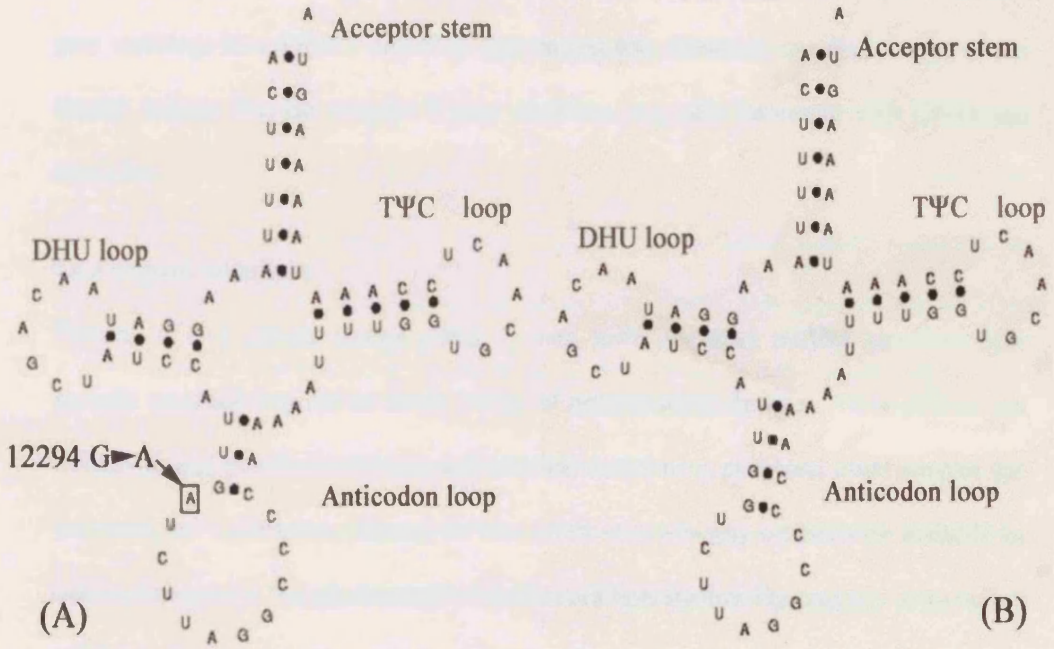
4.4.3 Chronic progressive external ophthalmoplegia

Chronic progressive external ophthalmoplegia (CPEO) is one of the common syndromes of mitochondrial encephalomyopathies. About two-third of the patients harboured large-scale rearrangements in the muscle mtDNA and further 15% are associated with the A3243G mutation. Several other tRNA point mutations were published in association with CPEO in single family or individuals and we reviewed these [Pulkes and Hanna 2001]. The author analysed the entire mtDNA in eight CPEO cases and partially analysed the mtDNA in six patients. Two new mutations have been identified, the G12294A mutation in the $tRNA^{Leu(CUN)}$ gene and the T11232C mutation in the ND4 gene. Patient 8 had progressive ptosis and complete ophthalmoplegia for 30 years. In addition she had severe exercise intolerance during the last 10

years. The G12294A mutation is in the $tRNA^{Leu(CUN)}$ gene, in which two other mutations , T12311C and G12315A mutations have been previously reported [Fu *et al.* 1996;Hattori *et al.* 1994].

The G12294A mutation is in the anticodon stem of the $tRNA^{Leu(CUN)}$ molecule. It may cause a conformational change of the secondary structure of the $tRNA^{Leu(CUN)}$ molecule (figure 4.8). The wild-type base forms a pairing bridge between G and C in the anticodon stem but the mutant base, which changes G to A, is predicted to disrupt this Watson-Crick base pairing. The predicted secondary structure of the mutant $tRNA^{Leu(CUN)}$ molecule has shorter anticodon stem and enlarged anticodon loop compared with wild-type. This change may affect translation resulting in a defect of respiratory chain function.

Figure 4.8 The predicted secondary structure of the mutant and wild-type $tRNA^{Leu(CUN)}$ molecule.



Biochemical studies on patient 8 (table 4.1) revealed multiple respiratory enzyme defects similar to the findings of previous mtDNA point mutations in tRNA genes [Chinnery *et al.* 1997a;Franceschina *et al.* 1998]. These data suggest that the mutation may cause a defect in protein synthesis. Each mtDNA-encoded protein subunits containing leucine (codon CUN) are generally affected. In contrast, mtDNA protein coding gene mutations are usually associated

with a specific respiratory chain activity corresponding to the protein coding genes [Hanna *et al.* 1998b].

In patient 9 the T11232C mutation results in an amino acid substitution, leucine to proline, at the highly conserved amino acid position, codon 158, in the ND4 gene (table 4.5C). The mutation may result in a conformational structural change leading to alter functional properties of complex I. We did not identify complex I deficiency from the muscle biopsy. This may be explained by a heterogeneous distribution of the mutant mtDNA in muscle tissue. Hence the studied mitochondria may be from muscle which contained a low proportion of mutant mtDNA.

