PHYLOGENETIC ANALYSIS OF MITOCHONDRIAL DNA

Detection of mutations in patients with occipital stroke

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Abstract

A mitochondrial disorder may be one of the rare aetiologies of occipital stroke. Clinical and molecular analysis has suggested that 10% of young patients with occipital stroke have a mitochondrial disorder and 6% harbour the mutation 3243A>G in mitochondrial DNA (mtDNA), causing the MELAS syndrome. To identify other possible mtDNA mutations involved, we studied mtDNA genotypes in patients who had suffered an occipital stroke and in whom the common pathogenic mutations in mtDNA had been excluded.

Since one systematic way of comparing mtDNA sequences is through phylogenetic analysis, a phylogenetic network for the Finnish mtDNA haplogroup U was constructed and used to identify differences in mtDNA between patients and controls. The usefulness of conformation sensitive gel electrophoresis (CSGE) for analysing differences within the coding sequence of mtDNA was also estimated.

We studied mtDNA genotypes of 29 patients with occipital stroke. The aetiology of the stroke was assessed using the criteria of the Baltimore-Washington Cooperative Young Stroke Study, and migraine was diagnosed in 18 patients according to the International Headache Society criteria. Moreover, we studied the mtDNA genotypes of 42 patients with migraine and a total of 480 population controls who reported that they themselves and their mothers were healthy with respect to common clinical manifestations of mtDNA disease. The mtDNA haplogroups were detected by restriction fragment analysis and the mtDNA structures of 14 patients with occipital stroke and 43 subjects belonging to haplogroup U were examined by CSGE. The data acquired by CSGE were then used to construct a phylogenetic network for the Finnish mtDNA haplogroup U.

We found CSGE to be a highly sensitive and specific method for screening mutations and polymorphisms in mtDNA. The sequence data on the 43 subjects belonging to the mtDNA haplogroup U were used to construct a phylogenetic network, which was found to be an unambiguous tree with few homoplasies that pointed to several previously unidentified common polymorphisms. The major branch of the network was U5, which seemed to be quite specific to the Finns. Branches representing haplogroups U2, U4, U7 and K could also be detected. Restriction fragment analysis of the patients with occipital stroke revealed that all those with migraine as a probable aetiology belonged to the mtDNA haplogroup U, suggesting that this genotype confers a risk of occipital stroke. In addition to the five patients with migrainous stroke, we analyzed the complete mtDNA coding sequences of nine other patients with occipital stroke belonging to haplogroup U by CSGE. Analysis of the phylogenetic network revealed an association of migrainous stroke with mtDNA haplogroup U5. Furthermore, the distribution of the mtDNA genotypes in the patients with stroke differed from that found in the controls. Four patients harboured potentially pathogenic mutations.

CSGE proved to be an effective method for use in mitochondrial genetics, enabling us to construct an unambiguous network for the Finnish haplogroup U. Similar phylogenetic networks are required for the purposes of both medical genetics and population genetics. Such networks were found to be helpful in deciding between a rare polymorphism and a pathogenic mutation in patients with occipital stroke. Likewise, they enabled more detailed comparisons to be made between and within populations and allowed more accurate phylogenetic relationships to be determined.

Keywords: population genetics, phylogenetic network, mutation analysis, mitochondrial encephalomyopathies

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Abbreviations

ADP	adenosine diphosphate
ANT	adenine nucleotide translocator
ATP	adenosine triphosphate
bp	base pair
CoQ	coenzyme Q_{10}
COX	cytochrome c oxidase
(C)PEO	(chronic) progressive external ophthalmoplegia
CSGE	conformation sensitive gel electrophoresis
cyt c	cytochrome c
D-loop	displacement loop
DNA	deoxyribonucleic acid
ETC	electron transfer chain
HSP	hereditary spastic paraplegia
HVS	hypervariable segment
LHON	Leber's hereditary optic neuropathy
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonus epilepsy with ragged-red fibers
MILS	maternally inherited Leigh syndrome
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mTERF	transcription termination factor
NADH	reduced nicotinamide adenine dinucleotide
NARP	neurogenic weakness, ataxia and retinitis pigmentosa
nt	nucleotide
O_H	origin of replication of the heavy strand of mtDNA
O_L	origin of replication of the light strand of mtDNA
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
P_H	mtDNA transcription initiation site in the heavy strand
P_i	inorganic phosphate

P_L	mtDNA transcription initiation site in the light strand
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RRF	ragged-red fiber
rRNA	ribosomal ribonucleic acid
SDH	succinate dehydrogenase
TCA	tricarboxylic acid
tRNA	transfer ribonucleic acid
YBP	years before present

List of original articles

I Majamaa K, Finnilä S, Turkka J & Hassinen IE (1998) Mitochondrial DNA haplogroup U as a risk factor for occipital stroke in migraine. Lancet 352: 455-456.

II Finnilä S, Hassinen IE & Majamaa K (1999) Restriction fragment analysis as a source of error in detection of heteroplasmic mtDNA mutations. Mutat Res 406: 109-114.

III Finnilä S, Hassinen IE, Ala-Kokko L & Majamaa K (2000) Phylogenetic network of mitochondrial DNA haplogroup U in northern Finland based on sequence analysis of the complete coding region by conformation sensitive gel electrophoresis. Am J Hum Genet 66: 000-000, in press.

IV Finnilä S, Hassinen IE & Majamaa K. Phylogenetic analysis of mitochondrial DNA in occipital stroke. Submitted for publication.

Contents

1. Introduction

A typical human cell has several hundred mitochondria. These are unique, because they are responsible for the major energy producing system of the cell, the oxidative phosphorylation enzyme pathway (OXPHOS). Furthermore, they are under dual genetic control. The vast majority of mitochondrial proteins are encoded by the nuclear DNA, but some are encoded by the mitochondrial DNA (mtDNA). The maternally inherited mtDNA is a closed, circular molecule 16,569 bp in length which codes for 13 polypeptides of the OXPHOS, two rRNAs and all the 22 tRNAs required for mitochondrial protein synthesis. Normally all the mtDNAs in a cell are identical, a condition known as homoplasmy, but patients with mitochondrial diseases due to mutations in mtDNA often harbour an intracellular mixture of mutant and normal molecules, a condition known as heteroplasmy.

The mutation rate of mtDNA genes is much higher than that of nuclear DNA genes, but most mtDNA mutations are neutral polymorphisms which have accumulated sequentially in maternal lineages. These polymorphisms create groups of related mtDNA haplotypes, or haplogroups. MtDNA sequence variation can be used to construct a phylogenetic network that displays the evolutionary relationships between individual sequences.

Mitochondrial dysfunction is one of the rare aetiologies of stroke, but it has been suggested that 10% of young adults suffering an occipital brain infarct have such a mitochondrial disorder, and the common MELAS mutation 3243A>G has been found in 6% of patients. In addition to pathogenic mutations, it is possible that the cumulative effect of many mildly deleterious sequence variants may synergistically contribute to the pathogenesis of the mitochondrial disorder. It has been shown that the expression of some pathological mtDNA mutations depends on the mtDNA background against which they occur. Such genotypes conferring risk have been found in Leber's hereditary optic neuropathy and Alzheimer's disease, for instance.

The aims of this work were to find new mtDNA mutations in patients who have suffered an occipital stroke and belong to mtDNA haplogroup U, and to investigate mitochondrial genotypes contributing to the risk of occipital stroke. The phylogenetic network of the Finnish mtDNA haplogroup U was constructed and used to identify differences in mtDNA between patients and controls.

2. Review of the literature

2.1. Mitochondria

2.1.1. Structure of mitochondria

The typical human cell has several hundred mitochondria, cytoplasmic organelles that convert energy to forms that can be used to drive cellular reactions. Without them cells would be dependent on anaerobic glycolysis for all their adenosine triphosphate (ATP). The mitochondria have a characteristic double membrane structure, in which the outer membrane contains large channel-forming proteins (called porin) and is permeable to all molecules of 5000 daltons or less, while the inner membrane is impermeable to most small ions and is intricately folded, forming structures called cristae. The large surface area of the inner mitochondrial membrane accommodates respiratory chain and ATP synthase enzymes involved in the process of oxidative phosphorylation (OXPHOS). The mitochondrial matrix contains hundreds of enzymes, including those required for the oxidation of pyruvate and fatty acids and those active in the tricarboxylic acid (TCA) cycle. The matrix also contains several identical copies of the mitochondrial DNA, mitochondrial ribosomes, tRNAs and various enzymes required for the transcription and translation of mitochondrial genes (see Alberts *et al*. 1994).

2.1.2. Energy production by mitochondria

Only a small fraction of the total free energy potentially available from glucose is released in glycolysis. The metabolism of carbohydrates is completed in the mitochondria when pyruvate is imported and oxidized by molecular oxygen (O_2) to CO_2 and water. The energy released is harnessed so efficiently that about 30 molecules of ATP are produced for each molecule of glucose oxidized, whereas only 2 molecules of ATP are produced by glycolysis alone. Oxidative metabolism in mitochondria is fuelled not only by pyruvate produced from carbohydrates by glycolysis in the cytosol but also by fatty acids. Pyruvate and fatty acids (from triglycerides) are selectively transported from the

cytosol into the mitochondrial matrix, where they are broken down into the two-carbon acetyl group on acetyl coenzyme A (acetyl CoA) by the pyruvate dehydrogenase complex and the β-oxidation pathway, respectively. The acetyl group is then fed into the tricarboxylic acid cycle for further degradation, and the process ends with the passage of acetyl-derived high-energy electrons along the respiratory chain.

The proteins involved in OXPHOS are located within the mitochondrial inner membrane and include the electron transport chain (ETC) components (complexes I to IV), F_OF_1 ATP synthase and the adenine nucleotide translocator (ANT) (Figure 1). High-energy electrons from TCA are combined with molecular oxygen by means of the ETC to generate water. These electrons, borne on NADH (nicotinamide adenine dinucleotide), are transferred to respiratory complex I (NADH dehydrogenase) and then to coenzyme Q_{10} (CoQ), while the electrons from succinate are transferred to complex II (succinate dehydrogenase, SDH) and CoQ. From CoQ, they are passed to complex III, and then to cytochrome c (cyt c), complex IV (cytochrome c oxidase, COX) and finally to ½ O_2 to give H_2O. The energy released is used to pump protons (H^+) out of the mitochondrial matrix, creating an electrochemical gradient across the inner membrane that is positive and acidic on the outside and negative and alkaline on the mitochondrial matrix side. This gradient creates a capacitor that can be depolarized by the transport of protons back into the matrix through a proton channel in the F_O membrane component of ATP synthase. This proton flux drives the condensation of adenosine diphosphate (ADP) and inorganic phosphate (P_i) to make ATP, which is then exported to the cytosol in exchange for ADP by the ANT (Figure 1). In this way oxygen consumption by the ETC is coupled to ADP phosphorylation by ATP synthase through the electrochemical gradient (DiMauro & Bonilla 1997, Wallace 1997).

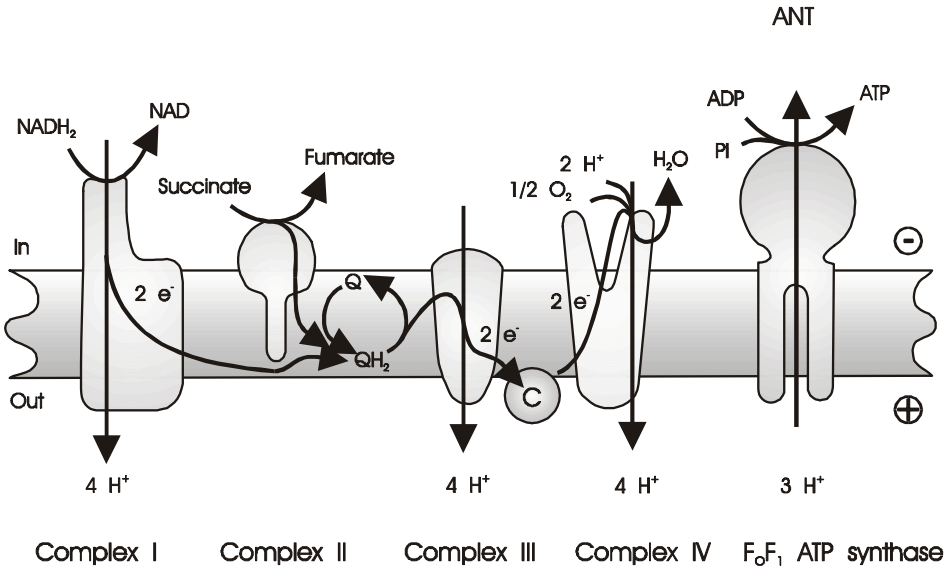

Fig. 1. The electron transport chain components involved in oxidative phosphorylation are located within the mitochondrial inner membrane. C, cytochrome c; Q, ubiquinone.

2.2. The mitochondrial genome

Mitochondria have been essential for the evolution of animals. It is generally believed that the energy-converting organelles of eucaryotes evolved from procaryotes that were engulfed by primitive eucaryotic cells and developed a symbiotic relationship with them about 1.5 x 10^9 years ago. This would explain why mitochondria have their own DNA (mtDNA), which codes for some of their proteins. Since their initial uptake by a host cell, these organelles have lost much of their own genome and have become heavily dependent on genes in the nucleus.

Extant mammalian mtDNAs have retained only 13 polypeptide genes, all of which encode essential components of OXPHOS. MtDNA also encodes the 12S and 16S rRNA genes and the 22 tRNA genes required for mitochondrial protein synthesis. The remaining mitochondrial OXPHOS proteins, the metabolic enzymes, the DNA and RNA polymerases, the ribosomal proteins and the mtDNA regulatory factors are all encoded by nuclear genes, synthesized in the cytosol and then imported into the organelle (Shoffner & Wallace 1995, Wallace *et al*. 1997a).

The transfer of mtDNA sequences to the nucleus is a continuous process (Wallace 1997, Hirano *et al*. 1997), but not all of the mtDNA genes transferred to the nucleus are functional. The presence of hundreds of mtDNA-like sequences or pseudogenes in the human nuclear genome has been well documented (Tsuzuki *et al*. 1983, Shay & Werbin 1992).

Furthermore, the genetic code in human mitochondria has come to differ from that used in the nucleus, and thus mtDNA genes are no longer intelligible to the nucleocytosolic system (Wallace 1982). UGA is read as tryptophan rather than ‘stop’, AGA and AGG as ‘stop’ rather than arginine, AUA as methionine rather than isoleucine, and AUA or AUU is sometimes used as an initiation codon instead of AUG (Anderson *et al*. 1981, Montoya *et al*. 1981).

