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INVITED EDITORIAL
Mitochondrial Genetics: Principles and Practice

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Introduction

In the past 4 years, pathogenic mitochondrial DNA (mtDNA) mutations have been associated with a broad spectrum of chronic degenerative diseases. The mtDNA plays a central role in assembly of the mitochondrial ATP generating pathway, oxidative phosphorylation (OXPHOS). OXPHOS consists of five enzyme complexes (I–V), assembled from polypeptides encoded by over 50 nuclear DNA genes and 13 mtDNA genes (table 1) (Shoffner and Wallace 1990). Electrons derived from the oxidation of carbohydrates and fats enter the pathway at complex I or complex II, are transferred to coenzyme Q_{10}, and then move sequentially to complex III, cytochrome c, complex IV, and finally to oxygen, which is the terminal electron acceptor for OXPHOS. The energy released by this electron transfer is used to pump protons across the inner mitochondrial membrane at complexes I, III, and IV. The resulting electrochemical gradient is used by complex V to condense ADP + Pi into ATP. ATP and ADP are then exchanged across the mitochondrial inner membrane by the adenine nucleotide translocator (ANT).

One of the most novel features of mtDNA diseases is their extraordinary phenotypic heterogeneity. Detailed analysis of mtDNA disease pedigrees has shown that this is in part due to five unique characteristics of mitochondrial genetics (Shoffner and Wallace 1990; Wallace 1992). First, mtDNA in humans is maternally inherited, with negligible contributions of mtDNA from the paternal lineage (Giles et al. 1980; Case and Wallace 1981; Gyllensten et al. 1991). Thus, disease manifestations caused by pathogenic mtDNA mutations are concentrated along the maternal lineage. Second, OXPHOS diseases are frequently caused by heteroplasmic mtDNA mutations in which both mutant and normal mtDNAs are present within each cell. Both cell division and mitochondrial proliferation result in a process called "replicative segregation" (Wallace 1986), where cells accumulate variable proportions of mutant and normal mtDNAs. Third, the impact of an mtDNA mutation on a tissue depends on the reliance of the component cells on mitochondrial ATP production (Wallace 1986). Each cell or tissue requires a minimum level of mitochondrial ATP production, its threshold, to maintain normal function. Thus, when mtDNA mutations cause a decline in ATP production, cellular degeneration and death occur as ATP levels fall below the expression thresholds of various tissues. Organ systems with the highest ATP requirements, such as the central nervous system (CNS), will have the most frequent manifestations. Fourth, the mtDNA fixes synonymous and replacement mutations approximately 10 times faster than nuclear OXPHOS genes (Neckelman et al. 1987; Wallace et al. 1987). Since the mtDNA is a highly constrained genome that occupies a pivotal role in energy metabolism, pathogenic mtDNA mutations are likely to be common. Finally, OXPHOS function declines with age (Muller-Hocker 1989, 1990; Trounce et al. 1989; Yen et al. 1991), in parallel with the accumulation of somatic cell mtDNA mutations (Cortopassi and Arnheim 1990; Corral-Debrinski et al. 1991, and in press; Hattori et al. 1991). The high mutation rate of the mtDNA must in part be due to its close proximity to free radicals generated by OXPHOS, its lack of protective histones, and its limited mtDNA repair mechanisms (Clark and Beardsley 1986; Richter et al. 1988; Breimer 1990; Lutz 1990). The accumulation of somatic mutations may augment the effects of inher-

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Table I

Oxidative Phosphorylation

Enzyme Complexes	Nuclear DNA Subunits	mtDNA Subunits
Complex I (NADH-ubiquinone oxidoreductase)	>18 Subunits	7 Subunits (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6)
Complex II (succinate—ubiquinone oxidoreductase)	4 Subunits	0 Subunits
Complex III (ubiquinone–cytochrome c oxidoreductase)	7–9 Subunits	1 Subunit (cytochrome b)
Complex IV (cytochrome c oxidase)	10 Subunits	3 Subunits (COI, COII, and COIII)
Complex V (ATP synthase)	10–16 Subunits	2 Subunits (ATPase 6 and ATPase 8)

ited OXPHOS defects by further impairing OXPHOS function.