The T11232C mutation is the first point mutation in a mtDNA protein-coding gene to be reported in association with CPEO. The first mutation in a mt complex I subunit gene in association with myopathy was previously described in a patient with exercise intolerance which was also in the ND4 gene [Andreu *et al.* 1999b]. Most patients with complex I subunit gene mutations have Leber's hereditary optic neuropathy. However, the data present in this chapter indicate that the complex I gene mutations may also associated with CPEO and myopathy.

4.4.4 Somatic mutations

Four out of five patients (except patient 7) with newly identified mtDNA mutations were sporadic cases and they had no family history of neuromuscular disorders. These patients had isolated skeletal muscle involvement and none had mutations in peripheral blood samples that were analysed. Furthermore, patient 8 for whom fresh muscle-biopsy samples were available for culture, the mutation was not detected in myoblast cell lines studied. The presence of the mutant mtDNA limited to skeletal muscle has been described in associated with other mtDNA point mutations. These included the A12320G in the $tRNA^{Leu(CUN)}$ gene [Weber *et al.* 1997], the G11832A in the ND4 gene [Andreu *et al.* 1999b] and eight other cytochrome *b* gene mutations associated with exercise intolerance [Dumoulin *et al.* 1996;Andreu *et al.* 1999a;Andreu *et al.* 1999c;Lamantea *et al.* 2002]. This observation may be explained by one of the following

mechanisms: 1) *Somatic mutations.* Since mitochondria and mtDNA in satellite cells turnover very slowly, the proportion of the mutant mtDNA in this cell population likely reflects the degree of heteroplasmy during early embryogenic development and changes little with age [Boulet *et al.* 1992]. The identified mutations may be spontaneous mutations in muscle after mesoderm differentiation into muscle, bone and vascular tissue; 2) *Germline mutations.* Mutations may occur in the germline, and then the mutant mtDNA randomly segregates with more into skeletal muscle through mitochondrial genetic bottleneck. Alternatively other cell types may have a lower threshold of expression and mutant mtDNA may cause cell death during embryogenic development which would result in a reduction of the proportion of mutant mtDNA in those tissues; 3) *Maternal transmissions.* Mothers may harbour very low amount of mutant mtDNA therefore they are asymptomatic. Genotypic change through one generation may be explained by the genetic bottleneck leading to decrease amount of mutant mtDNA in those tissues.

The presence of the mutant mtDNA in only skeletal muscle raise a possible approach to therapy suggested by Clark *et al.* 1997 and Taivassalo *et al.* 1999 [Clark *et al.* 1997;Taivassalo *et al.* 1999]. Their patients harboured mutant mtDNA only in skeletal muscle but not in the satellite cells therefore induction of the satellite cell proliferation would generate normal muscle fibres without mutant mtDNA. Methods of selection of patients and treatment remain to be studied to avoid complications from treatment and to be practical for regular patient care.

4.4.5 Evidence suggesting nuclear gene mutations.

Despite analysed the entire mtDNA, we have not identified any mtDNA mutations in the eleven remaining patients with both CPEO and isolated myopathy (patient 10-25). All patients exhibited characteristic clinical and histochemical findings of mitochondrial myopathies. These data suggest that they may have a nuclear genetic origin. Recently nuclear DNA mutations associated with autosomal dominant progressive external ophthalmoplegia (adPEO) have been described. All previously reported patients had multiple deletions of the mtDNA. Mutations in the adenine nucleotide translocator (*ANT1*) was the first nuclear gene identified in five families

and one sporadic patient with adPEO [Kaukonen *et al.* 2000]. The *ANT1* defect causes secondary accumulation of mtDNA mutations in postmitotic cells by an unknown mechanism. Subsequently mutations in two further genes namely the polymerase motif B of the mtDNA polymerase γ (*POLG*) and the Twinkle genes have simultaneously been reported both of which are believed to be involved in mtDNA replication [Van Goethem *et al.* 2001;Spelbrink *et al.* 2001].