2.2.1. Organization of the human mitochondrial genome

The human mitochondrial genome is 16,569 base pairs (bp) in length (Anderson *et al*. 1981), a closed, circular molecule (Figure 2) located within the mitochondrial matrix and present in thousands of copies per cell. Mitochondrial DNA has two strands, a guanine-rich heavy (H) strand and a cytosine-rich light (L) strand. The heavy strand contains 12 of the 13 polypeptide-encoding genes, 14 of the 22 tRNA-encoding genes and both rRNA-encoding genes. Introns are absent in mtDNA, and all of the coding sequences are contiguous (Anderson *et al*. 1981, Wallace *et al*. 1992, Zeviani *et al*. 1998). The only non-coding segment of mtDNA is the displacement loop (D-loop), a region of 1121 bp that contains the origin of replication of the H-strand (O_H) and the promoters for L and H-strand transcription. The mtDNA is replicated from two origins. DNA replication is initiated at O_H using an RNA primer generated from the L-strand transcript. H-strand synthesis proceeds two-thirds of the way around the mtDNA, displacing the parental H-strand until it reaches the L-strand origin (O_L), situated in a cluster of five tRNA genes. Once exposed on the displaced H-strand, O_L folds a stem-loop structure and L-strand

synthesis is initiated and proceeds back along the H-strand template. Consequently, mtDNA replication is bidirectional but asynchronous (Clayton 1982). MtDNA transcription is initiated from two promoters in the D-loop, P_L and P_H. Transcription from both promoters proceeds around the mtDNA circle, creating a polycistronic RNA. The tRNA genes which punctuate the larger rRNA and mRNA sequences then fold within the transcript and are cleaved out. The mRNAs and rRNAs liberated are post-transcriptionally polyadenylated and the tRNAs are modified and the 3' terminal CCA added (Attardi *et al.* 1982, Attardi & Montoya 1983, Clayton 1984, Wallace 1993, Taanman 1999).

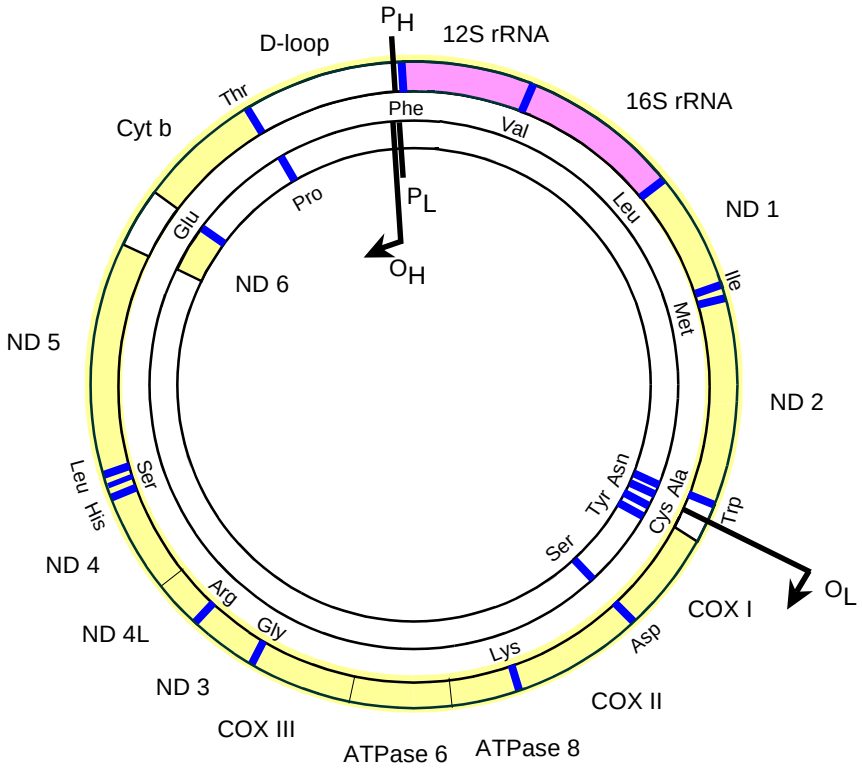

Fig. 2. The human mitochondrial genome encodes 13 subunits of respiratory chain complexes: seven subunits (ND 1–6 and 4L) of complex I, cytochrome b (Cyt b) of complex III, the COX I–III subunits of cytochrome oxidase or complex IV, and the ATPase 6 and 8 subunits of F_OF_1 ATP synthase. MtDNA also encodes 12S and 16S rRNA genes and 22 tRNA genes. The abbreviated amino acid names indicate the corresponding amino acid tRNA genes. The outer strand is heavy-chain DNA and the inner one light-chain DNA. O_H and O_L are the replication origins of the light and heavy chain, respectively, while P_H and P_L indicate the transcription sites.

2.2.2. Special features of mitochondrial genetics

The cytoplasmic location of mtDNA and the high copy number contribute to certain unique features of mitochondrial genetics. First, mtDNA is maternally inherited. Second, mtDNA genes have a much higher mutation rate than nuclear DNA genes. Third, mitochondria undergo replicative segregation at cell division. Fourth, many of the pathogenic mtDNA mutations are heteroplasmic. For expression of a disease it is required that a certain threshold level of mutant mtDNA should be exceeded. Fifth, somatic mtDNA mutations accumulate in post-mitotic tissues with age, reducing the ATP generating capacity.

2.2.2.1. Maternal inheritance

MtDNA is maternally inherited. The mammalian egg contains about 100,000 molecules of mtDNA, while the sperm contains of the order of 100–1500 mtDNAs (Chen *et al*. 1995b, Manfredi *et al*. 1997). Sperm mitochondria enter the egg during fertilization but they appear to be lost early in embryogenesis, soon after fertilization, between the two-cell and four-cell stages (Manfredi *et al*. 1997). Paternal mtDNA could not be detected in human neonates born after in vitro fertilization by intracytoplasmic sperm injection (Danan *et al*. 1999). This could be due either to destruction of sperm mitochondria or to impaired replication of sperm mtDNA in the cells (Manfredi *et al*. 1997). However, the presence of paternal mtDNA has been shown at the blastocyst stage in some abnormal (polyploid) human embryos generated by in vitro fertilization and intracytoplasmic sperm injection techniques (St John *et al*. 2000).

2.2.2.2. High mutation rate

Mitochondria seem to lack an efficient DNA repair system (Bogenhagen 1999). Moreover, protective proteins such as histones are missing and mtDNA is physically associated with the inner mitochondrial membrane, where highly mutagenic oxygen radicals are generated as by-products of OXPHOS (Richter 1988). Furthermore, abnormal mitochondrial metabolism may accelerate the rate of mtDNA mutation (Lightowlers *et al*. 1997). These unique features are probably the cause of the about 10 to 17 times faster accumulation of polymorphisms in mtDNA than in nuclear DNA (Neckelmann *et al*. 1987, Wallace *et al*. 1997a). The hypervariable sequences in the D-loop evolve even more rapidly than the coding regions (Howell *et al*. 1996).

2.2.2.3. Replicative segregation

Each cell has hundreds of mitochondria, each containing 2 to 10 copies of mtDNA molecules. Normally all mtDNAs in a cell are identical, a condition known as homoplasmy. At cell division, the mitochondria and their genomes are randomly distributed to the daughter cells, a process known as replicative segregation. When a mutation arises in mtDNA, it creates an intracellular mixture of mutant and normal molecules, a condition known as heteroplasmy. Despite the high mtDNA copy number in mature oocytes and the relatively small number of cell divisions in the female germline, mtDNA sequence variants segregate rapidly between generations (Poulton *et al*. 1998). This has been attributed to a genetic bottleneck.

A major component of the mtDNA bottleneck occurs by the time that oocytes are mature (Jenuth *et al*. 1996, Marchington *et al*. 1998). Jenuth *et al*. (1996) have estimated that in oogenesis the effective number of segregating units for mtDNA is approximately 200 in mice. The model used by Jenuth *et al*. (1996) assumes that the variance in genotypic ratios of the progeny or developing oocytes is caused by an identical random-sampling event that occurs during each of the 15 or so cell divisions during the later stages of oogenesis (i.e. repeated selection), in contrast to a more dramatic reduction in segregating units during a briefer period (i.e. single selection). On the basis of single selection model it is estimated that the most probable bottleneck size is 1–31 segregating units in humans (Marchington *et al*. 1998).

Both estimations of the number of segregating units are far below the number of mtDNA molecules in a cell, suggesting that there is first a restriction in the quantity of mtDNA to be transmitted, followed by amplification, and thereby constituting a genetic bottleneck (Poulton *et al*. 1998).

2.2.2.4. Threshold effect

Many but not all pathogenic mtDNA mutations are heteroplasmic. The phenotype is normal until a critical proportion of mutant mtDNA is present within the tissue and the threshold for genotype expression is exceeded (Wallace *et al*. 1997a). This threshold varies for different types of mtDNA mutation and is about 60% for deleted mtDNA (Hayashi *et al*. 1991). For the mutation 8344A>G, which causes the syndrome of myoclonic epilepsy and ragged-red fibers, the threshold level is about 85% mutated DNA (Chomyn 1998). Once this is exceeded, large changes in the phenotype can be observed with minor increases in the proportion of the mutant mtDNA.

Different phenotypes associated with the same genotype are determined mainly by the localized concentration and distribution of the mutation in affected tissues (Petruzzella *et al*. 1994). Furthermore, different tissues have different dependences on oxidative phosphorylation for normal function. Organs with the highest ATP requirements and the lowest regenerative capacities, such as the brain, heart and skeletal muscle, are the most sensitive to the effects of pathogenic mtDNA mutations (Wallace 1994, 1995).

2.2.2.5. Age-related somatic mtDNA mutations

Oxygen free radicals damage mtDNA, causing oxidative modifications of DNA bases, base substitutions and rearrangements. The cumulative accumulation of these somatic mutations during life may cause a bioenergetic deficit leading to cell death, or apoptosis, and normal ageing (Trounce *et al*. 1989, Simonetti *et al*. 1992, Ozawa 1995). In addition to the ageing or senescence process somatic mtDNA mutations may be important for determining the onset and progression of mtDNA diseases. Most inherited mutations are insufficient to suppress mitochondrial OXPHOS below the expression threshold and thus it is the accumulation of somatic mutations in postmitotic tissues that exacerbates the inherited OXPHOS defect and ultimately leads to phenotypic expression (Wallace *et al*. 1992, Wallace 1995).

Oxidative stress has been implicated in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. There is much evidence of increased oxidative stress and free radical damage in the substantia nigra in patients with Parkinson's disease, and there is also evidence for a defect in mitochondrial energy production, and especially reduced complex I activity, in the substantia nigra (Schapira 1999a).

2.3. Mitochondrial DNA sequence variation in human populations

The average number of base pair differences between two human mitochondrial genomes is estimated to be from 9.5 to 66 (Zeviani *et al*. 1998). The high mutation rate has resulted in the accumulation of a wide range of neutral, population-specific base substitutions in mtDNA. These have accumulated sequentially along radiating maternal lineages that have diverged approximately on the same time scale as human populations have colonized different geographical regions of the world. Thus the women that migrated out of Africa into the different continents about 130,000 years before present (YBP) harboured mtDNA mutations which today are seen as high-frequency, population-specific mtDNA polymorphisms creating groups of related mtDNA haplotypes, or haplogroups (Torroni & Wallace 1994, Wallace 1995).

2.3.1. Mitochondrial DNA haplogroups

The D-loop is the most variable region in the mitochondrial genome, and the most polymorphic nucleotide sites within this loop are concentrated in two 'hypervariable segments', HVS-I and HVS-II (Wilkinson-Herbots *et al*. 1996). Population specific, neutral mtDNA variants have been identified by surveying mtDNA restriction site variants or by sequencing hypervariable segments in the displacement loop. Restriction analysis using 14 restriction endonucleases allows screening of 15–20% of the mtDNA sequence for variations (Chen *et al*. 1995a). The large majority of mtDNA sequence data published to date are limited to HVS-I. The comparison of sequence variations in the

HVS-I with the restriction fragment length polymorphisms (RFLP) distributed throughout mtDNA has two major objectives. First, to corroborate the reliability of the analysis of these variations, and second, to differentiate the control region variants that are phylogenetically associated with a special haplotype, and thus relatively ancient and stable, from those that are recent and have been subject to repeated mutations or back mutations (Bandelt *et al*. 1995).

The coding and classification system used for mtDNA haplogroups refers to the information provided by RFLPs and the hypervariable segments of the control region. The principal clusters of polymorphisms or haplogroups are denoted by capital letters (Torroni *et al*. 1996a, Richards *et al.* 1998). About 76% of all African mtDNAs fall into haplogroup L, defined by a HpaI restriction site gain at bp 3592 (Chen *et al* 1995a, Graven *et al.* 1995). 77% of Asian mtDNAs are encompassed within a super-haplogroup defined by a DdeI site gain at bp 10394 and an AluI site gain at bp 10397 (Ballinger *et al.* 1992, Torroni *et al*. 1993a, 1993b, Chen *et al*. 1995a, Wallace 1995). Essentially all native American mtDNAs fall into four haplogroups, A–D (Torroni & Wallace 1994). Haplogroup A is defined by a HaeIII site gain at bp 663, B by a 9 bp deletion between bp 8271 to bp 8281, C by a HincII site loss at bp 13259, and D defined by an AluI site loss at bp 5176 (Torroni *et al.* 1993b, Torroni & Wallace 1994, Wallace 1995). Ten haplogroups encompass almost all mtDNAs in European populations (Torroni *et al*. 1996a).

2.3.1.1. European mtDNA haplogroups

Classical polymorphic markers (i.e. blood groups, protein electromorphs and HLA antigenes) have suggested that Europe is a genetically homogeneous continent with a few outliers such as the Saami, Sardinians, Icelanders and Basques (Cavalli-Sforza *et al.* 1993, Piazza 1993). The analysis of mtDNA sequences has also shown a high degree of homogeneity among European populations, and the genetic distances have been found to be much smaller than between populations on other continents, especially Africa (Comas *et al*. 1997).

The mtDNA haplogroups of Europeans are surveyed by using a combination of data from RFLP analysis of the coding region and sequencing of the hypervariable segment I. About 99% of European mtDNAs fall into one of ten haplogroups: H, I, J, K, M, T, U, V, W or X (Torroni *et al*. 1996a). Each of these is defined by certain relatively ancient and stable polymorphic sites located in the coding region (Torroni *et al.* 1996a).

In a phylogenetic analysis of European haplogroups (Torroni *et al*. 1996a) the first subdivision is based on the presence or absence of a DdeI site at bp 10394. A lack of that site is common to haplogroups H, T, U, V, W and X. Haplogroup H, which is defined by the absence of a AluI site at bp 7025, is the most prevalent, comprising half of all Europeans (Torroni *et al*. 1996a, Richards *et al.* 1998). Haplogroups J, T, K and U are also common, and are shared by all European populations. Haplogroup J is defined by a BstNI site loss at bp 13704, and haplogroup T by a BamHI site gain at bp 13366. Haplogroups K and U both have a transition 12308A>G, but haplogroup U lacks a DdeI site at bp 10394. The remaining haplogroups I, V, W and X are less common. Haplogroup V is defined by a NlaIII site loss at bp 4577, while haplogroups I, X and W are not so

clearly distinguished from each other. Haplogroups I and W both have an AvaII site gain at bp 8249, but haplogroups I and X both lack a DdeI site at bp 1715. Also, haplogroup W has a HaeIII site loss at bp 8994, and haplogroup I has an AluI site gain at bp 10028 (Torroni *et al*. 1996a).

