LETTER TO JMG

Sequence variation in mitochondrial complex I genes: mutation or polymorphism?

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Background: Defects of the mitochondrial genome are recognised as common causes of genetic disease. Sequencing of large portions or even the entire mitochondrial genome is routine in many laboratories for the investigation of mitochondrial disease. However, establishing whether a detected sequence change is polymorphic or pathogenic is still a major difficulty because of its highly polymorphic nature. This has major implications for the patient and the family.

Objective: To describe a scoring system for determining the likelihood that a given sequence variant in one of the seven mitochondrially encoded complex I (*MTND*) genes is truly pathogenic.

Results: The scoring system was applied to 50 reported *MTND* mutations. Using this system, 21 of the mutations analysed fell into the group of neutral sequence variants, 10 were classified as possibly pathogenic, three as probably pathogenic, and 16 as almost certainly pathogenic.

Conclusions: The proposed scoring system should advance the interpretation of sequence variants and ensure that candidate pathogenic mutations are rigorously investigated.

Mitochondrial DNA (mtDNA) disorders are an important group of genetic diseases with many different clinical manifestations.^{1,2} Symptoms range from a mild disturbance of eye movement in late adult life to neonatal death associated with profound lactic acidosis.¹ In the presence of such clinical heterogeneity, it is too much to hope that the investigation of mtDNA disease will be straightforward.³ Two main issues seriously complicate the genetic diagnosis of mtDNA disease: first, the presence of both heteroplasmic and homoplasmic mtDNA mutations; and second, the highly polymorphic nature of the mitochondrial genome. This latter property reflects the high mtDNA mutation rate.³

Population polymorphic variants have been extremely helpful in studies of human evolution.⁴ In addition, the polymorphic nature of mtDNA, combined with its high copy number in most cell types, has been valuable for the field of forensic medicine and in the identification of human remains.⁵ In contrast, the polymorphic nature of the mitochondrial genome has hindered diagnostic studies because there are relatively few pathogenic mutations that have been described in multiple independent families. The question arises, therefore, whether a mutation detected in the mitochondrial genome of a patient with a suspected mitochondrial disorder is a rare polymorphism or a pathogenic mutation. The difficulties posed in the diagnosis of mitochondrial disease were recognised by Walker *et al*⁶ and by DiMauro and Schon.⁷ DiMauro and Schon proposed five canonical criteria which they suggest should be met in order

to support a pathogenic role for a novel mtDNA mutation, but these criteria depend heavily on the presence of heteroplasmy. The discovery of an increasing number of homoplasmic mtDNA mutations means that other methods to define pathogenicity should be considered.

The seven complex I genes form a large proportion of the mitochondrial genome, and defects of these genes are increasingly being recognised as important causes of respiratory chain disease. Complex I deficiencies are the most common defects of the respiratory chain and the relative ease of mitochondrial DNA sequencing has inevitably led to an upsurge in the sequencing of these genes to search for mutations in patients with a suspected mitochondrial disorder. These sequence analyses have highlighted the variation within these genes and the importance of identifying factors that will differentiate pathogenic mutations from benign polymorphisms. We have recently explored the variation seen in the mitochondrial tRNA genes and identified important factors for pathogenicity.⁸ In this report, we describe a similar approach to the mitochondrial complex I genes. We have tried to factor in those characteristics that are most likely to determine pathogenicity, including the presence of a biochemical defect, functional studies in cell lines, heteroplasmy, segregation within a family, multiple independent reports, and evolutionary conservation. We believe that this scoring system will be valuable not only in defining which of the reported mutations are truly pathogenic, but also for setting guidelines for the effective investigation of patients with suspected mtDNA disease.

METHODS

A scoring system was devised to assess the pathogenicity of complex I (*MTND*) gene mutations listed on the MITOMAP database up to June 2004⁹ and the three recently published additional *MTND1* mutations.¹⁰ For each mutation listed, the references cited were collected and analysed. Each mutation was subsequently awarded a pathogenicity score (maximum score = 40) using the evidence provided in the references in support of pathogenicity. Table 1 shows the scoring system criteria.

In addition to the functional and canonical criteria described below, candidate pathogenic mutations that have been reported on two or more occasions by independent groups were scored 5 additional points.