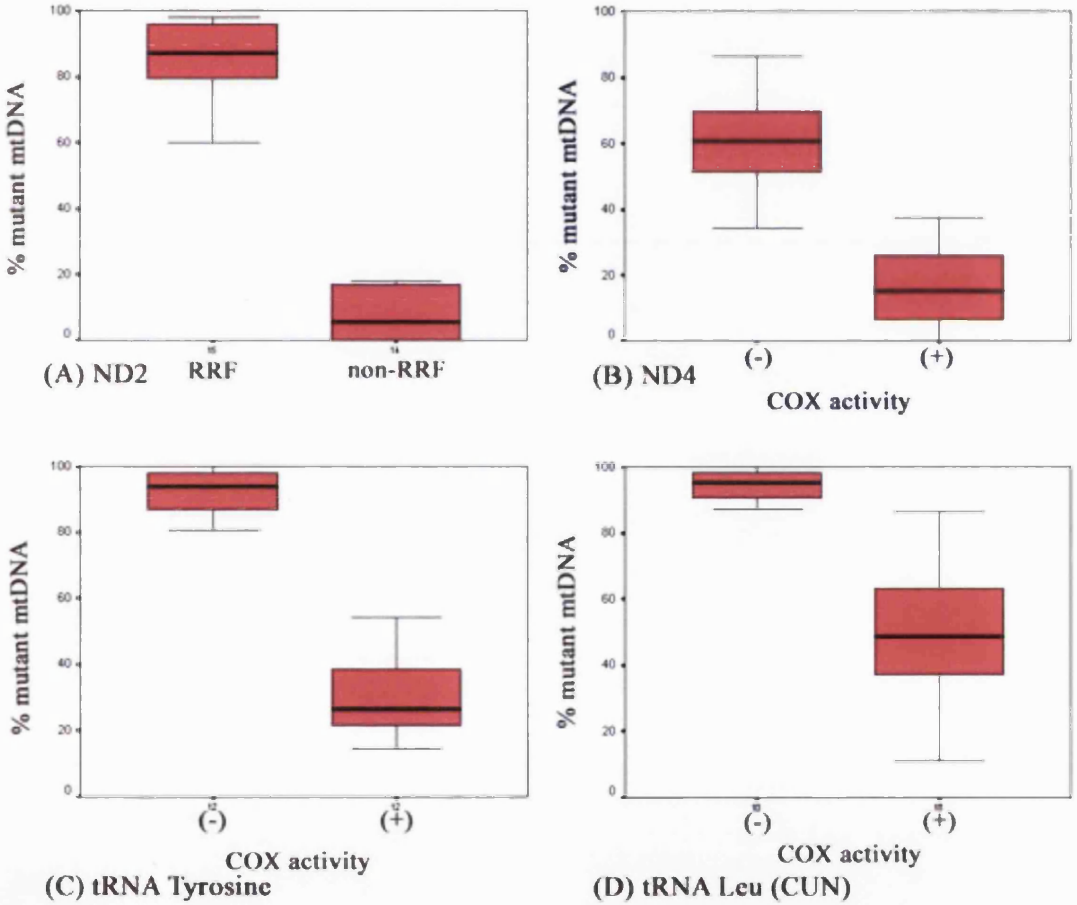
None of the patients in this study harbour mtDNA multiple deletions and most of them are sporadic suggesting that they may have different causative genes. Patient 20 had several affected family members with father to son transmission suggesting autosomal dominant transmission. Patient 23 had an affected mother suggesting that the mode of transmission may be maternal or autosomal dominant. Since the author excluded the possibility of an inherited mutation in the entire mtDNA in this patient these data suggest that autosomal dominant inheritance is likely. The ten remaining patients whom mtDNA mutations were excluded, autosomal recessive, x-linked recessive or new mutations could be the explanation. Five of the patients who had isolated complex I deficiency implying that nuclear complex I subunit genes might be candidate genes for further study.

4.4.6 Threshold of expression

Data from single fibre experiments gives indications of threshoreld of expression of the particular mtDNA mutations. Each mutation had significant higher proportion of the mutant mtDNA in the abnormal histochemical muscle-pathology fibres compared to normal fibre. These data confirmed the significant pathogenic role of each mutation. Furthermore we observed the lower threshold of expression in association with protein-coding gene mutations compared to the tRNA mutations. For the ND2 and ND4 mutations the lowest proportions of the mutant mtDNA in abnormal muscle fibres were 54 and 34 respectively. In contrast to the lowest proportions of the mutant mtDNA in abnormal muscle fibres were 75 and 81% in the tRNA mutations. The highest proportions of the mutant mtDNA in the protein-coding gene, which were observed in normal muscle fibre, were 20 and 37%. These proportions were much

lower than 54 and 87%, which were observed in the tRNA gene mutations (figure 4.9). These data confirm the previous observation that the proportion of the mutant mtDNA required to cause a respiratory chain defect is lower for the protein-coding gene mutations than in the tRNA gene mutations [Hanna *et al.* 1998b].

Figure 4.9 Comparison of the box-plot graphs demonstrate proportions of the mutant mtDNA in single muscle fibre in four different mutations. (A) G4810A, ND2 gene (B) T11232C, ND4 gene (C) A5874G, $tRNA^{Tyr}$ gene and (D) G12294A, $tRNA^{Leu(CUN)}$ gene.



Chapter 5 Mitochondrial encephalomyopathies without stroke-like episodes

5.1 New phenotypic diversity associated with the mitochondrial $tRNA^{Ser(UCN)}$ gene mutation: possible role of an intragenic suppressor polymorphism

5.1.1 Patients

Patient 26. This patient was a normal full term delivery and had normal motor and cognitive milestones. At the age of twelve years he developed generalised seizures and myoclonus. By the age of 16 years he had developed drug resistant epilepsy. Myoclonus persisted. From age 17 years, he developed progressive hearing loss and cognitive impairment. There was no family history of neurological disease or hearing loss. On examination at the age of 23 years he had a marked cerebellar syndrome including dysarthria and gait and limb ataxia. He had bilateral SNHL. There were myoclonic jerks in all limbs. There was no limb weakness or fatigue. Psychometric tests revealed a low verbal IQ of 82 and a low performance IQ of 74. Fasting plasma lactate and pyruvate were normal. An electroencephalography showed generalised spike wave discharge with photosensitivity. Muscle biopsy revealed scattered ragged red fibres in excess of 10%. Ragged red fibres showed enhanced activity for succinate dehydrogenase. A high proportion of non-ragged red fibres showed low COX activity. Respiratory chain enzyme activities in isolated mitochondria revealed a marked reduction in both complex I (8% of control value) and COX (9% of control value) (performed by Dr. M. Rose).