Six of the European haplogroups (H, I, J, K, T and W) are essentially confined to European populations (Torroni *et al*. 1994, 1996a), and probably originated after the ancestral Caucasoids became genetically separated from the ancestors of the modern Africans and Asians. Haplogroup U, although much more prevalent in Europe, is also found at a low frequency in the Japanese, the North-African Berber population, the Ethiopians and the Senegalese (Ozawa 1995, Torroni *et al*. 1996a, Passarino *et al*. 1998, Macaulay *et al*. 1999).

The European haplogroups have recently been defined in a more accurate manner, and their phylogenetic tree has been partly reconstructed. Specifically, haplogroup U has been enlarged to include haplogroup K as a subcluster (Hofmann *et al*. 1997, Richards *et al*. 1998, Macaulay *et al*. 1999).

It is estimated that haplogroups H, J, T and V may be of relatively recent origin, 8,000–30,000 years (Torroni *et al*. 1996a), and this supports the hypothesis that they originated after the genetic and geographical separation of the ancestral Caucasoids from the ancestors of modern Africans and Asians. On the other hand, haplogroup U appears to be much older than the others, with an estimated age of 51,000–67,000 yr (Torroni *et al*. 1996a), raising the possibility that it may have originated in Africa and subsequently expanded into the Middle East and Europe.

2.3.1.2. The Finns and their mtDNA haplogroups

The oldest archaeological evidence of settlement in Finland dates back to approximately 9,000 YBP, but the origin of the people is unknown. They were probably small groups which survived on hunting, fishing and gathering, and came from Europe and the southeast, possibly from the Ural Mountains area. They could be the ancestors of the Saami people (Pitkänen 1994). Finland has later received population influences from several other sources, but all the groups of settlers have been small (Pitkänen 1994).

The major route of colonization was over the Gulf of Finland, but settlement via both western and eastern routes has also occurred. Evidence for contacts with the Volga region can be found amongst archaeological remains of the Suomusjärvi culture dating from around 6000 YBP. Soon after that the Comb Ceramic Culture was introduced into Finland, and this was associated with the arrival of new, Finno-Ugric-speaking settlers. The native, linguistically unknown population already living in Finland partly acculturated to these settlers. The Battle-Axe Culture of Central and Northern Europe was introduced into Southwest Finland via the Baltic around 4,000 YBP and was incorporated into the native population. Finland had been divided into two cultures. The Combed Ware Culture adopted the agriculture, but north of the line Vaasa-Viipuri the Comb Ceramic Culture retained its the traditional hunting (Pitkänen 1994).

Finnish belongs to the Finno-Ugric group of languages, which form the largest non-Indo-European language group in Europe. Finno-Ugric languages are also spoken in Estonia, Karelia, Latvia, Lithuania and Hungary. The Saami languages and certain minor languages spoken in northern Russia are more distant members of the same group (Korhonen 1991).

Religious and linguistic barriers, geopolitical position and low population density have ensured that the Finns have remained in comparative local and national isolation, and therefore the spectrum of inherited diseases is different from that in neighbouring populations. Many recessive diseases are unique to Finland, whereas other diseases that are common elsewhere are rare (Norio *et al*. 1973, de la Chapelle 1993, Peltonen *et al.* 1995).

Previous studies on Finnish mtDNA variation have showed high homogeneity and a clear Caucasoid pattern of polymorphisms (Vilkki *et al*. 1988, Pult *et al*. 1994, Sajantila *et al*. 1996, Torroni *et al*. 1996a). These studies have indicated a close relationship between the Finns and the other Europeans. About 40% of the Finns belong to haplogroup H, which is the most common among Europeans, comprising half of each population (Torroni *et al*. 1996a, Richards *et al*. 1998). Other common haplogroups among the Finns include haplogroup U, with a frequency of 16%, haplogroup J, 14%, and haplogroup T, 6%. The remaining haplogroups (V, W, X, K, I, M) are less common, each with a frequency of 2–4 % (Torroni *et al*. 1996a). The Saami, however, have a mitochondrial gene pool which is distinct from that of other European populations (Sajantila *et al*. 1995, Lahermo *et al*. 1996).

An apparent discrepancy exists between the lack of a linquistic relation between the Finns and the other Europeans and the similarity between their mtDNA gene pools. On the other hand, the Saami speak a Finno-Ugric language and are genetically different from the surrounding European populations including the Finns. Based on this discrepancy it has been suggested that a language change has taken place in which an Indo-European population has adopted a Finno-Ugric language from the Saami without any substantial exchange of genetic material (Sajantila & Pääbo 1995).

As late as the 12th century only a minor part of Finland, the southwestern coast, the provinces of Häme, Southern Satakunta, Åland and the surroundings of Ladoga in eastern Finland, had permanent settlement. By the 16th century there was consistent settlement in southern Finland and a string of villages existed along the coasts of Ostrobothnia and its riversides, reaching the northern part of the Gulf of Bothnia. The major permanent colonization of Northern Ostrobothnia and Kainuu took place after the 16th century, when mainly people from Savo migrated under Crown orders or because of an increase in population and the adoption of agriculture. The Saami had to move to the north, but most of them had acculturated with the settlers (Pitkänen 1994).

2.3.2. Mitochondrial DNA as a phylogenetic tool

DNA sequence variation can be used to construct a phylogenetic tree, or several alternative trees arranged in a network, to display the evolutionary relationships between individual sequences. The tree will thus tell the phylogenetic story of a given gene. The

structure of this gene tree contains information which, in conjunction with a calibrated mutation rate for the DNA sequences under study, can be used to estimate a time-scale for events in human prehistory. Moreover, the geographical distribution of the lineages on a tree or network can be used to detect prehistoric movements from one region to another (Avise 1986).

Traditional tree-building methods are unsatisfactory when applied to human mtDNA data because of homoplasy, parallel mutation events or reversals (Bandelt *et al*. 1995). Maximum parsimony, maximum likelihood, and distance methods almost invariably fail to form nested sets of haplotypes, but instead exhibit incompatibility between pairs of characters. Excoffier and Smouse (1994) have calculated that the number of equally parsimonious trees for an RFLP data set of just 56 haplotypes exceeded 10^9.

Bandelt *et al*. (1995) argue that mtDNA data are best analyzed by a network based on a median algorithm. This approach distinguishes between unresolvable character conflicts, leaving a compact, intelligible representation of plausible solutions. The unmodified median network generated by partitioning the groups of haplotypes character by character is guaranteed to include all the most parsimonious trees. High rates of homoplasy might even lead to a single haplotype being independently derived from the same ancestor along different routes.

An approach using networks rather than trees has many advantages (Bandelt *et al.* 1995). The median network generated by using a table of binary data contains the same information as the table, yet in a much more comprehensible form. Furthermore, the network can predict haplotypes and tell us where homoplasy is located, which sites have frequently undergone mutation, where a consensus sequence is, whether recombination is likely to have occurred, where to look for sequence errors, which haplogroups may be distingquished, and so on. Since the median network harbours all the most parsimonious trees for the input data, it yields a more concise picture of the data than an exhaustive list of all maximum parsimony trees.

2.4. Mutations in mtDNA as causes of diseases

The biochemical and genetic complexity of the respiratory chain accounts for the extraordinarily wide range of clinical presentations of mitochondrial disorders (Table 1). In general, the organs with the highest aerobic demand and the lowest regenerative capacity, such as the brain, heart and skeletal muscle, are the ones that are most severely involved, but virtually any organ or tissue in the body can be affected, including the gastrointestinal tract (Bardosi *et al*. 1987), liver (Mazziotta *et al*. 1992), kidney (Manouvrier *et al.* 1995) and the endocrine systems (Shoffner *et al*. 1995a, Manouvrier *et al*. 1995). Each tissue can be affected alone, e.g. pure mitochondrial myopathy, encephalopathy or cardiomyopathy, or more often in combination, e.g. mitochondrial encephalomyopathy.

The two main biochemical features in most mitochondrial diseases are respiratory chain deficiency and lactic acidosis. Morphologically, patients often display ragged-red fibers (RRF) in the muscle due to the accumulation of structurally abnormal subsarcolemmal mitochondria (Rowland *et al*. 1991).

Some mitochondrial syndromes are well established, having a certain molecular genetic background and are nosologically defined entities. There are many disorders, however, that are defined on the basis of morphological or biochemical findings and still lack a molecular genetic definition. In addition, overlap syndromes and non-specific phenotypes mean that clinical data are not sufficient to provide a systematic classification of mitochondrial diseases (Zeviani *et al*. 1996).

Genetically, mitochondrial diseases can be divided into three groups: those characterized by the presence of sporadic or maternally inherited mtDNA mutations, those characterized by the association of mtDNA abnormalities with mendelian transmission of the trait, i.e. disorders believed to be due to mutations in nuclear genes that control mitochondrial biogenesis, and those that lack a mtDNA defect but are thought on the basis of biochemical findings to be caused by mutations in nuclear genes.

Table 1. Common clinical manifestations of mitochondrial disorders.

Neurological manifestations	Systemic manifestations
Ptosis, ophthalmoplegia	Metabolic acidosis
Optic neuropathy	Nausea and vomiting
Sensorineural hearing loss	Cardiomyopathy
Headache	Cardiac conduction defects
Seizures	Short stature
Stroke	Pigmentary retinopathy
Dementia	Hepatopathy
Myelopathy	Nephropathy
Peripheral neuropathy	Diabetes mellitus
Myopathy	Exocrine pancreatic dysfunction
Exercise intolerance, fatigability	Hypoparathyroidism
Ataxia	Intestinal pseudo-obstruction
Myoclonus	Cataract
Dystonia	Pancytopenia
	Sideroblastic anaemia

2.4.1. Point mutations

Many but not all pathogenic mtDNA point mutations are heteroplasmic. When the proportion of the mutant genome exceeds a certain threshold, the deleterious effects of the mutation will no longer be complemented by the coexisting wild-type mtDNA and will be expressed phenotypically as a cellular dysfunction leading to disease (Wallace *et al*. 1997a). Phenotypic expression will depend on the nature of the mutation, its tissue distribution and the relative reliance of each organ system on the mitochondrial energy supply (Schon *et al*. 1997). The influence of nuclear genes, coexisting mitochondrial

polymorphisms, the age and sex of the individual and environmental factors may also play an important, although poorly understood, role in the phenotypic expression of mtDNA point mutations (Wallace *et al*. 1997a).

Mitochondrial DNA point mutations are maternally inherited and can occur in rRNA or tRNA genes, or in genes coding for proteins of respiratory chain complexes. Although more than 50 deleterious point mutations have been identified to date, four mutations are by far the most frequent (Wallace *et al*. 1997a). These are the 3243A>G 'MELAS', the 8344A>G 'MERRF', the 8993T>G 'NARP' and the 11778G>A 'LHON' mutations. Others are found less often, while still others have been described only in single individuals or families.

The investigation of pathogenic mitochondrial DNA mutations has revealed a complex relation between patient genotype and phenotype (Schon *et al*. 1997). For unknown reasons, some mtDNA mutations lead to specific clinical manifestations, an example being 3243A>G, causing the MELAS syndrome, one of the classic mitochondrial syndromes. Moreover, the MELAS syndrome has a high prevalence in the adult population, suggesting that mitochondrial disorders constitute one of the largest diagnostic categories of neurogenetic diseases (Majamaa *et al*. 1998).

2.4.1.1. Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS)

Pavlakis *et al*. (1984) described two patients with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes. These two patients and nine others reported earlier (Gardner-Medwin *et al*. 1975, Shapira *et al*. 1975, Koenigsberger *et al*. 1976, Hart *et al*. 1977, Askanas *et al*. 1978, Skoglund 1979) presented with normal early development, short stature, seizures and alternating hemiparesis, hemianopia or cortical blindness. All these eleven patients had ragged-red fibers in their skeletal muscle, and most had elevated blood lactate concentrations. The cause of this syndrome was uncertain, but Pavlakis *et al*. (1984) presumed that there was an underlying biochemical defect in the mitochondria.

Genetics of the MELAS syndrome. MELAS is most commonly associated with a heteroplasmic point mutation in the $tRNA^{Leu(UUR)}$ gene, an A to G transition at position 3243 (Goto *et al*. 1990), and approximately 80% of the cases of MELAS harbour this mutation (Ino *et al*. 1991, Goto *et al*. 1990, 1991, Goto 1995). Another 7–15% of MELAS patients have been found to have a T to C transition mutation at position 3271 in the same $tRNA^{Leu(UUR)}$ gene (Goto *et al*. 1991, Togunaga *et al*. 1993, Marie *et al*. 1994, Goto 1995, Tarnopolsky *et al*. 1998). Other MELAS-associated point mutations have also been reported in the $tRNA^{Leu(UUR)}$ gene, at np 3252 (Morten *et al*. 1992), np 3256 (Moraes *et al*. 1993b, Sato *et al*. 1994) and np 3291 (Goto *et al*. 1994) (Figure 3). In addition, mutations elsewhere in mtDNA can give rise to a MELAS-like phenotype, including the 8344A>G MERRF mutation. Some tRNA mutations, such as the 3256C>T in $tRNA^{Leu(UUR)}$ and 7512T>C in $tRNA^{Ser(UCN)}$, can give rise to a MERRF/MELAS overlap syndrome (Moraes *et al*. 1993a, 1993b, Nakamura *et al*. 1995, Sato *et al*. 1994). One mis-sense mutation in a polypeptide-coding gene, at nt 11084 in ND4, has also been

associated with MELAS (Lertrit *et al*. 1992), but there is evidence that it is a rare polymorphism (Ozawa *et al*. 1991, Sakuta *et al*. 1993) rather than a disease-causing mutation.

The mitochondrial $tRNA^{Leu(UUR)}$ gene appears to be an aetiological hot spot for mtDNA mutations (Moraes *et al*. 1993b). In addition to mutations causing the MELAS syndrome, several others are located in this gene. Mutations at nt 3250 (Goto *et al*. 1992), nt 3251 (Sweeney *et al*. 1993) and nt 3302 (Bindoff *et al*. 1992) are associated with myopathy, while mutations at nt 3260 (Zeviani *et al*. 1991) and nt 3303 (Silvestri *et al*. 1993) are thought to cause both myopathy and cardiomyopathy. A 2 bp nucleotide pair deletion in the $tRNA^{Leu(UUR)}$ gene involving positions 3271 to 3273 is expressed in severe intracerebellar calcifications with complex neurological manifestations (Shoffner *et al*. 1995a).

All mutations associated with the MELAS syndrome are heteroplasmic, with a high proportion (>80%) of mutant mtDNAs in muscle (Goto *et al*. 1992, Ciafaloni *et al*. 1992). It has been suggested that the onset of the MELAS syndrome is precipitated by proportions of mutant genome in muscle exceeding a threshold of about 60% (Miyabayashi *et al*. 1992). The percentage is always lower in blood than in muscle (Ciafaloni *et al*. 1992, Inui *et al*. 1992), but there is no correlation between the percentage of blood heteroplasmy and the phenotypic expression of the disorder (Tarnopolsky *et al*. 1998).