Myoclonic Epilepsy and Ragged-Red Fiber (MERRF) Disease and the Mitochondrial Genetics Paradigm

MERRF is a maternally transmitted disorder associated with OXPHOS impairment, myoclonic epilepsy, dementia, ataxia, and mitochondrial myopathy in severely affected individuals (Fukuhara et al. 1980; Rosing et al. 1985; Wallace et al. 1988*b*). However, significant degrees of phenotypic heterogeneity are frequently observed in the maternal lineage family members of MERRF pedigrees. The first clear indication of the role that mitochondrial genetics can play in chronic degenerative diseases came from molecular genetic studies of MERRF. This disease is caused by an A-to-G point mutation at nucleotide pair (np) 8344 in the $tRNA^{Lysine}$ gene (MTTK*MERRF8334) (Wallace et al. 1988*b;* Shoffner et al. 1990). The MTTK*MERRF-8334 mutation impairs mitochondrial protein synthesis when present in high concentrations (Wallace 1986; Chomyn et al. 1991). The MERRF mutation is invariably heteroplasmic, and the percentage of mutant mtDNA, together with the age of family members, has been found to correlate with the severity of the resulting OXPHOS defect and with the extent of clinical manifestations (Wallace et al. 1988*b;* Shoffner et al. 1990).

Additional insights into the relationship between genotype and phenotype in MERRF are provided by three papers presented in this issue of the *Journal* (Boulet et al. 1992; Larsson et al. 1992; Silvestri et al. 1992). Severely affected MERRF patients were found to have skeletal muscle levels of the MTTK*MERRF-8334 mutation ranging from 80% to almost 100% (Boulet et al. 1992; Larsson et al. 1992), values that are typical of severely affected patients (Shoffner et al. 1990). In other maternal lineage family members, who were less severely affected, percentages of the MTTK*MERRF8334 mutation ranged from 0% to 66% in fibroblasts, 0% to 82% in lymphocytes, and 0% to 92% in skeletal muscle (Larsson et al. 1992). Moreover, for each individual, fibroblasts have the lowest levels of the MTTK*MERRF8334 mutation, lymphocytes have intermediate levels, and skeletal muscle has the highest levels, suggesting that the normal mtDNAs impart a selective advantage to rapidly replicating cells, though the extent of this effect may vary between different cell types.

Consistent with the higher proportion of MTTK*-MERRF8334 mutant mtDNAs in muscle, explanted myoblast clones were frequently found to be homoplasmic for the mutant mtDNA, with only a small number of clones being heteroplasmic or homoplasmic for the normal mtDNA (Boulet et al. 1992). Boulet et al. hypothesized that this skewed distribution might be due to a nonrandom mechanism of mtDNA segregation, one that favored selection and fixation of the MTTK*MERRF8334 mutation in myoblasts. They predicted that since myoblasts harboring normal mtDNAs had a growth advantage over those harboring the mutants, patient muscle should be derived from a myoblast population that contained predominantly normal mtDNAs. An alternative explanation could be that MERRF patients develop from ova containing very high proportions of the MTTK*MERRF8334 mutation. Hence, both myoblasts and muscle may have high proportions of mutant mtDNAs. This concept is more likely, since during early development there would be less of a selective advantage for cells with normal mtDNAs. In the fetus, oxygen levels are limited by factors such as a deficiency in the oxygen transporter myoglobin (Weller et al. 1986) and a reduction in oxygen tensions in fetal blood, to about

50% of that in adult arterial blood. Low oxygen tensions are associated with increased glycolytic enzyme transcripts and decreased OXPHOS transcripts (Webster et al. 1990). Hence, during fetal development, the reliance on glycolysis for ATP production could remove any selective growth advantage imparted by normal mtDNAs, allowing myoblasts with high percentages of mutant mtDNAs to persist and participate in myogenesis.

Myotubes with varying percentages of the MTTK*-MERRF8334 mutation demonstrated a sharp decline in mitochondrial protein synthesis levels when the intracellular concentration of normal mtDNAs fell below 15% of the total mtDNAs (Boulet et al. 1992). At this point, the translation efficiency of COI and the cytochrome c oxidase enzyme activity were dramatically decreased. This indicates that the MTTK*MERRF-8334 mutation is functionally recessive, which is consistent with our observations in members of a large MERRF pedigree. When the mutant mtDNAs increased above 85% in our MERRF pedigree, large changes in clinical phenotype occurred with only small changes in genotype (Shoffner et al. 1990). Because of the precipitous nature of the expression thresholds, patients born with high percentages of mutant mtDNAs would be particularly sensitive to further inhibition of OXPHOS function. Therefore, even relatively small reductions in OXPHOS function (Muller-Hocker 1989, 1990; Trounce et al. 1989; Yen et al. 1991) that are associated with the age-related accumulation of somatic mtDNA mutations (Cortopassi and Arnheim 1990; Corral-Debrinski et al. 1991, and in press; Hattori et al. 1991) could be sufficient to take a patient who is near his or her OXPHOS threshold and reduce ATP generation enough to produce clinical manifestations.