Patient 27 was a normal full term delivery with normal cognitive and motor milestones. She developed exercise intolerance from the age of 11 years. After this age walking even short distances produced muscle fatigue. There was no history of hearing impairment, muscle pain or myoglobinuria. There was no family history of neurological disease. On examination at the age of 15 years she had mild proximal upper and lower limb weakness (MRC grade 4/5). Electromyography showed a myopathic pattern and nerve conduction studies were normal. A CT brain scan was normal at the age of 13 years. Six maternal family members were examined

but had no abnormal neurological signs. Muscle biopsy showed florid ragged red change in the majority of muscle fibres which were stained strongly for succinate dehydrogenase. The majority of the muscle fibres were also markedly deficient in COX activity, only a few scattered fibres showing a normal or near normal activity. Respiratory chain activities (table 5.1) showed a marked reduction in activities of complex I (19% of control value) and COX (7% of control value) (performed by Dr. S. Heales).

Table 5.1 Muscle mitochondrial respiratory chain enzyme activities in patient 27.

Enzyme	Result (ratio to citrate synthase)	Normal range (ratio to citrate synthase)
NADH Ubiquinone Reductase	**0.019**	0.104-0.268
Succinate Cytochrome *c* Reductase	**0.013**	0.040-0.204
Cytochrome *c* Oxidase	**0.001**	0.014-0.034

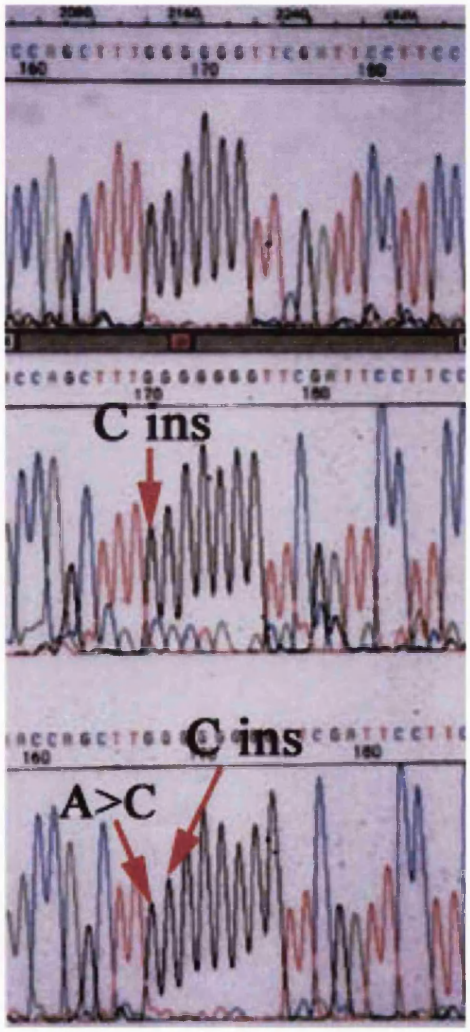
5.1.2 Results

Large-scale mtDNA rearrangements and common mtDNA point mutations were excluded in both patients. Sequence analysis identified a C insertion at nucleotide position (np) 7472 (7472insC) in the $tRNA^{Ser(UCN)}$ gene in both patients (Figure 5.1). The $tRNA^{Ser(UCN)}$ gene is on the heavy strand therefore the mutation should actually be insertion G rather than C. The position of the mutation is in the 6-G homopolymeric run spanning from np 7471-7466 therefore the exact position of the G insertion cannot be determined. Since this mutation has been previously described as the 7472insC mutation this nomenclature is used here. This change was heteroplasmic in the patient's blood and muscle samples and in the blood of all available maternal relatives (table 5.2). The 7472insC mutation was not found in 22 patients with mitochondrial encephalomyopathy who lacked any known mutations and over 300 previously reported controls [Tiranti *et al.* 1995]. In addition patient 27 also harboured a T to G transition (A to C on the light strand) at np 7472 in the same gene. The T7472G change was homoplasmic in the muscle and blood mtDNA of the patient and in the blood mtDNA from all available maternal relatives. The T7472G change was absent in 92 control samples, however we did not

have ethnic-matched control for this patient. (The patient is from Malta.)

Quantitation of the proportion of the mutant mtDNA in muscle of patients 26 and 27 revealed mutant loads of 87% and 91% respectively (table 5.2). The proportion of mutant mtDNA in blood of maternal family members of patient 27 ranged from 0% to 42%.

Figure 5.1 Electropherogram demonstrates the sequence of the normal control and patient 26 (B) and 27 (C). Patient 26 had a 7472insC mutation (7C run) and patient 27 had a 7472insC and an A7472C transition (8C run) compared to 6C run in normal control.