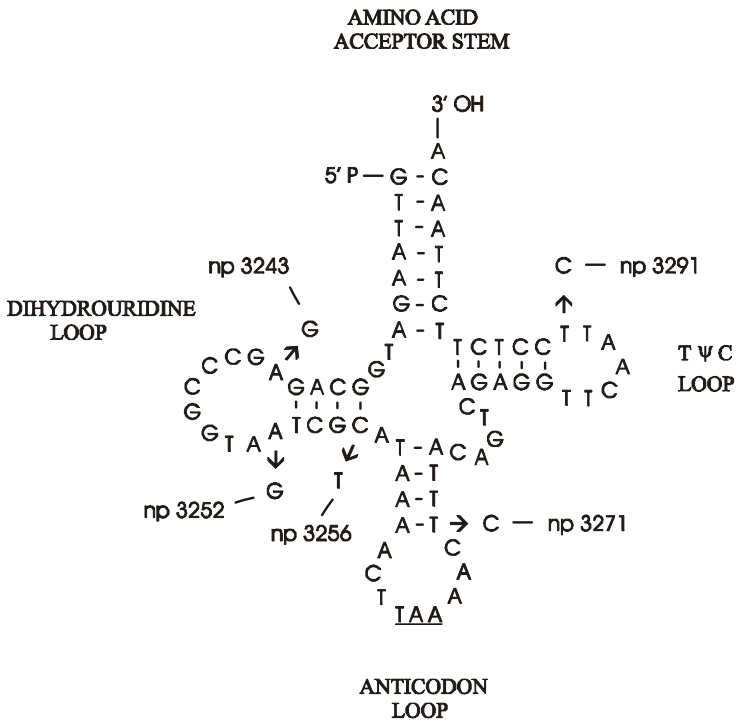

Fig. 3. $tRNA^{Leu(UUR)}$. The nucleotide transitions 3243A>G, 3252A>G, 3256C>T, 3271T>C and 3291T>C are associated with the MELAS syndrome.

Phenotype of the MELAS syndrome. The genotype to phenotype correlation of the 3243 A>G mutation is fairly loose, since not all patients with this mutation have the full-blown MELAS syndrome. For instance, the 3243 A>G mutation has been detected in several patients and families with maternally inherited Kearns-Sayre or chronic progressive external ophthalmoplegia (CPEO) syndromes, mitochondrial encephalomyopathies with or without ragged-red fibers, isolated myopathy, cardiomyopathy, or pedigrees with maternally inherited diabetes mellitus and deafness (Goto *et al*. 1990, Hammans *et al*. 1991, Ciafaloni *et al*. 1992, Reardon *et al*. 1992, van den Ouweland *et al*. 1992, Hirano *et al*. 1992, Mariotti *et al*. 1995).

The MELAS syndrome is first suspected when the patient has focal or generalized seizures, recurrent migraine headaches, vomiting, and stroke (Pavlakis *et al*. 1984). Recurrent strokes, perhaps the sole exclusive clinical manifestation, may be a late feature of the disease in the majority of cases (Morgan-Hughes *et al*. 1995). The posterior cerebral hemispheres are particularly vulnerable and the stroke commonly causes hemianopia and cortical blindness. The aetiology of the stroke is unknown.

Other manifestations in MELAS patients may include a ragged-red fiber myopathy, lactic acidaemia, sensorineural hearing loss, short stature, dementia, pigmentary retinal degeneration, hypertrophic cardiomyopathy, cardiac conduction abnormalities, renal failure, diabetes mellitus and basal ganglia calcifications (Kobayashi *et al*. 1990, Goto *et al*. 1990, 1992, Hammans *et al*. 1991, Ciafaloni *et al*. 1992, Moraes *et al*. 1992, Inui *et al*. 1992, Hirano *et al*. 1992, Togunaga *et al*. 1993, Sakuta *et al*. 1993, de Vries *et al*. 1994, Manouvrier *et al*. 1995).

The phenotype associated with the 3271T>C mutation is very similar, but expression may occur at a later age (Sakuta *et al*. 1993, Tarnopolsky *et al*. 1998).

The highest levels of mutant mtDNA occur in patients with an early onset of the disease (Morgan-Hughes *et al*. 1995). Furthermore, the proportion of mutant mtDNA is generally higher in patients with recurrent strokes than in those without strokes (Ciafaloni *et al*. 1992). Differences in the proportions of mutant mtDNA may not be the sole determinants of disease expression, however, and additional genetic mechanisms may be involved in defining the range of clinical and biochemical phenotypes associated with this aberrant mitochondrial genome (Morgan-Hughes *et al*. 1995).

Biochemical pathophysiology of the 3243A>G mutation. The mechanism by which the 3243 A>G transition leads to disease remains unclear. It alters a highly conserved dihydrouridine stem region in $tRNA^{Leu(UUR)}$ (Goto *et al*. 1990), and thus may possibly alter the stability and functioning of the tRNA molecule. Furthermore, the mutation is located within a 13-nucleotide segment which binds the factor (mTERF) that promotes termination of transcription at the 16S rRNA/$tRNA^{Leu(UUR)}$ gene boundary (Hess *et al*. 1991, Daga *et al*. 1993). Cybrids harbouring 3243A>G accumulate a precursor RNA 19, which corresponds to a transcript containing the contiguous 16S rRNA + $tRNA^{Leu(UUR)}$ + ND1 genes (King *et al*. 1992, Koga *et al*. 1995). Furthermore, decreased 5' and 3' processing of $tRNA^{Leu(UUR)}$ has been observed in these cybrids (King *et al*. 1992).

The complex I subunits ND6 and ND3 are mtDNA encoded polypeptides with the highest proportion of leucine residues translated by means of the UUR codon. Complex I deficiency is the most common respiratory chain defect observed in patients with the 3243A>G mutation (Goto *et al*. 1992). A deficiency in complex I or IV (Kobayashi *et al*. 1991, Chomyn *et al*. 1992, King *et al*. 1992, Dunbar *et al*. 1996) will lower proton

pumping, reduce the electrochemical potential gradient across the mitochondrial inner membrane (Moudy *et al*. 1995, James *et al*. 1996) and lead to decreased respiration and a lower rate of ATP synthesis.

Inhibition of the respiratory chain increases the production of reactive oxygen species. The severity of complex I deficiency has been correlated with the production of superoxide anions and the induction of mitochondrial Mn superoxide dismutase (Pitkänen & Robinson 1996). Chronic generation of reactive oxygen species can result in oxidative damage to mitochondrial and cellular proteins, lipids and nucleic acids (Wallace & Melov 1998, Wallace 1999). Moreover, increased oxidative stress and decreased energy levels could activate the mitochondrial permeability transition pore, leading to apoptosis (Petit *et al*. 1996, Green & Reed 1998, Zoratti & Szabo 1995).

2.4.1.2. Other mtDNA point mutations

Together with the MELAS syndrome, Leber's hereditary optic neuropathy (LHON), myoclonic epilepsy with ragged-red fibers (MERRF) and neurogenic weakness, ataxia, and retinitis pigmentosa (NARP) are by far the most frequent mitochondrial diseases caused by mtDNA point mutations.

Several other point mutations have been detected in single patients or in pedigrees affected with various phenotypes (Servidei 1997). For instance, myopathy and cardiomyopathy is associated with a number of tRNA gene mutations (Zeviani *et al.* 1991, Servidei 1997). One interesting phenomenon is aminoglycoside-induced or spontaneous, non-syndromic progressive deafness, which has been associated with a homoplasmic 1555A>G transition in the 12S RNA gene. It has been postulated that the 1555 mutation elongates the region where the tRNA binds to the ribosome, thus facilitating the binding of aminoglycosides and thereby potentiating their effects on the fidelity of the translation of the mRNA (Prezant *et al.* 1993). The cochlear cell death observed in these patients is thought to be due to misreading of mRNA during mitochondrial protein synthesis in this highly energy-dependent organ.

Leber's hereditary optic neuropathy (LHON). Leber's hereditary optic neuropathy is a maternally inherited form of blindness predominantly affecting men and with onset in the second or third decade of life. Painless loss of vision begins in the central visual field, usually in one eye, and subsequently affects the other eye weeks or months later. Recovery of vision has been reported in some patients (Mackey & Howell 1992) and seems to depend on the particular pathogenic mtDNA mutation present.

LHON was the first mitochondrial disease to be defined at the molecular level. Wallace *et al*. (1988a) found a G>A transition at position 11778 in the ND4 gene of complex I in pedigrees with maternally inherited LHON, and since then 18 novel mis-sense mutations have been associated with the disease. Only a few of them are 'primary' mutations: 14459G>A in the ND6 gene (Jun *et al*. 1994, Shoffner *et al*. 1995b), 11778G>A in the ND4 gene (Wallace *et al*. 1988a), 3460G>A in the ND1 gene (Howell *et al*. 1991, Huoponen *et al*. 1991, 1993) and 14484T>C in the ND6 gene (Johns *et al.* 1992, Mackey & Howell 1992). The remaining mutations may either increase the probability of expressing the phenotype or are polymorphisms frequently linked to one of

the clinically important mutations rather than contributing substantially to the potential for developing blindness. The 3460G>A mutation is associated with a variety of mtDNA haplotypes, demonstrating that this mutation has arisen independently on multiple occasions, each resulting in LHON (Brown *et al*. 1995, Howell *et al*. 1995). Similarly, all 14459G>A pedigrees have different mtDNA haplotypes (Jun *et al*. 1994, Shoffer *et al*. 1995b). By contrast, most patients harbouring the 11778G>A or 14484T>C mutation belong to a European haplogroup J (Torroni *et al*. 1994, 1997, Brown *et al*. 1997, Lamminen *et al*. 1997). This implies that the mtDNA haplogroup J may contribute to expression of the LHON phenotype in the presence of the 11778G>A or 14484T>C mutation. Furthermore, several additional mutations of unknown pathogenic significance have been found, and LHON may be a result of the cumulative effects of such 'secondary' mutations (Mackey & Howell 1992).

Analyse of the LHON mutations have provided a new insight into the phenotypic expression of mtDNA mutations. It appears that specific mutations may act together, perhaps synergistically, to produce the LHON phenotype (Howell *et al*. 1991, Mackey & Howell 1992, Brown *et al*. 1992), in addition to which the mtDNA haplogroup J may contribute to its expression (Torroni *et al*. 1996b).

Myoclonus epilepsy with ragged-red fibers (MERRF). MERRF is a maternally inherited neuromuscular disorder characterized by progressive myoclonus, epilepsy, muscle weakness and wasting, cerebellar ataxia, deafness and dementia (Wallace *et al*. 1988b). The most commonly observed mutation is an A>G transition at nt 8344 in the $tRNA^{Lys}$ gene in mtDNA (Shoffner *et al*. 1990), and a second mutation has been reported in the same gene, at position 8356 (Silvestri *et al*. 1992, Zeviani *et al*. 1993). The genotype to phenotype correlation with the 8344A>G mutation is stronger than in the case of the other mtDNA mutations (Silvestri *et al*. 1992). There is a positive correlation between the severity of the disease, age at onset, mtDNA heteroplasmy (Chinnery *et al*. 1997) and reduced activity of the respiratory chain complexes I and IV in skeletal muscle (Boulet *et al*. 1992).

Neurogenic weakness, ataxia and retinitis pigmentosa (NARP). NARP is a maternally inherited, adult-onset syndrome associated with a heteroplasmic T>G transversion at position 8993 in the ATPase 6 subunit gene (Holt *et al*. 1990). A transition 8993T>C was described later in other patients with NARP (de Vries *et al*. 1993). RRFs are absent in a muscle biopsy. The degree of heteroplasmy is correlated with the severity of the disease, so that when the proportion of mutant mtDNA is more than 95%, patients show clinical, neuroradiological and neuropathological findings of Leigh syndrome (see chapter 2.5). Hence it is called maternally inherited Leigh syndrome (MILS) (Tatuch *et al*. 1992). The two phenotypes, NARP and MILS, may coexist in the same family. Impairment of ATP synthesis has been reported in cell cultures harbouring the 8993T>G mutation, possibly because of defective assembly of the enzyme complex (Houstek *et al*. 1995).

2.4.2. Large-scale rearrangements of mtDNA

Large-scale rearrangements of mtDNA can be either mtDNA deletions or, more rarely, duplications. Both types of mutation are heteroplasmic, and they can occasionally exist simultaneously in patient tissues (Ballinger *et al*. 1992). More than 120 mtDNA deletions have been identified, most within direct repeats of 3–13 nucleotides in length (Kogelnik *et al*. 1998). The most common large-scale deletions are between a 13-base pair direct repeat from nt 8470 to nt 8482 in the ATPase8 gene and from nt 13447 to nt 13459 in the ND5 gene (Moraes *et al*. 1989). The resulting 4997 bp deletion (the common deletion) has occurred independently over 200 times and accounts for perhaps 50% of ocular myopathy patients (Kogelnik *et al*. 1998). Because virtually all deletions eliminate at least one tRNA, it is likely that they result in a generalized translational defect. Deletions are usually sporadic, non-transmittable mutagenic events (Brown & Wallace 1994).

The three main clinical phenotypes associated with large-scale mtDNA deletions are the Kearns-Sayre syndrome, chronic progressive external ophthalmoplegia (CPEO) and the Pearson syndrome (Zeviani *et al*. 1988, Moraes *et al*. 1989, Poulton *et al*. 1989, Rötig *et al*. 1990, McShane *et al*. 1991). The Kearns-Sayre syndrome is characterized by an invariant triad of PEO, pigmentary retinopathy and onset before 20 years of age. Frequent additional symptoms are a progressive cerebellar syndrome, heart block and increased protein content in the cerebrospinal fluid. CPEO is characterized by bilateral ptosis and ophtalmoplegia, frequently associated with variable degrees of proximal muscle weakness and wasting, and exercise intolerance. The Pearson bone marrow-pancreas syndrome is a rare disorder of early infancy characterized by sideroblastic anaemia with pancytopaenia and exocrine pancreatic insufficiency. Infants surviving into childhood may develop the clinical features of Kearns-Sayre syndrome (McShane *et al*. 1991, Rötig *et al*. 1990).

Partial duplications of mtDNA has been detected in ocular myopathy and Pearson's syndrome patients, although duplications are much rarer in these patients than deletions. Duplications can be sporadic (Poulton *et al*. 1991) or maternally transmitted (Rötig *et al*. 1992).

2.4.3. MtDNA genotypes conferring increased risk of disease

The high mutation rate has resulted in the accumulation of a wide range of population-specific base substitutions in mtDNA. While most of these variants are neutral, some are mildly deleterious. The latter, although they do not significantly reduce fitness, may interact with nuclear and environmental factors, predisposing individuals to an increased risk of developing neurodegenerative diseases late in life (Shoffner *et al*. 1993, Wallace 1994). Moreover, mildly deleterious polymorphisms may synergistically compromise mitochondrial function and contribute to the pathogenesis of a mitochondrial disorder (Lertrit *et al*. 1994).