In myoblasts and myotubes that contained similar quantities of the MTTK*MERRF8334 mutation, the efficiency of protein synthesis was greater in the myotubes (Boulet et al. 1992). When myoblasts fuse to form myotubes, an induction of muscle-specific genes occurs that coincides with induction of contractile proteins, mitochondrial proliferation, the differentiation of OXPHOS-dependent type I fibers or the glycolysis-dependent type II fibers, and a 2.4–4-fold increase in mtDNA content (Webster et al. 1990). Prominent changes occur in the transcription of OXPHOS genes, including the nuclear-encoded ATP synthase b subunit and the mtDNA-encoded 16S rRNA, ATPase 6, and COIII mRNAs whose expression is high in myoblasts, falls during exit from the cell cycle, and increases again in myotubes (Webster et al. 1990). Transcripts for the heart-muscle and skeletal muscle–specific isoform of the ANT (ANT1) are not present in myoblasts and are specifically induced in myotubes (Webster et al. 1990; Stepien et al. 1992). Hence, the changes in protein synthesis observed by Boulet et al. (1992) are likely to reflect changes in the regulatory mechanisms for mitochondrial mRNA translation that are induced by cell differentiation.

Although the MTTK*MERRF8334 mutation accounts for approximately 80%–90% of MERRF cases, other mtDNA mutations or even nuclear DNA mutations are likely to cause the remaining cases. The third article in this issue of the *Journal* (Silvestri et al. 1992) describes one such mutation, a heteroplasmic T-to-C point mutation at position 8356 of the $tRNA^{Lysine}$ gene (MTTK*MERRF8356). This point mutation may disrupt base pairing at the first np of the TyC stem and has been associated with two independent MERRF pedigrees (Silvestri et al. 1992; Zeviani et al., in press). The clinical description of the patient, by Silvestri et al. (1992), was indistinguishable from the clinical presentations of patients who harbor the MTTK*MERRF-8334 mutation.

All of these observations indicate that there is much left to learn about the relationship between genotype and phenotype in mtDNA diseases. By defining the relationships between the clinical, biochemical, and genetic variability in MERRF pedigrees and the principles of mitochondrial genetics, it became clear that mtDNA mutations could be responsible for an enormous range of disease manifestations that depended on which cell types experienced reductions in ATP-generating capacity to below their threshold. For example, some mtDNA mutations can produce clinical manifestations that are systemic, such as in Kearns-Sayre and many chronic progressive external ophthalmoplegia (CPEO) syndromes, MERRF, mitochondrial encephalomyopathy lactic acidosis and strokelike episodes (MELAS), Leigh disease, and retinitis pigmentosa plus CNS degeneration. In contrast, other mtDNA mutations produce clinical manifestations that are confined primarily to specific tissues, such as the optic nerve in Leber hereditary optic neuropathy (LHON), the extraocular muscles and levator palpebrae in some CPEO cases, the pancreas and auditory nerve in diabetes mellitus and deafness, the cardiac and skeletal muscle in hypertrophic cardiomyopathy and mitochondrial myopathy, and the skeletal muscle in isolated mitochondrial myopathies (table 2). These differences among OXPHOS diseases are not entirely accounted