Abbreviation as follow: C ins = C insertion

Table 5.2 Proportions of mutant mtDNA in Patients 26 and 27 and Maternal Relatives of Patient 27

Patient	Tissue	Proportion of mutant mtDNA (%)
26	Muscle	87
	Blood	62
27	Muscle	91
	Blood	77
Maternal relatives of patient 27		
Mother	Blood	42
Aunt 1	Blood	2.5
Aunt 2	Blood	1.6
Aunt 3	Blood	2
Aunt 4	Blood	0

5.1.3 Discussion

This chapter described the clinical, histochemical, biochemical and molecular genetics features of two new unrelated patients harbouring the heteroplasmic 7472insC mutation in the mitochondrial $tRNA^{Ser(UCN)}$ gene. The first patient had a MERRF-like phenotype similar to previous reports [Jaksch *et al.* 1998;Schuelke *et al.* 1998;Tiranti *et al.* 1995]. However, the second patient exhibited a new phenotype for this mutation, characterised by early onset myopathy with exercise intolerance but without central nervous system disease.

Several lines of evidence indicate that the 7472insC is likely to be a pathogenic mutation: (1) The 7472insC is heteroplasmic, a characteristic feature associated with pathogenic mtDNA mutations, (2) The base composition in the TΨCG loop in the mitochondrial $tRNA^{Ser(UCN)}$ molecule is highly evolutionarily conserved, (3) It was absent in a large number of controls as described in previous reports [Tiranti *et al.* 1995], (4) It has previously been identified in several families with similar phenotype [Tiranti *et al.* 1995;Verhoeven *et al.* 1999].

The present study adds to the evidence that the 7472insC mutation is an important cause of progressive sensorineural hearing loss in combination with ataxia, myoclonic epilepsy and cognitive impairment. The neurological features are similar to classical MERRF although

sensorineural hearing loss is more commonly observed in association with the 7472insC mutation than with the A8344G mutation [Hammans *et al.* 1993].

The two patients we have described exhibited some histochemical differences compared to previous reports of 7472C-insertion cases. First, our patients had ragged red fibres in their muscle biopsies. Although ragged red fibres are a characteristic feature of A8344G-associated MERRF patients [Sweeney *et al.* 1994], they have not previously been found in 7472insC cases with a MERRF-like phenotype [Jaksch *et al.* 1998;Schuelke *et al.* 1998;Tiranti *et al.* 1995]. Secondly, the observation that the overwhelming majority of the muscle fibres were COX deficient has not been reported before in association with tRNA mutations. This observation of a similar absence of COX reactivity in muscle biopsies has previously reported in patients identified COX subunit gene point mutations [Hanna *et al.* 1998b;Rahman *et al.* 2000].

A mitochondrial tRNA mutation may theoretically affect transcription and/or translation as shown in the A3243G and A8344G mutations which generally affected multiple tissues [Hanna *et al.* 1995;King *et al.* 1992]. The 7472insC and other mutations in the $tRNA^{Ser(UCN)}$ gene frequently cause sensorineural hearing loss [Reid *et al.* 1994;Sue *et al.* 1999] at high levels of heteroplasmic or homoplasmic indicating of tissue-restricted pathological states by modest effects on mitochondrial function. The 7472insC mutation caused only a mild abnormality of mitochondrial protein synthesis in osteosarcoma cell cybrids despite a 65% drop in the steady-state level of the $tRNA^{Ser(UCN)}$ molecules suggesting that pathogenesis may be due to $tRNA^{Ser(UCN)}$ instability rather than its processing [Toompuu *et al.* 1999]. The authors suggested that the clinical phenotype could be the result of a functional insufficiency of mitochondrial $tRNA^{Ser(UCN)}$ gene to which some cells, such as those in the auditory system, might be vulnerable. Recently further study in details by the same group including tRNA half-life and synthesis rates, effects on aminoacylation and nucleotide base modification were performed [Toompuu *et al.* 2002]. They found that the mutation effected on $tRNA^{Ser(UCN)}$ synthesis rather than its molecular stability. The mutation had only minimal effects on $tRNA^{Ser(UCN)}$ structure although the presence of the additional G (C on light strand) lies within a homopolymeric tract spanning from the variable loop to the TΨCG stem. There were 25% decrease in aminoacylation

of the mutant mtDNA in the 143B cybrids and no effects on half-life of the $tRNA^{Ser(UCN)}$ molecules. Synthesis of the $tRNA^{Ser(UCN)}$ was impaired but absence of polarity effects on downstream of the light strand [Toompuu *et al.* 2002].

Patient 27 had a completely different phenotype from the phenotype previously reported by other groups and that of patient 26. This patient had only myopathy with no auditory or brain involvements despite harbouring very high proportions of the mutant mtDNA. Furthermore, no one in her family had hearing loss. The muscle histochemistry revealed florid ragged red fibres which were COX deficient. This finding was also different from previously reported patients in whom were reported to harbour ragged red fibres. It is interesting to speculate upon the basis of the phenotypic diversity described. In this context it is possible that the T7472G may in someway limit extra-muscle tissue expression. This possible role as an intragenic suppressor mutation requires further study.