Three functional criteria were included in our system. The first was whether the mutation results in a measurable biochemical defect within tissues: low complex I activity, low oxidation of complex I substrates, or abnormalities of complex I using blue native (BN)-PAGE. Eight points were awarded if a biochemical defect was detected in affected

Abbreviations: LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; mtDNA, mitochondrial DNA

Table 1 Complex I mutation pathogenicity scoring system criteria

Criteria	Maximum score
Biochemical defect	Demonstrated in affected tissues 8
	Demonstrated in multiple tissues 2
Functional studies	Single fibre PCR and/or cybrid studies 7
Reports from two or more independent laboratories of the mutation in patients with clinical disease	5
Heteroplasmy	5
Segregation of variant with disease within a family	3
Conservation	10
	-1 if a variant is seen in a mammal -1 if a variant is seen in a primate -1 if there are ≥ 2 variants or there are ≥ 4 mammals with variants Up to -3 for variation in any of the four adjacent residues -2 if variants are from different amino acid classes -2 if variant is seen on databases in the absence of mtDNA disease
Total score	40

mtDNA, mitochondrial DNA; PCR, polymerase chain reaction.

tissues, and a further two points were added if this defect was demonstrated in more than one tissue. This category was weighted heavily, because a mutation should be associated with a biochemical defect to be considered pathogenic. The total score of 10 was divided inequitably because in many cases only one tissue is available for investigation. Points were added for both isolated complex I defects and for complex I defects combined with other respiratory chain deficiencies in order to reflect current thinking that the complexes of the mitochondrial respiratory chain may interact.

The second criterion included the use of functional studies such as *transmitochondrial* cybrids and single muscle fibre polymerase chain reaction (PCR) studies on histochemically abnormal muscle biopsies, to demonstrate pathogenicity.^{11–13} This category was awarded seven points. Functional studies provide high quality evidence in support of pathogenicity, and *transmitochondrial* cybrids can be applied to both homoplasmic and heteroplasmic mutations. It was appreciated that the techniques required are not universally available, and that many mutations that are accepted as pathogenic predate the development of these techniques.

Three of the original canonical criteria⁷ were included in the complex I scoring system. The first was heteroplasmy, which was awarded five points. This category was weighted moderately because, although many pathogenic mutations are heteroplasmic, a significant number are homoplasmic. The second canonical criterion was that the mutation should segregate with the disease within a family. This finding was worth three points and included mtDNA mutations that arise *de novo*. The third canonical criterion and the one weighted most heavily was the evolutionary conservation of the amino acid residue affected by a mutation. The extent of conservation was assessed with the mtSNP Database mtSAP evaluation approach¹⁴ and was awarded a maximum of 10 points. In this approach, a panel of 61 mammals was considered and a point deduction system was used. One point was deducted from the maximum score if any variant was seen in any mammal, and a further point was deducted if any variant was seen in a primate. If there were two or more amino acid variants seen in the panel, or if variants were seen in four or more mammals, another point was subtracted. If the variants were from different amino acid classes, a further two points were deducted. If the mutation was seen on the MitoKor database¹⁵ or in other available databases¹⁴ in the absence of

mitochondrial disease, two more points were subtracted. Finally, up to three points were deducted for any variation in any of the four amino acid residues that bracket the residue of interest. For those mutations that induce a significant truncation of a protein, we awarded a score based on the amino acids lost. The 11832G→A was the only nonsense mutation in complex I genes reported on MITOMAP. Finally, we did not score the mutations based on their site within the tertiary structure of the protein. While pathogenic mutations commonly involve the transmembranous domains, there are well recognised examples outside these regions (such as the 3460G→A Leber's hereditary optic neuropathy (LHON) mutation).

RESULTS

In all, 47 point mutations within the *MTND* genes had been reported on the MITOMAP database up to June 2004.⁹ In addition, three recently published mutations were analysed.¹⁰ Therefore a total of 50 mutations (listed in table 2 and in the supplementary table, which can be viewed on the journal website: www.jmedgenet.com/supplemental) were scored using the complex I pathogenicity scoring system. Pathogenic mutations have been reported in all of the *MTND* genes: 16 in *MTND1* (including the three recently published mutations), five in *MTND2*, two in *MTND3*, one in *MTND4L*, seven in *MTND4*, nine in *MTND5*, and 10 in *MTND6*.