It has been shown that the expression of some pathogenic mtDNA mutations depends on the mtDNA background against which they occur. The risk of expression of LHON in the presence of the primary mutation 14484T>C is eight-fold higher when this mutation

occurs in the specifically European haplogroup J (Torroni *et al*. 1996b). The 11778G>A mutation was observed in a wide range of mtDNA haplogroups but has shown an almost six-fold preferential association for haplogroup J (Torroni *et al*. 1997). The mutation 4336T>C is associated with late-onset Alzheimer disease (Shoffner *et al*. 1993, Hutchin & Cortopassi 1995), and has arisen as a single mutational event in the European haplogroup H (Torroni *et al*. 1994).

The association of a particular mtDNA sequence variant with a particular disease is not an unambiguous indicator of aetiological significance (Chinnery *et al*. 1999), however, as the mtDNA sequence may act as a surrogate marker for a nuclear gene defect, particularly in isolated populations that have experienced a marked founder effect (Heyer 1995, Jorde *et al*. 1995). Similarly, a particular mtDNA haplotype may signal, through a founder effect, a population subgroup that has inherited a group of detrimental or protective nuclear genes.

2.5. Defects in nucleo-mitochondrial signalling and nuclear gene defects

Most of the genes involved in mitochondrial functions reside in the nucleus. These include the majority of genes encoding polypeptides for respiratory chain complexes and ATP synthase, and the genes that control the biogenesis of the mitochondrial genome and mitochondrial metabolism. Mitochondrial disorders that still lack molecular genetic characterizations but are defined biochemically as defects of the respiratory chain complexes or are inherited as mendelian traits indicate the existence of mutations in nuclear genes or faulty interactions between nuclear and mitochondrial genes. Virtually any nuclear mutation that alters a pathway in intermediate metabolism, which interfaces with the mitochondria could also interact with OXPHOS gene polymorphisms and lead to disease (DiMauro 1999).

The Leigh syndrome is the most common mitochondrial encephalomyopathy of infancy or childhood (Rahman *et al*. 1996), with the characteristic neuropathology of focal, bilaterally symmetrical spongiform lesions, especially in the thalamus and brain stem regions (Dahl 1998). It is much more commonly transmitted as an autosomal recessive trait than as a maternally inherited trait, and is occasionally transmitted as an autosomal dominant or X-linked trait. The Leigh syndrome is one of the most commonly recognized disorders of mitochondrial energy production and can be regarded as one part of the spectrum of these disorders. For example, some mutations in mtDNA, such as 8993T>G or 8993T>C, when present in high proportions in affected tissues, will cause Leigh syndrome, whereas lower levels of mutant mtDNA will cause milder phenotypes (Tatuch *et al*. 1992, Santorelli *et al*. 1993). In a similar manner, mutations in some nuclear genes appear to result in Leigh syndrome, but functionally less severe mutations in the same genes give rise to different clinical presentations (Dahl 1998). The first pathogenic mutation in nuclear genes was found in two sisters with Leigh syndrome and complex II deficiency (Bourgeron *et al*. 1995). Complex II is entirely encoded by nuclear DNA, and the mutation was detected in the gene encoding the flavoprotein subunit. A few mutations have also been found in nuclear DNA encoding complex I subunits in patients with Leigh

syndrome (Loeffen *et al*. 1998, van den Heuvel *et al*. 1998, Triepels *et al*. 1999), and some have been identified in a nuclear SURF1 gene encoding a protein needed for proper COX assembly (Zhu *et al*. 1998, Tiranti *et al*. 1998).

Certain abnormalities of mtDNA have been shown to be secondary to nuclear mutations. Autosomal dominant chronic progressive external ophthalmoplegia is a genetically heterogeneous disorder, with at least three distinct genomic loci causing very similar phenotypes (Suomalainen *et al*. 1995, Kaukonen *et al*. 1996, 1999). All affected patients have multiple mtDNA deletions (Kaukonen *et al*. 1999). Furthermore, two other autosomally inherited diseases with multiple mtDNA deletions, Wolfram syndrome and a recessively inherited mitochondrial neurogastrointestinal encephalomyopathy, have been mapped to distinct nuclear regions (Barrientos *et al*. 1996, Nishino *et al*. 1999).

Friedreich's ataxia and autosomal recessive hereditary spastic paraplegia (HSP) are good examples of diseases of secondary OXPHOS deficiency. HSP is characterized by progressive weakness and spasticity of the lower limbs due to degeneration of the corticospinal axons. HSP patients have two major morphological hallmarks of mitochondrial myopathy, RRF and COX-negative fibers (Casari *et al*. 1998). HSP is a genetically heterogeneous group of neurodegenerative disorders. An autosomal recessive form is associated with mutations in the gene encoding paraplegin, a novel mitochondrial protein highly homologous to the yeast mitochondrial ATPases AFG3, RCA1 and YME1, which have both proteolytic and chaperone-like activities at the inner mitochondrial membrane (Banfi *et al*. 1999).

Friedreich's ataxia is an adolescent autosomal recessive disorder, the main clinical features of which include progressive ataxia, dysarthria, skeletal deformities, hyporeflexia, pyramidal features and hypertrophic cardiomyopathy (Schapira 1999a). Muscle biopsies from patients with Friedreich's ataxia also show typical mitochondrial changes. Genetic analysis has revealed an expanded intronic GAA repeat in the gene encoding frataxin (Koutnikova *et al*. 1997, Rötig *et al*. 1997). This frataxin deficiency results in mitochondrial iron accumulation, which probably results in oxidative stress and damage, which in turn may cause a decrease in mtDNA levels and severe deficiencies in the activities of complexes I–III (Schapira 1999b).

Wilson's disease is an inherited disorder of copper homeostasis characterized by an abnormal accumulation of copper in several tissues, particularly the liver, brain and kidney. The disease-associated gene encodes a copper-transporting P-type ATPase located in the mitochondria (Lutsenko & Cooper 1998). Mitochondrial function has not been investigated in Wilson's disease but one would predict that an accumulation of copper may promote oxidative damage and result in an OXPHOS defect predominantly affecting complex IV (Lutsenko & Cooper 1998).

2.6. Migraine

Migraine is a common neurovascular disorder characterised by attacks of severe headache and autonomic and neurological symptoms (Ferrari 1998). Ten percent of the general population (6% of men, 15% of women) are active migraineurs (Russell & Olesen 1996). From age 16 onwards, migraine is 2–3 times more frequent in women than

in men, and women around age 40 have the highest prevalence (Stewart *et al*. 1994, Abu-Arefeh & Russell 1994). Migraine and other headache syndromes should be diagnosed according to the criteria of the International Headache Society (1988). The two main types of migraine are migraine without aura, occurring in 75% of migraineurs, and migraine with aura. One-third of migraineurs experience both types of attack during their lifetime (Russel *et al.* 1995, Bille 1997).

There is some experimental evidence for mitochondrial dysfunction in migraine. Biochemical analysis has revealed that OXPHOS is depressed in the skeletal muscle and platelets of patients suffering from migraine with prolonged auras or with stroke-like episodes (Montagna *et al*. 1989, Barbiroli *et al.* 1990). Sangiorgi *et al*. (1994) demonstrated abnormal platelet mitochondrial function in patients affected by migraine with or without aura. Several studies employing phosphorus 31 magnetic resonance spectroscopy have detected impaired oxidative metabolism in brain and muscle in patients with various forms of migraine, both during and between the attacks (Welch *et al.* 1989, Barbiroli *et al*. 1990, 1992, Bresolin *et al*. 1991, Montagna *et al*. 1994).

First-degree relatives of migraine patients have up to a four-fold increase in the risk of migraine compared with the general population (Russell *et al*. 1993, Russell & Olesen 1995, Haan *et al*. 1996, Terwindt *et al*. 1998). The familial aggregation of migraine suggests that in addition to environmental factors, hereditary factors are of significance in its aetiology, although the mode of transmission and the molecular basis of migraine have not yet been defined. Epidemiological studies have detected a bias towards maternal as opposed to paternal transmission (Couch *et al*. 1986, Mortimer *et al*. 1992), a feature suggesting mtDNA involvement.

Migrainous headache is common in the MELAS syndrome (Hirano *et al.* 1992). In fact, many patients were originally diagnosed as having malignant migraine before the mitochondrial encephalomyopathies were defined as distinct clinical entities (Andermann *et al*. 1986). Moreover, migraine may be a major feature in oligosymptomatic patients with 3243A>G, and in the maternal relatives of MELAS patients (Ciafaloni *et al*. 1992). Episodic hemicranial headache is also a feature of the MERRF syndrome (Hammans *et al*. 1993).

Migraine with a defined mitochondrial aetiology appears to be rare, however, nor have deletions of mtDNA or the common point mutations associated with the MELAS and MERRF syndromes been found in migraine with aura (Klopstock *et al*. 1996) or in cluster headache (Seibel *et al*. 1996).

Patients with migraine show an increased risk of ischaemic stroke, especially females aged <35 years (Buring *et al*. 1995, Carolei *et al*. 1996, Lipton & Stewart 1997). Furthermore, severe migraine typically precedes the development of stroke-like episodes in the MELAS syndrome (Andermann *et al*. 1986, Hirano *et al*. 1992).

2.7. Occipital stroke

The brain receives blood from four large arteries. The two internal carotid arteries divide into branches of the anterior and middle cerebral artery that supply most of the supratentorial part of the brain, while the two vertebral arteries combine to form an

unpaired basilar artery which supplies the cerebellum, medulla and pons. In the majority of people, the two posterior cerebral arteries are the terminal branches of the basilar artery and they supply the occipital lobes and basal parts of the temporal lobes. Approximately one-fourth of all cerebral infarcts are located in the vertebrobasilar territory (Bogousslavsky *et al*. 1988), and approximately one-third of these are exclusively occipital lobe infarcts (Bogousslavsky & Regli 1987, Bogousslavsky *et al*. 1993), suggesting an overall frequency of 8% for occipital infarcts.

The aetiology of stroke is quite different in young adults from that in older patients. Atherosclerosis and cardiogenic embolus make up approximately 40 to 50% of all etiologies in the younger age group (Nencini *et al*. 1988, Carolei *et al*. 1993, Kappelle *et al*. 1994, Adams *et al*. 1995). The aetiology of occipital infarction may be somewhat different from that of ischaemic stroke in the carotid territory (Pessin *et al*. 1987), however, with cardiac embolism (29%) and vertebrobasilar atheroma with local embolism (17%) as the two most common aetiologies. Migraine has been considered to be the cause of stroke in 14% of patients and in a fairly large proportion (31%) the aetiology has remained unknown (Pessin *et al*. 1987). Systemic illness has been deemed to be an aetiological factor in 9% of cases.

A mitochondrial disorder may be one of the rare aetiologies of stroke. One patient out of 41 younger than 30 years of age and with ischaemic stroke of any localization had a clinically diagnosed mitochondrial disease, suggesting a frequency of 2% (Bogousslavsky & Regli 1987). Clinical and molecular analysis of patients suffering an occipital brain infarct at 18 to 45 years of age has suggested that 10% had a mitochondrial disorder and 6% harboured the common MELAS mutation 3243A>G (Majamaa *et al*. 1997).

2.7.1. Stroke-like episodes in the MELAS syndrome

The stroke-like episodes in the MELAS syndrome most commonly involve the posterior part of the cerebrum, as the location ratio for the occipitoparietal-to-temporal-to-frontal areas is approximately 8:4:2 (Allard *et al*. 1988). Occipital episodes resemble acute stroke in their onset and their symptoms, but there is some dispute as to whether a vascular pathology with smooth muscle energy crisis and subsequent vasospasm, or a primary downturn in neuronal oxidative energy generation is responsible for these events (McKelvie *et al*. 1991).

It has been suggested that structural changes in brain vasculature may play a role in producing the focal ischaemic lesions involved in the MELAS syndrome (Kobayashi *et al*. 1982). Electron microscopy of cerebral blood vessels has shown swelling and an increase in the number of mitochondria in the smooth muscle and endothelial cells of the pial arterioles and small arteries (Ohama *et al*. 1987, Ohama & Ikuta 1987, Sakuta & Nonaka 1989, Muller-Höcker *et al*. 1993, Togunaga *et al*. 1993), and pronounced succinate dehydrogenase (complex II) activity has been observed in the wall of the intramuscular arteries (Hasegawa *et al*. 1991, Togunaga *et al*. 1993). Impaired autoregulation secondary to impaired metabolic activity of the mitochondria in the endothelial and smooth muscle cells of blood vessels has been suggested as a cause of the stroke-like episodes in the MELAS syndrome (Clark *et al*. 1996), but the lesions seen in

CT or MRI resemble those of an ischaemic stroke, although they do not necessarily conform to the vascular territories (Castillo *et al*. 1995, Barkovich *et al*. 1993), nor does the lesion observed in the MELAS syndrome resemble an ordinary small-vessel disease. A metabolic basis for the lesion would seem more likely (Ooiwa *et al*. 1993, Barbiroli *et al*. 1994, Castillo *et al*. 1995). Indeed, generalized hyperperfusion, highest in infarcted areas, has been observed days before, during and several months after the stroke-like episode. This generalized hyperaemia in the brain may be a consequence of local lactic acid production (Gropen *et al*. 1994, Miyamoto *et al*. 1997, Takahashi *et al*. 1998). Furthermore, serial imaging has shown a vasodilatation localized in the affected cerebral cortexes during stroke-like episodes, without any reduction in regional cerebral blood flow (Ooiwa *et al*. 1993). These findings confirm that the stroke-like episodes are the result of a metabolic dysfunction in neural tissue.

Hirano and Pavlakis (1994) stated that mitochondrial disease manifests itself when there is a mismatch between energy requirements and availability. The affected mitochondria try to meet the energy demands of the cell at times when these energy demands are especially high, e.g. during a febrile illness, and the relative deficiency in cellular ATP may result in “metabolic strokes”.

It has been shown by positron emission tomography that patients with mitochondrial encephalopathy have a diffuse reduction in cerebral oxygen metabolism and a reduced metabolic molar ratio, and that they also exhibit less cerebral blood flow in response to hypercapnia (Shishido *et al*. 1996). These results indicate impaired oxidative energy metabolism in the brain and mild dilatation of the cerebral vessels due to mild lactic acidosis.

3. Aims of the present research

Mitochondrial dysfunction may be one of the rare aetiologies of stroke. It has been suggested that 10% of young patients suffering an occipital brain infarct have a mitochondrial disorder. The common MELAS mutation 3243A>G has been found in 6% of the patients, but in addition to pathogenic mutations, it is possible that the cumulative effect of a number of mildly deleterious sequence variants may synergistically contribute to the pathogenesis of mitochondrial disorder.

Studies of mitochondrial DNA require the population controls to be accurately matched with the patients. A knowledge of mtDNA variants and their associated haplotypes is of great importance in the search for possible pathogenic mutations in mtDNA. Furthermore, it has been shown that the degree of penetrance of certain pathogenic mtDNA mutations depends on the genomic background against which they occur. Such genotypes conferring risk have been found in Leber's hereditary optic neuropathy and in Alzheimer's disease, for instance.