5.2 Mitochondrial encephalomyopathies without any mutations in the mitochondrial DNA

5.2.1 Patients

Patient 28. Clinical, muscle histochemical and biochemical studies were previously reported on this 48-year-old Indian woman [Morgan-Hughes *et al.* 1982]. No information about her birth and early development was available. At the age of 33 years, she developed myoclonus in her hands and arms. She also had intermittent unsteady gait and proximal muscle weakness which fluctuated but slowly progressively worse. At the age of 46 years she became deaf and suddenly became confuse and disorientation. Her mental state was abnormal since then. Apart from her father she was only surviving member of her family. Her mother died aged 38 years from suspected liver disease and her brother died in early infancy from unknown causes. Two older sisters died in middle life, one following cardiac surgery and the other from the stroke. On examination she had board-based waddling gait and unable to walk heel to toe. Fundoscopic examination showed sight pallor and cupping of optic discs and patchy pigmentary retinopathy. Ocular movements were normal. Mild generalised muscle weakness was observed in all limbs which proximal muscles were more affected. Cerebellar incoordination was observed in both upper and lower limbs. Muscle biopsy revealed ragged red fibres in 37% of the muscle fibres. Muscle biochemical studies on isolated muscle mitochondria showed decrease amount of cytochrome *b*.

Patient 29. A 37-year-old female has suffered from progressive myoclonic epilepsy since childhood. She also had mental retardation. Muscle biopsy at the age of 31 year old revealed few ragged red fibres and COX-negative fibres. Electron microscope showed a mass of accumulations of abnormal mitochondria at the periphery of numerous muscle fibres in which contained large numbers of paracrystalline inclusions.

Patient 30. A 66-year-old male has suffered from progressive drooping eyelids and external ophthalmoplegia for 18 years. At the age of 51 years he began to experience slowly progressive

unsteadiness, nasal voice and difficulty swallowing. He had no family history of neurological diseases. On examination showed bilateral ptosis and mild limitation of ocular movements all directions. Horizontal nystagmus was observed. He had limbs wasting and weakness which was marked distally. Electrocardiogram revealed intraventricular conduction defect and Q wave in V4-6. Resting serum lactate was normal. CSF protein was in normal range. Muscle biopsy showed ragged red fibres in 25% of the muscle fibres.

5.2.2 Results

Large-scale mtDNA rearrangements and common mtDNA point mutations were excluded in all patients. Automated sequencing of the entire mtDNA in these patients identified several changes from the standard sequence. Ten of these changes were not previously published (table 5.3). These are nine silent mutations in the protein-coding genes and one mutation in the $tRNA^{Thr}$ gene.

Table 5.3 The unpublished nucleotide changes from standard mtDNA sequence [Anderson *et al.* 1981].

Patient	Nucleotide change	Gene	Amino acid change	Heteroplasmy
28	T5201C	ND2	Glu (silent)	-m
	G9755A	COX III	Ile (silent)	-m
29	A3384G	ND1	Lys (silent)	-m
	T7759C	COX I	Ala (silent)	-m
	C9449T	COX III	Tyr (silent)	-m
	A9494G	COX III	Gly (silent)	-m
	T13215C	ND5	Leu (silent)	-m
	C15194T	Cyt *b*	Leu (silent)	-m
	A15937G	$tRNA^{Thr}$	-	-m, -b
30	G5177A	ND2	Lys (silent)	-m

Abbreviations as follow: ND = NADH dehydrogenase, Cyt = cytochrome, COX = cytochrome oxidase, tRNA = transfer RNA, Ala = alanine, Glu = glutamic acid, Gly = glycine, Ile = isoleucine, Leu = leucine, Lys = lysine, Tyr = tyrosine, +m = heteroplasmic in muscle, +b = heteroplasmic in blood, -m = homoplasmic in muscle, -b = homoplasmic in blood, ND = not determine.

5.2.3 Discussion

This section describes three patients with mitochondrial encephalopathies with typical muscle histochemical features. First two patients (patient 28 and 29) were consistent with the diagnosis of MERRF and the last patient was **K**earn-**S**ayre **S**yndrome (KSS). After analysed the entire mtDNA, the author did not identify any pathogenic mtDNA mutations in these patients. These data suggest that they may have a nuclear genetic origin. Patient 28 had several affected family members having phenotypes possibly due to mitochondrial disorder. This pedigree was consistent with maternal inherited or autosomal dominant transmission. Exclusion of the pathogenic mtDNA mutations was suggested that the mode of transmission in this family might be autosomal dominant. Two other patients were sporadic which may be autosomal recessive, x-linked recessive (in patient 30) or new mutations.

Nuclear DNA mutations associated with mitochondrial encephalopathies have been reported in association with mainly infantile onset encephalopathic syndrome [Schuelke *et al.* 1999;Tiranti *et al.*1998]. Patients often presented with rapidly progressive encephalopathy and lactic acidosis sometimes accompanied with cardiomyopathy, hepatic failure or anemia [Allikmets *et al.* 1999;Loeffen *et al.* 2001;Papadopoulou *et al.* 1999]. All patients developed symptoms at very early age, often in the first year of life and they exhibited autosomal recessive inheritance. In contrast to other mitochondrial phenotypes such as MERRF and MELAS, ages of onsets are often in adulthood or adolescent which are thought to be purely associated with primary mtDNA defects. This is the first indirect evidence that the adult onset mitochondrial encephalopathies including KSS and MERRF phenotypes may associate with mutations in the nuclear DNA.