Table 2

Pathogenic mtDNA Mutations

mtDNA Mutation	Phenotype	Reference(s)
I. Deletions and duplications:		
Spontaneously occurring mtDNA deletions	Kearns-Sayre and CPEO syndromes, Pearson syndrome, malignant migraine	Holt et al. 1988; Rotig et al. 1989; Bresolin et al. 1991; Wallace et al. 1991
Maternally inherited mtDNA deletion	Diabetes and deafness	Ballinger et al. 1992
Spontaneously occurring mtDNA duplications ...	Kearns-Sayre and CPEO syndromes with diabetes mellitus	Poulton et al. 1989
Maternally inherited duplications	Complex Kearns-Sayre and CPEO syndromes which include proximal renal tubulopathy, diabetes mellitus, and cerebellar ataxia	Rotig et al. 1992
II. Point mutations:		
MTCYB*LHON15257: (G to A), aspartate to asparagine	LHON	Brown et al. 1992*b*
MTND4*LHON11778: (G to A), arginine to histidine	LHON, rare pedigrees with LHON and CNS degeneration	Wallace et al. 1988*a;* Larsson et al. 1991
MTCOI*LHON7444: (G to A), termination codon to lysine	LHON	Brown et al. 1992*a*
MTND1*LHON4160: (T to C), leucine to proline	LHON plus CNS degeneration	Howell et al. 1991*b*
MTND1*LHON3460: (G to A), alanine to threonine	LHON	Howell et al. 1991*a;* Huoponen et al. 1991
MTND1*LHON3394: (T to C), tyrosine to histidine	LHON	Brown et al. 1992*a*
Synergistic mtDNA mutations: MTCYB*LHON-15812 (G to A), MTND6*LHON14484 (T to C), MTND5*LHON13708 (G to A), MTND2*LHON5244 (G to A), MTND1*-LHON4917 (A to G), and MTND1*-LHON4216 (T to C)	LHON can occur when these mutations are present in various combinations	Brown et al. 1991, 1992*a,* 1992*b,* 1992*c;* Johns and Berman 1991; Johns and Neufeld 1991; Mackey and Howell 1992
MTND4*MELAS11084: (A to G), threonine to alanine	MELAS	Lertrit et al. 1992
MTATP6*NARP8993: (T to G), leucine to arginine	Retinitis pigmentosa, Leigh disease, highly variable manifestations in single- or multiple-organ systems	Holt et al. 1990; Tatuch et al. 1992; Shoffner et al., in press
MTTK*MERRF8344: (G to A)	MERRF, Leigh disease, highly variable manifestations in single- or multiple-organ systems	Wallace et al. 1988*b;* Berkovic et al. 1989; Shoffner et al. 1990
MTTK*MERRF8356: (T to C)	MERRF, highly variable manifestations in single- or multiple-organ systems	Silvestri et al. 1992; Zeviani et al., in press
MTTL1*MELAS3243: (T to C)	MELAS, highly variable manifestations in single- or multiple-organ systems	Goto et al. 1991
MTTL1*MMC3260: (A to G)	Hypertrophic cardiomyopathy plus mitochondrial myopathy	Zeviani et al. 1991
MTTL1*MM3250: (T to C)	Mitochondrial myopathy	Goto et al. 1990, 1992*a,* 1992*b;* unpublished observation
MTTL1*MELAS3243: (A to G)	MELAS, CPEO syndromes, Leigh disease, diabetes mellitus, highly variable manifestations in single- or multiple-organ systems	

NOTE.—Human Gene Mapping (HGM) designations are given for each point mutation (Wallace et al. 1991).

for by replicative segregation of mutant and normal mtDNAs. Since these clinical manifestations are found in many commonly encountered disease classes, such as diabetes mellitus (Ballinger et al. 1992) and cardiomyopathies (Shoffner and Wallace 1992), mtDNA mutations are likely to contribute to the genetic heterogeneity of these disorders. As our understanding of the molecular genetics of OXPHOS diseases matures, valuable insights into common mechanisms for disease are likely to emerge. Clearly, one relevant variable is the interaction between nuclear and cytoplasmic genes and the cumulative effects of multiple mutations. In this issue of the *Journal,* Mackey and Howell (1992) provide further support for the relationship between multiple mtDNA mutations and LHON.

LHON

LHON is a maternally inherited OXPHOS disease in which individuals experience acute, painless loss of vision in the central visual field (Newman et al. 1991). The typical ophthalmoscopic features of acute LHON include circumpapillary telangiectatic microangiopathy and swelling of the nerve fiber layer around the optic disk. Once visual loss has occurred, spontaneous recovery is uncommon but has been reported in a few patients (Lessell et al. 1983; Stone et al. 1992). Approximately 40%–60% of LHON cases are caused by a G-to-A point mutation in the ND4 gene at position 11778 (MTND4*LHON11778) (Wallace et al. 1988*a*), and approximately 20% of cases are caused by a G-to-A point mutation in the ND1 gene at position 3460 (MTND1*LHON3460). Mutations in the ND1 gene at position 4160 (MTND1*LHON4160) and in the cytochrome b gene at position 15275 (MTCYB*LHON15257), as well as a growing cohort of synergistic mutations, account for many of the remaining cases (table 2). To date, all LHON mutations have been missense mutations in OXPHOS subunits of complexes I, III, and IV.

Synergistic mtDNA mutations may produce a pathogenic reduction in ATP-generating capacity by their cumulative effect on OXPHOS function. For example, the missense mutation at position 13708 of the ND5 gene (MTND5*LHON13708) has been found in conjunction with almost all of the other synergistic point mutations and appears to be an important premutation for the development of the LHON phenotype (Brown et al. 1991, 1992*b;* Johns and Berman 1991; Johns and Neufeld 1991). The MTND5*LHON-13708 mutation is a G-to-A transition that changes a leucine in ND5 at amino acid 458 to a polar threonine. This mutation is present at a prevalence rate of 5% (16/320) in the Caucasian population (Johns and Berman 1991; Brown et al. 1992*b*). However, an increase in the probability for the expression of LHON occurs when an mtDNA lineage with the MTND5*LHON-13708 mutation also harbors a homoplasmic missense mutation in the cytochrome b gene at np 15257 (MTCYB*LHON15257), which changes a highly conserved aspartate to an asparagine. Additional confounding mutations can include a second cytochrome b mutation at np 15812 (MTCYB*LHON15812), which changes a valine to a methionine, and a heteroplasmic missense mutation in the ND2 gene at np 5244 (MTND2*LHON5244), which changes a glycine to a serine (Brown et al. 1992*b*).