The specific aims of this work were:

1. to investigate a possible mitochondrial genotype conferring risk of occipital stroke (I, IV),
2. to construct a phylogenetic network for the Finnish mitochondrial DNA haplogroup U to be used in identifying differences in mtDNA between controls and patients (II, III), and
3. to identify mtDNA mutations in patients belonging to mtDNA haplogroup U who have suffered an occipital stroke (IV).

4. Subjects and methods

4.1. Patients (I and IV)

Patients with occipital stroke treated at Oulu University Hospital over the 19-year period 1976–1995 were identified retrospectively using the discharge files (Majamaa *et al*. 1997). Inclusion required that patients should have been aged 18 to 45 years at the time of the diagnosis and that the onset of the disorder had been acute and not related to intracranial traumatic lesions. Furthermore, patients had to have been residents of Northern Ostrobothnia at the time of onset of the disease. The total number of patients was 38 (23 men, 15 women), of whom four men had died and five men were not available for investigation. The remaining 29 patients (14 men, 15 women) were assessed clinically and interviewed about their history of migraine. Migraine was diagnosed in 18 of them (5 men, 13 women) according to the International Headache Society (1988) criteria. The aetiology of the stroke was assessed using criteria from the Baltimore-Washington Cooperative Young Stroke Study (Johnson *et al*. 1995). Migraine was deemed to be a probable aetiology for stroke in five cases and a possible aetiology in one.

Patients with possible migraine were evaluated clinically at Oulu University Hospital, and 42 (6 men, 36 women) were diagnosed as having migraine according to the criteria of the International Headache Society (1988). Their mean age was 42 ± 7 years. Fifteen women (42%) had migraine with aura, whereas all the men and the remaining women had migraine without aura.

The research protocol was approved by the Ethics Committee of the Medical Faculty, University of Oulu. Blood samples from patients (designated with ”#”) were taken after obtaining informed consent.

4.2. Population controls (I–IV)

Two groups of population controls were used. The first group (designated with ”P”) was collected from health care centres in the provinces of Northern Ostrobothnia and Kainuu. Blood samples were obtained from 83 persons, who reported that they themselves and

their mothers were healthy with respect to common clinical manifestations of an mtDNA disease such as diabetes mellitus, sensorineural hearing impairment and neurological ailments. Furthermore, it was required that their matrilineal ancestors had been born in northern Finland, i.e. in the province of Northern Ostrobothnia or Kainuu, before the year 1900. Recent relationship between the persons was excluded by reference to the time and place of birth of the matrilineal ancestors. After obtaining this information the samples were anonymized. Six samples were obtained late and they were not included paper III.

The second group of controls included 403 healthy blood donors, and the samples were obtained at Finnish Red Cross offices in the capitals of the provinces of Northern Ostrobothnia, Central Ostrobothnia, Kainuu and Northern Savo (designated with “PO”, “PL”, “PK” and “PS”, respectively). The donors and their mothers were required to be healthy with respect to diabetes mellitus, sensorineural hearing impairment and neurological ailments and their mothers to have been born in the same province.

4.3. DNA extraction (I–IV)

Total DNA was isolated from blood cells using a QIAamp Blood Kit (Qiagen, Hilden, Germany). Platelet mitochondria were isolated from 40 ml of venous blood by fractionating centrifugation (Ausenda & Chomyn 1996) and DNA was purified from the isolated mitochondria with the QIAamp Blood Kit.

4.4. Primers for polymerase chain reactions (I–IV)

The primers used in polymerase chain reaction (PCR) amplifications were numbered according to the Cambridge reference sequence (Anderson *et al*. 1981). The mean primer length was 22 nt.

4.5. Restriction fragment analysis of mtDNA (I–IV)

4.5.1. Detection of the 5656A>G by NheI digestion and inhibition of the enzyme by NaCl (II)

The 5656A>G transition in mtDNA was detected by restriction fragment analysis. The mtDNA region was amplified by PCR in the presence of ^{35}S-dATP using a forward primer L5548 (5548–5569, 5’-AGC CCT CAG TAA GTT GCA ATA C-3’) and a reverse primer H5677 (5677–5657, 5’-GTT TAA GTC CCA TTG GGC TAG-3’). The underlining of the nucleotide in the latter primer indicates a mismatch from C to G at position 5661, creating a novel NheI cleavage site when the mutant variant is present. The amplified DNA fragment (130 bp) was then digested overnight at 37°C with 5 U of NheI (New England Biolabs, Beverly, MA, USA) and electrophoresed through a 10%

polyacrylamide gel. The intensities of the bands on autoradiography films were quantified by image analysis (Bioimage, Millipore, Ann Arbor, MI, USA) and the percentage of apparent heteroplasmy was calculated (Shoffner *et al*. 1990).

To study whether nuclear pseudogenes are the source of the apparent heteroplasmy of 5656A>G, the mutation was detected in mtDNA purified from isolated platelet mitochondria, as described above, and a nuclear encoded gene was amplified using the forward primer 5'-ACC TAG GAT TGG CCT CCT ATA C-3' and the reverse primer 5'-GAG GAC TAG GAG GAG GGG GTG ATG G-3', corresponding to the exon 5 of the type XI collagen α2 gene residing in chromosome 6 (Vuoristo *et al*. 1995).

The effect of the concentration of NaCl on the activity of NheI was studied by using amplified mtDNA fragments spanning between nt's 5548 and 5677 and containing 5656A>G. The amplified fragments were first purified using the QIAquick PCR Purification Kit (Qiagen) and were then eluted with a non-salt buffer. The sample was digested with NheI in the presence of various concentrations of NaCl and the degree of the digestion was verified by gel electrophoresis on 3% MetaPhor agarose (FMC BioProducts, Rockland, ME, USA).

4.5.2. Detection of common mtDNA mutations (I)

The most frequent mtDNA point mutations were detected by RFLP analysis. DNA fragments encompassing the nucleotide positions for the MERRF mutation 8344A>G (Wallace *et al*. 1988b) and the MELAS mutations 3243A>G (Goto *et al*. 1990, Kobayashi *et al*. 1990) and 3271T>C (Goto *et al.* 1991) were amplified in the presence of ^{35}S-dATP and then digested overnight at 37°C with BglI, ApaI, and DraI, respectively, and electrophoresed through 6–10% polyacrylamide gels (see above). The NARP 8993T>G and the LHON 11778G>A mutations were also detected by RFLP. An amplified fragment spanning between the nts 8648 and 9199 was digested with AvaI at 37°C overnight and electrophoresed through 1.5% agarose (Holt *et al*. 1990), a cleavage being observed when the 8993T>G mutation was present. The 11778G>A mutation was detected by digestion of an amplified fragment with the MaeIII enzyme at 51°C overnight, and the digested fragments were then electrophoresed through 2% Metaphor (Wallace *et al.* 1988a). All the enzymes used were from New England Biolabs (Beverly, MA, USA).

The common 4997 bp deletion was detected by PCR amplification through 35 cycles using primers L8198 and H13851. When the deletion was present a fragment of 657 bp was amplified and was visualized on a 1.5% agarose gel (Holt *et al*. 1989).

4.5.3. Analysis of mtDNA haplogroups (I–IV)

The mtDNA haplogroups of the patients and population controls were determined by restriction digestion in order to identify informative polymorphic sites (Table 2) (Torroni *et al*. 1996a). The amplified fragments were digested overnight at +37°C with DdeI, NlaIII, HaeIII (New England Biolabs), AluI (Pharmacia Biotech), or MvaI (Boehringer Mannheim) and then electrophoresed through 1.5% agarose or 2–3% MetaPhor agarose

gel as appropriate. 12308A>G was detected by DdeI digestion of a fragment amplified in the presence of a mismatched (underlined nucleotide) forward primer L12279 (5'-AAC AGC TAT CCA TTG GTC TTA GGC CCT AA-3'). A cleavage was observed when 12308A>G was present.

Table 2. Identification of European specific mtDNA haplogroups by restriction fragment analysis.

Haplogroup	Variant nucleotide	Restriction site, loss (–), gain (+)	10394 DdeI	Primers used	Frequency in Finland (%)[b]
H	7028	–7025 AluI	–	L6869, H7608	40.8
T	15607	+15606 AluI	–	L15409, H15701	6.1
U	12308	+12305 DdeI	–	L12279[a], H12386	16.3
V	4580	–4577 NlaIII	–	L3951, H4739	4.1
W	8994	–8994 HaeIII	–	L8648, H9199	4.1
X	1719	–1715 DdeI	–	L1615, H1900	4.1
I	1719	–1715 DdeI	+	L1615, H1900	2
J	13708	–13704 MvaI	+	L13570, H14007	14.3
K	12308	+12305 DdeI	+	L12279[a], H12386	4.1
M	10400	+10397 AluI	+	L9911, H10700	2

[a] A mismatched primer which creates a Dde I cleavage site when 12308A>G is present.

[b] The frequency of mtDNA haplogroups in Finland is according to Torroni *et al*. (1996a).

4.6. Conformation sensitive gel electrophoresis (CSGE) (III and IV)

4.6.1. PCR for CSGE

Sixty-three pairs of primers were designed for the amplification of the mtDNA coding region (nts 523–16090). The mean size of the amplified fragments was 354 bp, and neighbouring PCR products were designed to overlap by at least 80 bp at both ends. The template DNA was amplified in a total volume of 50 µl by PCR in 30 cycles through denaturation at 94°C for 1 min, annealing at a temperature specific to the primer for 1 min and extension at 72°C for 1 min, and with a final extension at 72°C for 10 min. The quality of the amplified fragment was estimated visually on a 1.5% agarose gel and then a suitable amount of the PCR product, usually 3–10 µl, was taken for heteroduplex formation. Each amplified fragment of haplogroup U was mixed with the corresponding fragment amplified on a haplogroup H or I template. The amplified fragments were denatured at 95°C for 5 min and the heteroduplexes were subsequently allowed to anneal at 68°C for 30 min.

Poorly visualized bands were observed in CSGE of heteroduplexes from seven fragments covering regions of the 12SRNA, 16SRNA (two fragments), COX1, ATPase, ND4 and ND5 genes. Homologous sequences corresponding to these regions have been

shown to exist as pseudogenes in the nuclear genome (Wallace *et al.* 1997b). The use of DNA from isolated platelet mitochondria as the template did not improve the resolution, whereas a shift in primer location by 50–100 nucleotides resulted in sharp bands in CSGE.

4.6.2. CSGE

CSGE was carried out essentially as described earlier (Körkkö *et al.* 1998). A 1 mm thick gel with a 36-well comb was prepared with 15% polyacrylamide, a 99:1 ratio of acrylamide to 1,4-bis(acryloyl)piperazine (Fluka, Buchs, Switzerland), 10% ethylene glycol, 15% formamide (Gibco BRL, MA), 0.1% ammonium persulphate and 0.07% TEMED in 0.5 x TTE buffer (44.4mM Tris, 14.25mM taurine, 0.1mM EDTA, pH 9.0). The loading buffer stock for the PCR products was a 10 x solution of 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol FF. The PCR products were analyzed on a standard DNA sequencing gel apparatus using 0.5 x TTE as the electrode buffer. The gel was pre-electrophoresed for 30 min and the samples electrophoresed through the gel at a constant voltage of 400V overnight at room temperature. After electrophoresis, the gel was stained on the glass plate in 150 µg/l of ethidium bromide for 5 minutes followed by destaining in water. The gel was then transferred to a UV transluminator and photographed (Grab-IT Annotating Grabber 2.04.7, UVP Inc, Upland, CA).

4.7. Sequencing (III and IV)

Selected PCR fragments covering the coding region and the entire D-loop in the patients and population samples belonging to haplogroup U were analyzed by automated sequencing (ABI PRISM^{TM} 377 Sequencer using the Dye Terminator Cycle Sequencing Ready Kit, Perkin Elmer, Foster City, CA) after treatment with exonuclease I and shrimp alkaline phosphatase (Werle *et al.* 1994). The primers used for sequencing of the coding region were the same as those used in the amplification reactions for CSGE. The D-loop sequence was amplified in two fragments spanning the nts 15714–16555 and 16449–725 and the sequence was determined between nts 16024 and 576. The reference samples belonging to haplogroup H and I were sequenced between nts 568-16400.

4.8. Cloning (II and IV)

The apparent heteroplasmy of the 5656A>G mutation and the possible heteroplasmies of the tRNA^{Ile} (4295A>G), tRNA^{Cys} (5773A>G) and tRNA^{Lys} (8296A>G) mutations were examined by cloning amplified fragments of mtDNA spanning nts 5467–5917 from the 5656A>G positive population control P47, nts 3969–4508 from patient #282 and nts 5467–5917 and 8100–8748 from patient #148, into a pCR® 2.1-TOPO vector (TOPO TA Cloning Kit; Invitrogen, Leek, The Netherlands). Positive colonies were cultured

overnight in 2 ml of LB medium containing 50 μg/ml ampicillin, and a portion of 1μl from 50 (5656A>G) or 80 (tRNA mutations) cultures was then incubated for 10 min at 94^{o}C in order to lyse the cells and inactivate the nucleases and amplified in the presence of the primers L4266–H4508 (4295A>G), L5548–H5799 (5656A>G and 5773A>G) and L8100–H8323 (8296A>G). To detect mutations, the amplified DNA fragments were digested with the enzymes NheI (5656A>G), Nla III (4295A>G), Hinf I (5773A>G) and Sac II (8296A>G) (New England Biolabs) and cleavage was verified on 3% MetaPhor agarose. For sequencing, the plasmid-specific M13 forward and reverse primers were used to amplify the template, the sequencing being carried out with plasmid-specific and insert-specific primers.

4.9. Phylogenetic analysis (III and IV)

The phylogenetic networks were based on the median algorithm (Bandelt *et al*. 1995).

5. Results

5.1. Association of migrainous occipital stroke with mtDNA haplogroup U (I)

Twenty-nine patients with an occipital stroke were assessed clinically and interviewed for a history of migraine. This was diagnosed in 18 patients (5 men, 13 women). Migraine was deemed to be a probable aetiology for stroke in five patients and a possible aetiology in one. The median age at stroke onset in these six patients was 29 years. Migraine with aura was present in four of these patients and three had a family history of migraine. Interestingly, an increased amount of fat or accumulations of subsarcolemmal or interfibrillar mitochondria were seen in electron microscopy of the muscle in three out of four patients with migraine-associated stroke examined in this way. The mtDNA mutations 3243A>G, 8344A>G, 8993T>G and 11778G>A, and also the common 4977 bp deletion were excluded in these patients.

All five patients with a probable migraine-associated stroke were found to belong to the mtDNA haplogroup U, whereas the patient with a possible migraine-associated stroke belonged to haplogroup V, thus suggesting a frequency of 83% for haplogroup U. This frequency differed significantly from that of 17% found among healthy controls in the same population, and from that of 25% found among the remaining 12 patients with migraine and unrelated stroke. Furthermore, the frequency of mtDNA haplogroup U was 19% among 42 patients who met the International Headache Society criteria for migraine but had not suffered a stroke. None of these patients harboured the 3243A>G mtDNA mutation.