Chapter 6 Summary and conclusions

This thesis describes studies on thirty patients with typical mitochondrial encephalomyopathies. At the start of this study, none of these patients was defined at a molecular genetic level. The author identified pathogenic mutations in eleven patients, of which were five new mutations, and two had previously been reported. Of the nineteen remaining patients, the entire muscle mtDNA was analysed in thirteen and they did not harbour pathogenic mutations in the mtDNA. A study of the possible role of polymorphism in the $tRNA^{Leu(CUN)}$ gene influencing phenotypic expression of the A3243G MELAS mutation was also carried out.

The author concludes seven important points from this study:

1) All seven mutations identified in this study have fulfilled criteria supporting pathogenicity.

2) The G13513A mutation is one of the common mutations associated with MELAS and it may be the second commonest cause of MELAS in the U.K. Routine diagnosis DNA screening should be considered in A3243G-negative MELAS cases

3) The G13513A mutation can exhibit a MELAS/LHON overlap phenotype. This mutation appears to frequently affect the optic nerve. Optic atrophy was identified in half of our patients and in a newly reported patient [Corona *et al.* 2001]. However, to date there is no evidence of an association between the G13513A mutation and LHON in isolation.

4) Sporadic cases of isolated myopathy or progressive external ophthalmoplegia without large-scale rearrangements of the mtDNA are often associated with unique mtDNA point mutations. These mutations can be in any genes including tRNA and protein coding genes. There is evidence that they may be somatic mutations.

5) The A12308G polymorphism in the $tRNA^{Leu(CUN)}$ gene may double the risk of stroke in patients harbouring A3243G mutation. If this observation is confirmed in a larger group of patients this may be useful in patient prognostication.

6) Intragenic polymorphism or polymorphism in other genes in the mtDNA (mtDNA

background) may influence the phenotypic expression of primary pathogenic mtDNA point mutations.

7) Classical cases of mitochondrial encephalomyopathies with phenotypes such as CPEO, isolated myopathy, exercise intolerance, KSS and MERRF may associate with mutations in nuclear genes as yet unidentified.

There are now over one hundred point mutations in mtDNA described in association with human diseases. The author has reviewed the reported clinical features and tissue distribution of each of these defects. It is suggested that three categories of mtDNA point mutations exist based on tissue distribution and phenotype. It is possible that this classification may have implications for therapy.

A possible new classification of mtDNA point mutations [Pulkes and Hanna 2001]

1) Restricted tissue – restricted phenotype

This group refers to most of the recently described protein encoding gene mutations and to a few of the tRNA gene mutations: A12320G mutations in the $tRNA^{Leu(CUN)}$ gene [Weber *et al.* 1997] and the A5874G mutation in the $tRNA^{Tyr}$ gene [Pulkes *et al.* 2000b]. These mutations are confined to skeletal muscle disease. Patients predominantly present with exercise intolerance with or without muscle weakness. These patients may be labelled as 'chronic fatigue' cases. All these mutations were detected in only muscle tissue and were absent in either blood of patients or blood of available family members. Where studied these mutations are also absent from the muscle of maternal relatives [Pulkes *et al.* 2000b;Weber *et al.* 1997]. In some cases the mutant mtDNA was also absent in myoblast cultures derived from satellite cells [Andreu *et al.* 1999a]. Since mutant DNA was not present in satellite cells, this group may be the best candidates for satellite cell therapies [Weber *et al.* 1997]. The reason these point mutations should be confined to muscle is unclear. One possibility is that the mutational event occurred late in development e.g. after the mesoderm stage.

2) Unrestricted tissue – restricted phenotype

This applies to the three common primary LHON mutations (G11778A, T14484C and G3460A) and to the non-syndromic or aminoglycoside-induced sensorineural deafness mutations (A1555G and A7445G). Despite these mutations being virtually homoplasmic in all tissue analysed the disease phenotype is remarkably tissue specific. Both diseases are maternal inherited and not associated with RRF. The underlying mechanisms of such tissue specific expression remain unknown. It is suggested that the mtDNA defect is necessary but not sufficient to cause disease. Nuclear genes remain a strong candidate for influencing expression.

3) Unrestricted tissue – unrestricted phenotype

This group includes most of the tRNA mutations and a few of the protein encoding gene mutations. These mutations are detected in many tissues but in varying heteroplasmic proportions. Patients frequently exhibit multi-system and central nervous system involvement. They are usually maternal inherited.

Most protein encoding mutations fall into the first and second groups with relatively few in the third groups. In contrast, most tRNA mutations fall into the third group with only a few in the first and second group.

It is evident that there is a bewildering away of clinical phenotypes associated with mutations in mtDNA. Virtually any organ systems may be involved, although brain and skeletal muscle are frequently most affected. Perhaps this is not surprising when one consider the central role of respiratory chain function in cell physiology. Whilst most works agree that the mtDNA defects are central to the disease process, many additional factors must operate to influence phenotype. Nuclear genes remain strong candidate. At least three groups of mtDNA point mutations exist as outlined above. It seems possible that different factors are operating in each of these groups to differentiate phenotypes. A more precise understanding of these factors requires further study, but should aid therapeutic strategies.