Additional evidence that synergistic interactions among mtDNA mutations can cause LHON is presented in this issue of the *Journal* (Mackey and Howell 1992). Two large maternal LHON pedigrees are reported that are distinctive in showing significant recoveries of visual acuity in affected individuals who were less than 30 years old. Sequencing the mtDNA-encoded subunits of complex I and III from these two families revealed 10 missense mutations. The pathological significance of these mutations is difficult to evaluate, because of the small numbers of population controls analyzed, the limited phylogenetic comparisons performed, and the availability of sequence data on only half of the mitochondrial genome. However, of these mutations, two homoplasmic mutations and one heteroplasmic mutation showed interesting associations with LHON in these two pedigrees. The MTND5*LHON13708 (Brown et al. 1991, 1992*b;* Johns and Berman 1991) and MTND1*LHON4216 mutations (Johns and Berman 1991) were homoplasmic in both Australian pedigrees. These mutations are found together at increased frequencies in LHON pedigrees, relative to controls (Johns and Berman 1991). A missense mutation was identified at position 14484 of ND6 (MTND6*LHON14484), which changed a poorly conserved methionine to a valine. The MTND6*LHON-14484 mutation has been found in five LHON pedigrees: three Australian pedigrees (Howell et al. 1991*b;* Mackey and Howell 1992), a singleton case of LHON (Mackey and Howell 1991), and one American pedigree (Brown et al. 1992). This mutation was heteroplasmic in only one of these pedigrees (Vic2 in Mackey and Howell 1992). In the two Australian pedigrees presented in this issue of the *Journal* (Mackey and Howell 1992), the cumulative effect of the

MTND5*LHON13708, the MTND1*LHON4160, and the MTND6*LHON14484 mutations on OXPHOS function may produce sufficient impairment of ATP generation in the optic nerve to result in LHON. Sequence data on the remaining portions of the mtDNA from these Australian pedigrees will be important for assessing whether other relevant nucleotide substitutions are present.

Practical Applications

The numerous complexities in correlating phenotype with genotype that have been discussed above make it particularly difficult to manage patients and counsel family members. Therefore, it is essential that a coordinated approach to patient diagnosis, mutation identification, genetic counseling, and institution of metabolic therapies be utilized. Our diagnostic approach to these patients focuses on six elements: (1) clinical evaluation, (2) pedigree analysis, (3) metabolic testing, (4) enzymological analysis of OXPHOS function in skeletal muscle, (5) histochemistry and electron microscopy of skeletal muscle, and (6) mtDNA mutation analysis in appropriate tissues. In many individuals, clinical evaluation, pedigree analysis, and metabolic testing permit recognition of phenotypes that are associated with specific mtDNA mutations. For most mtDNA point mutations, blood cell DNA can then be tested, and, if the expected pathogenic mtDNA mutation is identified, no further diagnostic testing is required. Other maternal lineage family members can then be tested for the mutation. However, most mtDNA deletions are not readily detected in blood (Holt et al. 1988), and failure to detect an mtDNA point mutation in blood does not preclude its presence in muscle and brain. By contrast, muscle, being a stable tissue, tends to retain all mtDNA mutations. Hence, additional analyses to address these issues necessitates muscle biopsy. We divide the skeletal muscle into three portions: one for histochemistry and electron microscopy, one for OXPHOS enzymology, and one for mtDNA analysis. In order to perform OXPHOS enzymology, mitochondria are immediately isolated from fresh muscle to reduce artifacts associated with sample handling and freezing (Zheng et al. 1991). Each respiratory complex is then assayed by our optimized procedures and compared with a group of controls. This has proved to be a highly sensitive approach for establishing whether a patient's clinical manifestations represent an OXPHOS disease. Further analyses of muscle mtDNA can then be conducted to identify causal mutations. The development of this integrated clinical and laboratory protocol has greatly enhanced our ability to diagnose, counsel, and treat patients with OXPHOS disorders, which are increasingly being recognized as a common cause of chronic, degenerative diseases.

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