5.2. 5656A>G is not a pathogenic mutation but a common variant in haplogroup U (II)

The nucleotide at position 5656 in mtDNA is a single non-coding nucleotide between the tRNA genes for alanine and asparagine (Anderson *et al*. 1981). The position is conserved between species, and in most cases it is occupied by an A. The transition 5656A>G has

been reported at a low frequency in patients with diabetes mellitus (Thomas *et al*. 1996) and in a patient with familial progressive tubulointerstitial nephritis (Zsurka *et al*. 1997). The transition has been thought to be homoplasmic (Thomas *et al*. 1996). Interestingly, a heteroplasmic 5656A>G has been found in association with another mutation at nt 10010 in a patient with severe mitochondrial encephalomyopathy (Bidooki *et al*. 1997). The heteroplasmy at nt 5656 was verified by demonstrating a novel NheI restriction site in the presence of the mutant variant 5656G when DNA was amplified in the presence of a mismatched oligonucleotide primer (Bidooki *et al*. 1997).

We found that three out of six patients with migraine-associated occipital stroke harboured 5656A>G, but it was also found in 14 out of 83 healthy population samples from northern Finland, suggesting a population frequency of 17%. 5656A>G was exclusively associated with mtDNA haplogroup U and was found in 58% of all the haplogroup U samples. It appeared to be heteroplasmic when studied by restriction fragment analysis following NheI digestion. Surprisingly, we failed to detect the wild type genome when cloning DNA with apparent heteroplasmy. The nuclear-embedded mtDNA pseudogenes did not contribute to the erroneous identification of 5656A>G heteroplasmy. Finally, the apparent heteroplasmy was found to be due to inhibition of NheI by NaCl.

5.3. Use of CSGE to analyse mtDNA (III)

In order to estimate the usefulness of CSGE for analysing differences within the coding sequence of mtDNA, DNA from 22 healthy controls belonging to haplogroup U were examined. Sequence differences within 63 overlapping fragments were screened by CSGE. Heteroduplexes were allowed to form between an amplified fragment from each person belonging to haplogroup U and the corresponding fragment amplified from a reference DNA sample belonging to either haplogroup H (heteroduplex U/H) or haplogroup I (heteroduplex U/I).

A total of 24 amplified fragments migrated in one band and could therefore be regarded as identical in sequence to the 22 samples representing haplogroup U and the two reference samples representing mtDNA haplogroups H and I. Among the remaining 39 fragments there was at least one sample that yielded more than one band in either the heteroduplex U/H or the heteroduplex U/I, suggesting a difference between the sequences.

The two reference DNA samples belonging to haplogroups H and I were sequenced in their entirety with the exception of the HVS II region, and a total of 104 haplogroup U samples from the 63 fragments were sequenced, including at least one sample from each heteroduplex that had migrated in one band and one sample from each heteroduplex that had differed in migration pattern.

Sequence data were obtained for 104 U/H and U/I heteroduplexes, 87 of which differed in the nucleotide sequence of the two fragments. In the case of the U/H heteroduplexes, CSGE suggested a similar primary structure in 45 fragments and a dissimilar primary structure in 42 fragments, while in the case of the U/I heteroduplexes CSGE suggested a similar primary structure in 44 fragments and a dissimilar primary

structure in 43 fragments. Three fragments turned out to be false negatives, suggesting that a sensitivity of 0.96 had been achieved by CSGE in detecting sequence differences in mtDNA. The specificity was found to be 1.0.

The CSGE was reproducible, as no inter-assay variation was found when a given heteroduplex PCR fragment was loaded on separate gels. Furthermore, identical migration was observed in each lane of a gel when a given heteroduplex PCR fragment was electrophoresed in multiplicates, suggesting that there is negligible intra-assay variation.

5.4. Phylogenetic networks for the Finnish haplogroup U based on coding region and HVS-I (III)

Sequence information on the complete coding region covering 15,567 nucleotides was obtained in 22 samples belonging to haplogroup U. Using this information, a novel phylogenetic network for haplogroup U was outlined (Figure 2a, III). This was found to be a perfect tree with no homoplasy and provided several previously unidentified common polymorphisms. The median network constructed for the HVS-I sequence data (Figure 2b, III) included three reticulations, but in general correlated well with the network obtained for the coding region. Comparison of these two networks suggests that the reticulation involved in sample P17 may be resolved by assuming a back mutation at nt 16270. Furthermore, comparison of the two networks favours the assumption that the lineage of P60 consists of 16270C>T and 16256C>T.

The network was compared with one based on a compilation of European samples (Macaulay *et al*. 1999) and was found to be concordant with haplogroup U5 in the median network based on the HVS-I sequence, in accordance with previous observations (Torroni *et al*. 1996a, Macaulay *et al*. 1999). Furthermore, four complete mtDNA coding region sequences containing 12308A>G were available for comparison (Ozawa 1995; Arnason *et al*. 1996; Ohlenbusch *et al*. 1998). Two sequences, P-8 (Ozawa 1995) and MS128 (Ohlenbusch *et al*. 1998), harbour many common polymorphisms with the haplogroup U network and share additional polymorphisms at nts 1811 and 9698, but lack the polymorphisms at nts 3197, 9477 and 13617, suggesting that the genotypes are incompatible with the network. The two other sequences could be placed in the network.

5.5. Analysis of mtDNA in patients with occipital stroke using the phylogenetic network for the mtDNA haplogroup UK (IV)

Nucleotide sequence information on the complete coding region and the HVS I in mtDNA was obtained from 14 patients who had suffered an occipital stroke and harboured 12308A>G. Ten of these patients could be positioned in the phylogenetic network of U5 (Figure 2a, III). Six of their haplotypes were located within the network, whereas those of patients #153, #207, #277 and #285 differed from the nearest

neighbouring haplotype by one substitution in the coding region. All five patients with migrainous stroke belonged to U5, suggesting an association between migrainous stroke and this haplogroup.

Since four out of the 14 patients with occipital stroke could not be positioned in the network of U5, the network was expanded using sequence data from 21 samples harbouring 12308A>G but not belonging to U5 (Figure 1, IV). The network enlargement included branches of the haplogroups U*, U2, U4 and K, and was found to be a perfect tree (Figure 4). With the exception of a reticulation composed of 1811A>G and 3197T>C, ambiguities were not involved. The network enabled the identification of 5773A>G in the $tRNA^{Cys}$ gene that has arisen twice in this population, and the polymorphisms in HVS I could also be positioned in it by assuming five pairs, two triplicates and one quadruplicate of parallel mutations. The previous networks (Richards *et al*. 1998, Macaulay *et al*. 1999) have included a group of poorly defined haplotypes designated as cluster U* that was the most probable designation here for the five controls and two patients. We defined that part of U* belongs to the subcluster U4 by the presence of 1811A>G, 16519T>C and the wild type nt A at position 9698. The rest of U* is suggested to form subcluster U7, which is defined by 1811A>G, 9698A>G and the wild type nt T at position 16519. The branches of the enlarged network included haplogroup U5 with a frequency of 83 %, haplogroups U2, U4 and U7 with a combined frequency of 7.3 % and haplogroup K with a frequency of 9.7 % (Figure 1, IV).

The four stroke patients not belonging to U5 were found to belong to haplogroups U4, U7 or K (Figure 1, IV). Interestingly, the genotypes in haplogroups U2, U4 and U7 were quite distinct in harbouring a considerable number of polymorphisms. The genotypes of patients #148, #260 and #282 could not be detected among 480 population controls, but that of patient #411 was identical to that in three controls. The genotype of patient #148 diverged most and differed by 11 coding region substitutions from the major branch in the network and by 16 substitutions from the nearest neighbouring haplotype. The mtDNA of patient #282 differed from the nearest neighbouring haplotype by nine substitutions in the coding region, whereas that of patient #260 differed only by two substitutions. The combined frequencies of haplogroups U2, U4 and U7 were three-fold higher among the stroke patients than among the controls harbouring 12308A>G.

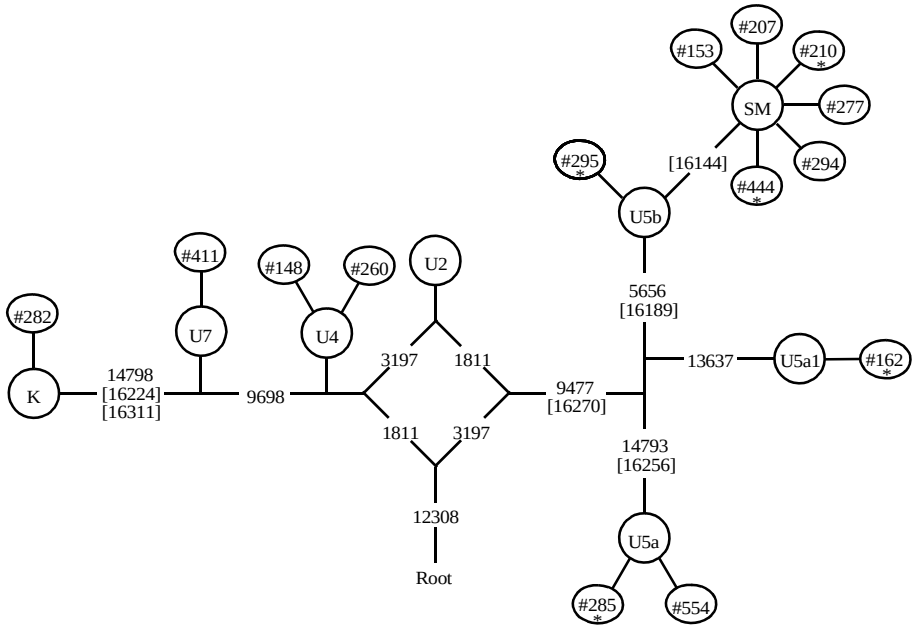

Fig. 4. Outlines of phylogenetic network of Finnish mtDNA haplogroup UK (see Figure 1, IV). Circles denote subgroups of haplogroup U with the exception of the Saami motif (SM). Ellipses denote patients with occipital stroke. Patients with migrainous stroke are marked by asterisks.

5.6. Possible pathogenic mutations in patients with occipital stroke (IV)

Several mutations which differed from the network were detected in the patients with occipital stroke. The mutations leading to amino acid replacements and mutations in rRNA and tRNA genes were considered further (Table 3). Four patients, #148, #153, #260 and #282, harboured mutations that were not found among 480 controls.

The 14 patients with occipital stroke harboured three mutations in the genes encoding tRNAs and two mutations immediately adjacent to tRNA genes. One of the latter was 5656A>G (II) and the other was a transversion 12135C>A that leads to Ser>Tyr in the last codon of the ND4 gene preceeding the histidine tRNA. To assess heteroplasmy, PCR amplified fragments encompassing the three tRNA mutations were subcloned and the clones were analyzed for the mutations by restriction fragment analysis. Examination of 80 subclones revealed that 5773G>A in patient #148 was a homoplasmic substitution. Furthermore, this mutation was found in a control sample belonging to haplogroup K. Analysis of the 80 subclones for 8296A>G also revealed only the mutant variant, suggesting that the mutation detected in patient #148 is a homoplasmic one. On the other hand, both the mutant genome and the wild type genome were detected in subclones

containing a segment encompassing nt 4295. The wild type genome, however, was found only in 2 out of 79 clones, suggesting that the degree of heteroplasmy in the blood of patient #282 was approximately 97 %.

Table 3. Mutations in the coding region of mtDNA found in patients with occipital stroke.

Patient	Mutation	Gene	Present in controls	Comment	Reference
#148	1700T>C	16S rRNA	no	Located in a base-paired structure within a region stabilizing the secondary structure	Glotz *et al.* 1981
	4025C>T	ND1	no	Secondary mutation in LHON	Huoponen *et al.* 1993
	5773G>A	$tRNA^{Cys}$	yes	Invariant nucleotide in the pseudouridine loop	Sprinzl *et al.* 1989
	8296A>G	$tRNA^{Lys}$	no	Located in the amino acid acceptor stem	Kameoka *et al.* 1998a
	8921G>A	ATPase6/8	no	Lys>Asp	
	8938A>G	ATPase6/8	no	Ile>Val	
	12361A>G	ND5	no	Ala>Thr, non-conserved	Obayashi *et al.* 1992
#153	1850T>C	16S rRNA	no	Highly conserved	Glotz *et al.* 1981
#207	10907T>C	ND4	yes	Phe>Leu	
#260	3736G>A	ND1	no	Val>Ile	
#277	1341C>T	12S rRNA	yes		
#282	4295A>G	$tRNA^{Ile}$	no	3'-nucleotide flanking the anticodon	Merante *et al.* 1996
	15884A>G	Cyt b	no	Rare polymorphism in African haplogroup L1	Chen *et al.* 1995a

6. Discussion

6.1. CSGE is an effective means of resolving mtDNA sequences

The pathogenic mutation is typically buried within a background of multiple sequence changes, and the high frequency of benign mtDNA polymorphisms makes the finding of new mutations by sequencing a laborious task, whereas restriction fragment analysis lacks sensitivity. In addition, polymorphisms may be relatively rare themselves and cosegregate with the disease, confounding identification of the pathogenic mutation.

We found CSGE to be an ideal method for screening mutations and polymorphisms in mtDNA. Comparison of the CSGE data with the actual sequence data suggested that the sensitivity of CSGE was 96%. SSCP may not detect all mutations in mtDNA (Thomas *et al*. 1994) and its reported sensitivity is 84% (Jordanova *et al*. 1997). Methods based on low-stringency single specific primer PCR (Pena *et al.* 1994) and single strand conformation analysis in a mutation detection enhancement gel matrix (Alonso *et al*. 1996) have been used for mtDNA genotyping, but no analysis of sensitivity was presented in either case.

The effectiveness of CSGE was demonstrated by the identification of several new, informative polymorphisms determining haplogroup U and its subclusters. Furthermore, the method has the advantage of being non-radioactive and of large capacity. Sequence information was obtained with relatively little effort, and the data acquired by CSGE enabled a phylogenetic network to be constructed for haplogroup UK. A systematic way of comparing different mtDNA sequences, those of patients with healthy population controls, is through phylogenetic analysis (Chinnery *et al*. 1999).

6.2. A phylogenetic network for the Finnish mtDNA haplogroup UK

Haplogroup U is determined by a transition 12308A>G in the $tRNA^{Leu(CUN)}$ gene. Haplogroup U is almost specific to Europeans, and it is found only at a low frequency in the Japanese, the North African Berber population, the Ethiopians and the Senegalese (Ozawa 1995, Torroni *et al.* 1996a, Passarino *et al.* 1998, Macaulay *et al.* 1999).