Publications arising from the present work

Papers

Pulkes T, Eunson L, Patterson V, Siddiqui A, Wood NW, Nelson IP, Morgan-Hughes JA, Hanna MG. The mitochondrial DNA G13513A transition in ND5 is associated with a MELAS/LHON overlap syndrome and may be a frequent cause of MELAS. *Ann Neurol* 1999;46:916-919.

Andreu AL, Hanna MG, Reichmann H, Bruno C, Penn AS, Tanji K, Pallotti F, Iwata S, Bonilla E, Lach B, Morgan-Hughes J, DiMauro S. (other authors: Shanske S, **Pulkes T**, Siddiqui A, Clark JB, Land J, Iwata M, Schaefer J, Sue CM) Exercise intolerance due to mutations in the cytochrome *b* gene of mitochondrial DNA. *N Engl J Med* 1999;341: 1037-1044.

Pulkes T, Siddiqui A, Morgan-Hughes JA, Hanna MG. A novel mutation in the mitochondrial $tRNA^{Tyr}$ gene associated with exercise intolerance. *Neurology* 2000;55:1210-1212.

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Pulkes T, Eunson L, Patterson V, Siddiqui A, Hanna MG. Leber hereditary optic neuropathy/ MELAS overlap syndrome: a case report. Submitted to *Neurol Rev J.*

Pulkes T, Eunson LH, Rose M, Nelson IP, Rahman S, Poulton J, Marchington DR, Landon DN, Debono AG, Morgan-Hughes JA, Hanna MG. New phenotypic diversity associated with the mitochondrial $tRNA^{Ser(UCN)}$ gene mutation: possible role of an intragenic suppressor polymorphism. Submitted to *Brain.*

Pulkes T, Liolitsa D, Nelson IP, Hanna MG. Mitochondrial DNA analysis in adult-onset mitochondrial encephalomyopathies: evidence for nuclear gene involvement. *In preperation.*

Pulkes T, Wills AJ, Hargreaves I, Heales S, Hanna MG. A novel nonsense mutation in the mitochondrial ND2 gene associated with mitochondrial myopathy. *In preparation.*

Abstracts

Pulkes T, Eunson L, Patterson V, Wood NW, Nelson IP, Hanna MG. MELAS: a mitochondrial DNA complex I (ND5) subunit gene mutation identified in three families. (abstract) *J Neurol Neurosurg Psychiatry* 1999;66:263.

Pulkes T, Siddiqui A, Nelson IP, Sweeney MG, Schapira AHV, Cooper JM, Morgan-Hughes JA, Wood NW, Hanna MG. A novel change in the mitochondrial ND1 subunit gene associated with pure mitochondrial myopathy in two British families. (abstract) *J Neurol Neurosurg Psychiatry* 1999;67:273.

Pulkes T, Siddiqui A, Morgan-Hughes JA, Wood NW, Hanna MG. A novel nonsense mutation in the mitochondrial cytochrome *b* gene associated with exercise intolerance and complex III deficiency. (abstract) *J Neurol* 1999;246:S165.

Siddiqui A, **Pulkes T**, Nelson IP, Stephens D, Francis M, Wood NW, Reardon W, Hanna MG. A genetic study of a maternal pedigree with sensorineural deafness. (abstract) *J Neurol Neurosurg Psychiatry* 1999;67:273-274.

Pulkes T, Andreu Al, Siddiqui A, Morgan-Hughes JA, Dimauro S, Hanna MG. Exercise induced myalgia associated with five new mutations in the mitochondrial DNA cytochrome *b* genes: an important cause of muscle aches, pains, and cramps? (abstract) *J Neurol Neurosurg Psychiatry* 2000;68:268.

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Pulkes T, Wood NW, Sweeney MG, Hanna MG. A polymorphism in the mitochondrial $tRNA^{Leu(CUN)}$ gene influences A3243G-associated phenotype in humans. (abstract) *Ann Neurol* 2000;48:438.

Presentations

Pulkes T, Eunson L, Patterson V, Wood NW, Nelson IP, Hanna MG (1998).
MELAS: a mitochondrial DNA complex I (ND5) subunit gene mutation identified in three families.
Oral presentation at the Autumn Meeting of Association of British Neurologists, London, UK.

Pulkes T, Siddiqui A, Nelson IP, Morgan-Hughes JA, Hanna MG (1999).

A novel mutation in the mitochondrial ND1 subunit gene associated with pure mitochondrial myopathy in two British families.
Poster presentation at the Meeting of Association of British Neurologists, Southampton, UK.

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Pulkes T, Wood NW, Sweeney MG, Hanna MG (2000).
A polymorphism in the mitochondrial $tRNA^{Leu(CUN)}$ gene influences A3243G-associated phenotype in humans.
Poster presentation at the 125th Annual Meeting of the American Neurological Association, Boston, USA.

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Mitochondrial encephalomyopathies - new mtDNA mutations and evidence for nuclear gene defects.
Poster presentation at the World Congress of Neurology, London, UK.

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Poster presentation at the 5th European meeting on mitochondrial pathology, Venice, Italy.

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A novel nonsense mutation in the mitochondrial ND2 gene is associated with mitochondrial myopathy.
Poster presentation at the 5th European meeting on mitochondrial pathology, Venice, Italy.

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Mitochondrial DNA analysis in mitochondrial encephalomyopathies: evidence for nuclear gene involvement.
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