Haplogroup U is composed of subclusters termed U1-U6 and U*, in addition to which haplogroup K has been included as a subgroup (Richards *et al*. 1998). Estimated minimum divergence times have suggested that haplogroup U is ancient, and the oldest subgroup U5 dates back to about 52,000 YBP (Richards *et al*. 1998). The divergence times of other European haplogroups vary a lot: 12,500–18,500 YBP for haplogroups V, K and W and about 20,500–28,000 YBP for haplogroups H, X and J. Haplogroups I and T are among the oldest, with divergence times of 35,000 and 46,500 YBP, respectively (Richards *et al*. 1998).

The phylogenetic network of haplogroup UK (see Figure 4) was constructed in two parts. The first part was constructed using sequence data on 22 population samples from the provinces of Northern Ostrobothnia and Kainuu. This turned out to be exclusively a U5 network (Figure 2a, III). An analysis of 21 control samples with 12308A>G but not belonging to haplogroup U5 was used to create a complete network for haplogroup UK (Figure 1, IV). Comparison of this haplogroup UK network with four previously published complete mtDNA sequences containing 12308A>G (Ozawa 1995; Arnason *et al*. 1996; Ohlenbusch *et al*. 1998) revealed that all four sequences could be unambiguously placed in the network suggesting that we have detected all the important polymorphisms that characterize haplogroup UK. Our network turned out to be almost a perfect tree, because only three parallelisms could be identified in the coding sequence. Two of them, transitions 1811A>G and 3197T>C, existed between U5 and other subgroups. Several parallelisms were found in the sequence of HVS-I, however.

Comparison of the expanded network with that based on a compilation of European samples revealed subcluster U2 by identification of polymorphisms at nts 15907, 16051, 16129, 16189 and 16362 (Macaulay *et al*. 1999). Similarly, subcluster U4 was identified on the basis of substitutions at nts 4646, 11332, 16356 and 16362. Cluster K has been defined by polymorphisms at nts 16224 and 16311 (Macaulay *et al*. 1999), but we found seven additional common polymorphisms determining this cluster. Substitutions 10398A>G and 11299T>C have been thought to create separate branches (Macaulay *et al*. 1999), but we found exclusive co-occurrence of these two polymorphisms among Finns. The previous networks (Richards *et al*. 1998, Macaulay *et al*. 1999) have also included a group of poorly defined haplotypes designated as cluster U*, which was the most probable designation here for five controls and two patients. The HVS I motifs determining subclusters U1, U3 and U6 (Macaulay *et al*. 1999) were not found among the Finns examined.

The haplogroup UK network enabled the relationships between the various clusters to be identified. Contrary to CRS, clusters U and K had six coding region variants in common, and thereafter, a reticulation composed of the transitions 1811A>G and 3197T>C divided the haplogroup UK into three groups. 3197T>C was found in subcluster U5, 1811A>G was found in K, U4 and U*, and both transitions were found in subcluster U2. Cluster K was fairly distant from subcluster U5, as the most recent common node was separated from its central node by nine coding region substitutions. A striking feature in the genomes belonging to K, U2, U4 and U* was the considerable number of polymorphisms and the paucity of branching. This may be due to the possibility that branching haplotypes are extinct or to the rarity of these haplotypes in the population.

The character of subcluster U* was then considered further. The Hae III restriction site at position 16517 is a variable one, and a restriction site gain suggesting the presence of 16519T>C has been found in samples belonging to clusters U2, U3, U4, U* and K, whereas it is absent in U1 and U5 and no data have been reported for U6 (Macaulay *et al.* 1999). We found that 16519T>C had arisen together with 1811A>G and was thus present in all the samples in the network with the exception of cluster U5. Furthermore, a back mutation had taken place in the cluster including patient #411, three controls and the previously reported patient P-8 (Ozawa 1995). Using this additional information, we suggest that there is an additional star-like cluster in the UK network that is defined by the presence of 1811A>G, 16519T>C and the wild type nt A at position 9698 and includes patients #148 and #260 and four population controls. As this cluster includes patient #260, who belongs to subcluster U4, we would like to term this subcluster U4. Furthermore, the subcluster defined by 1811A>G, 9698A>G and the wild type nt T at position 16519 and includes patient #411 and three controls may be proposed as U7. This subcluster is quite interesting in that it is found in populations as diverse as the Finns and the Japanese (Ozawa 1995) and, on the basis of the HVS I polymorphisms at nts 16146 and 16342, in the Basques as well (Richards *et al*. 1998).

We found that 83% of the controls with 12308A>G belonged to subgroup U5 (Richards *et al*. 1998, Macaulay *et al*. 1999), suggesting restricted variation in haplogroup U in northern Finland. The frequency of U5 was found to be 5 to 6-fold higher than in the Germans (Hofmann *et al*. 1997). The upper branch of U5 is characterized by 5656A>G, and the HVS-I sequence suggests that it is similar to the previously defined subcluster U5b (Richards *et al*. 1998), which may be quite specific to the Finns, as 5656A>G is detected among Finns approximately 34 times more frequently than in the Hungarians or the English (Thomas *et al*. 1996, Zsurka *et al*. 1997, II). Interestingly, the transition 5656A>G was first associated with maternally inherited progressive tubulointerstitial nephritis in a Hungarian family and was not detected among the Hungarian controls (Zsurka *et al*. 1997). These differences in frequencies suggest that the Finns and the Hungarians are genetically distant in spite of the fact that the Hungarian language belongs to the Finno-Ugric group in the same way as Finnish and Saami languages (Korhonen 1991). Furthermore, part of the U5b branch is characterized by the HVS-I motif, including 16144T>C, 16189T>C and 16270C>T, which has been considered to be fairly specific to the Saami and is not found among other Europeans except for the Finns (Sajantila *et al*. 1995, Lahermo *et al*. 1996). The branch characterized by nt 13637A>G appears to be fairly rare in Finns, whereas its most probable counterpart, subcluster U5a (Richards *et al*. 1998), is not uncommon among other Europeans. These findings suggest that the haplotype characterized by the coding region variants 5656A>G and 12618G>A may be highly specific to the Finns and the Saami.

Geographical, linguistic, and cultural factors have contributed to the isolation of the Finnish population (de la Chapelle 1993, Peltonen *et al*. 1995) and this may partly explain the limited variation within haplogroup U. It has been shown that the frequency of a mtDNA genotype is higher when it has been introduced into the population earlier (Heyer 1995). Moreover, the rate of mtDNA loss in an expanding population may be as high as 72% within 150 years or in six to eight generations (Heyer 1995), which may explain the different haplogroup frequencies between populations. Moreover, the current frequencies of haplogroups in a population do not necessarily reflect the frequencies a few

generations ago, as shown by the example of haplogroup V in the Basques. This haplogroup is observed nowadays only in northwestern Europe and in North Africa. It reaches high frequencies in some Iberian populations and is also very common among the Berbers of North Africa, but showed its highest frequencies (about 41%) among the Saami (Torroni *et al*. 1998). This observation has been thought to indicate that haplogroup V originated in Europe or North Africa, most likely in the Iberian peninsula, and that it reflects a major late Palaeolithic population expansion from southwestern Europe towards the northeast (Torroni *et al*. 1998). Dental samples from prehistoric Basques nevertheless show an absence of haplogroup V (Izagirre & de la Rua 1999). Therefore, the date of origin of haplogroup V might be more recent than that proposed (Torroni *et al*. 1998), or else the observation reflects the high rate of mtDNA loss in the expanding population (Heyer 1995).

6.3. Genotypes of the patients with occipital stroke and an association of migrainous stroke with U5

Clinical evaluation of 29 patients who had suffered an occipital stroke revealed migraine in 18 patients, and migraine was deemed to be a probable aetiology for stroke in the case of five patients and a possible etiology in one. The mtDNA genotype was studied by RFLP and an association of migrainous stroke and mtDNA haplogroup U was found. In addition to the five patients with migrainous stroke, we analyzed the complete mtDNA coding sequence and the HVS I from nine other patients with occipital stroke.

Sequence analysis of the entire coding region by CSGE revealed that all five patients with migrainous stroke and five others belonged to U5, one to haplogroup K and the remaining three to the subgroups U4 and U7 intervening between U5 and haplogroup K, suggesting a ratio of 10:1:3, whereas the corresponding ratio in the general population is 10:1:0.75. This result suggests that if mtDNA haplogroup U5 poses a risk for migrainous stroke, this risk may be defined by clusters of polymorphisms at nt 11467–12308–12372 or 3197–9477–13617 (Figure 1, IV). None of these substitutions may be considered pathogenic as such, but mild pathogenicity can not be ruled out. There is molecular evidence for Muller's ratchet in mtDNAs (Lynch 1996). The hypothesis that non-recombining organelle genomes are subject to gradual loss of fitness due to the cumulative chance fixation of mildly deleterious mutations has been tested in LHON, for example (Brown *et al.* 1997, Hofman *et al*. 1997, Torroni *et al*. 1997), and it has been proposed that sets of variants rather than single mutations may underlie certain degenerative diseases (Brown *et al*. 1992).

The mitochondrial genetic background which may contribute to disease has been previously found in Leber's hereditary optic neuropathy. The 3460G>A mutations are distributed randomly along the phylogenetic trees without any preferential association with European haplogroups, whereas the 11778G>A and 14484T>C mutations show a strong preferential association with haplogroup J (Brown *et al*. 1997, Lamminen *et al*. 1997, Torroni *et al*. 1997), findings which suggest that the combination of polymorphisms specific to haplogroup J increases both the penetrance of the two primary mutations 11778G>A and 14484T>C and the risk of disease expression (Torroni *et al*. 1997).

Another association has been found in Alzheimer's disease, where the mutation 4336T>C in the $tRNA^{Gln}$ gene has arisen as a single mutational event in haplogroup H (Shoffner *et al*. 1993, Hutchin & Cortopassi 1995, Torroni *et al*. 1994). This situation is very rare, however (Bonilla *et al*. 1999).

6.4. mtDNA mutations in patients with occipital stroke

Four patients with occipital stroke harboured mutations that were not found among the controls, and three of these represented rare mtDNA genotypes, two of which could not be found in 480 Finnish controls. Two of the mutations were in transfer RNA genes. The 4295A>G mutation in $tRNA^{Ile}$ has been considered aetiologically related to cardiomyopathy in a seven-month-old child (Merante *et al*. 1996) and the 8296A>G mutation in $tRNA^{Lys}$ has previously been associated with diabetes mellitus and hearing impairment. This mutation is thought to explain 0.9 % of diabetes mellitus cases and 2.3 % of those of sensorineural hearing impairment in Japan (Kameoka *et al*. 1998a, 1998b).

Heteroplasmy has been considered one criterion for the pathogenicity of a point mutation. Both 4295A>G and 8296A>G have been shown to be heteroplasmic by restriction analysis and by dot blot hybridisation, respectively (Merante *et al*. 1996, Kameoka *et al*. 1998a). In the light of experience with the apparent heteroplasmy of transition 5656A>G, we checked the heteroplasmy of the two tRNA mutations by subcloning the PCR amplified fragments encompassing them. The 8296A>G mutation turned out to be homoplasmic in blood, but both the mutant genome and the wild type genome were detected in subclones containing a segment encompassing nt 4295. The wild type genome was found only in 2 out of 79 clones, however, suggesting that the degree of heteroplasmy in the blood of the patient was approximately 97 %.

Other point mutations found only in patients have to be considered against the background of the genotype in the network. Patients #148 and #282 (IV) represent unique genotypes in the total of 480 genotypes in the Finnish population. Although a synergistic mutation in LHON and other point mutations leading to amino acid replacements were found in patient #148, it is hard to evaluate their significance for the pathogenesis of occipital stroke because of the unique haplotype. The homoplasmic transition 5773G>A in patient #148 is interesting even though it was also found in a healthy control. It is an invariant nucleotide (Sprinzl *et al*. 1989) in the pseudouridine loop of $tRNA^{Cys}$, and was found to exist in two distinct branches of the haplogroup U network.

Patient #153 harboured the mutation 1850T>C in the gene coding for 16S rRNA. This mutation was located in a dinucleotide pair that is highly conserved between species, but its pathogenic nature is not known. Interestingly, the homoplasmic 1555A>G transition in the 12S rRNA gene has been associated with aminoglycoside-induced or spontaneous non-syndromic progressive deafness. It has been postulated to elongate the region in which the tRNA binds to the ribosome, thus facilitating the binding of aminoglycosides and potentiating their effects on the fidelity of mRNA translation (Prezant *et al*. 1993).

7. Conclusions

We found CSGE, as used here to study the mtDNA genotypes of 43 population samples belonging to haplogroup U, to be an ideal method for screening mutations and polymorphisms in mtDNA. It turned out to be a rapid method with a high capacity and it dramatically alleviated the laborious task of sequencing. A sensitivity of 0.96 was achieved in detecting sequence differences in mtDNA, with a specificity of 1.0. The phylogenetic network for the Finnish mtDNA haplogroup U constructed on the basis of these findings proved to be an unambiguous tree with few homoplasies and pointed to several previously unidentified common polymorphisms.

Ten percent of young patients with an occipital brain infarct are thought to have a mitochondrial disorder and 6% to have the common MELAS mutation 3243A>G. considering the mitochondrial genetics of patients who suffer from an occipital stroke and do not harbor 3243A>G, we found that all those with migraine as a probable aetiology for stroke belonged to mtDNA haplogroup U, suggesting that this genotype confers a risk of occipital stroke. More specific investigations revealed an association of migrainous stroke with haplogroup U5.

In addition to the five patients with migrainous stroke, we analyzed the complete mtDNA coding sequence by CSGE and the HVS I in nine other patients with occipital stroke. The network analysis showed all five patients with migrainous stroke and five others to belong to the cluster U5, one to haplogroup K and the remaining three to the haplotypes U2, U4, and U* intervening between U5 and haplogroup K, suggesting a ratio of 10:1:3, whereas the corresponding ratio in the general population is 10:1:0.75.

Sequence analysis of the entire coding region revealed that the haplotypes in the five patients with migrainous stroke differed from those observed in the controls in only one case (#285). Therefore, if mtDNA poses a risk of migrainous stroke, this risk may be defined by clusters of polymorphisms at nt 11467-12308-12372 or 3197-9477-13617. None of these substitutions may be considered pathogenic as such, but mild pathogenicity cannot be ruled out.

Four patients harboured potentially pathogenic mutations. Two of these were in transfer RNA genes and one in the 16S ribosomal RNA gene. Three mutations led to amino acid replacements. The 4295A>G in $tRNA^{Ile}$ has previously been considered part of the aetiology of cardiomyopathy in a child. In our patient it was found to be

heteroplasmic with a proportion of 97% of mutant DNA in blood. The 8296A>G in $tRNA^{Lys}$ proved to be homoplasmic in blood, but it has previously been associated with diabetes mellitus and hearing impairment. The mutation 1850T>C in the gene coding for 16S rRNA is located in a dinucleotide pair that is highly conserved between species.

Similar phylogenetic networks will be required for the purposes of medical genetics as well as population genetics. Such networks would help in distinguishing between a rare polymorphism and a pathogenic mutation in clinically affected persons. Likewise, they would enable more detailed comparisons to be made between and within populations and allow more accurate phylogenetic relationships to be determined.

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