The putative mutations attained scores ranging from 0 to 39. Twenty one mutations attained scores of 0 to 10, 10 attained scores of 11 to 20, three attained scores of 21 to 29, and 16 attained scores of 30 to 40. Of the 47 mutations listed on MITOMAP, 28 (60%) were given a "provisional" pathogenic status, meaning that they had been reported either on one occasion only or by one group only. Three (6%) had been given the status of "unclear" pathogenicity, and 13 (28%) were listed as "confirmed" pathogenic mutations. Eight mutations (17%) appear not only in the MITOMAP list of pathogenic mutations but also in their list of 457 neutral sequence variants that have been reported in the *MTND* genes. Of these eight, three were assigned polymorphic status, two were listed as "unclear pathogenicity", two as "provisional", and one as a "confirmed" pathogenic mutation on MITOMAP. Thus one cannot rely solely on the available databases when attempting to decide if a sequence change is pathogenic or benign.

Table 2 *MTND* gene mutations analysed with the complex I pathogenicity scoring system

Gene	Mutation	Status*	Amino acid substitution	Disease association	Score	MITOMAP status
<i>MTND1</i>	3308T→C	Het	M→T	MELAS	5	Polymorphism
	3316G→A	Hom	A→T	NIDDM, LHON	5	Unclear
	3394T→C	Hom	Y→H	LHON, NIDDM	3	Unclear
	3397A→G	Hom	M→V	AD/PD	5	Provisional
	3460G→A	Hom/Het	A→T	LHON	39	Confirmed
	3496G→T	Hom	A→S	AD/PD and LHON	2	Provisional/secondary
	3497C→T	Hom	A→V	LHON	0	Provisional/secondary
	3635G→A	Hom	S→N	LHON	9	Provisional
	3697G→A	Het	G→S	MELAS	32	Reference 10
	3796A→G	Het	T→A	Adult onset dystonia	13	Provisional
	3946G→A	Het	E→K	MELAS	32	Reference 10
	3949T→C	Het	Y→H	MELAS	32	Reference 10
	4136A→G	Hom	Y→C	LHON	6	Provisional
	4160T→C	Hom	L→P	LHON	5	Provisional
	4171C→A	Hom/Het	L→M	LHON	17	Provisional
	4216T→C	Hom	Y→H	LHON	5	Confirmed
	4640G→A	Hom	I→M	LHON	12	Provisional
	4917A→G	Hom	N→D	LHON	0	Confirmed
	5244G→A	Het	G→S	LHON	9	Provisional
	5460G→A	Hom/Het	A→T	AD	0	Polymorphism
<i>MTND2</i>	5460G→T	Hom/Het	A→S	AD	7	Provisional
	10158T→C	Het	S→P	Leigh	33	Confirmed
<i>MTND3</i>	10191T→C	Het	S→P	ESOC/Leigh-like	32	Confirmed
<i>MTND4L</i>	10663T→C	Hom	V→A	LHON	31	Provisional
<i>MTND4</i>	11084A→G	Hom/Het	T→A	MELAS	6	Polymorphism
	11232T→C	Het	L→P	CPEO	24	Provisional
	11696G→A	Het	V→I	LHON	17	Provisional
	11777C→A	Het	R→S	Leigh	32	Provisional
	11778G→A	Hom/Het	R→H	LHON	34	Confirmed
	11832G→A	Het	W→Ter	Exercise intolerance	33	Provisional
	12026A→G	Hom	I→V	DM	2	Provisional
	12706T→C	Het	F→L	Leigh	30	Confirmed
	12770A→G	Het	E→G	MELAS	17	Provisional
	13045A→C	Het	M→L	MELAS/LHON/Leigh overlap syndrome	25	Provisional
<i>MTND5</i>	13084A→T	Het	S→C	MELAS/Leigh	26	Provisional
	13513G→A	Het	D→N	MELAS/Leigh	39	Confirmed
	13514A→G	Het	D→G	MELAS	37	Confirmed
	13528A→G	Hom	T→A	LHON-like	1	Provisional
	13708G→A	Hom	A→T	LHON	7	Unclear
	13730G→A	Het	G→E	LHON	9	Provisional
	14453G→A	Het	A→V	MELAS	19	Provisional
	14459G→A	Hom/Het	A→V	LDYT/Leigh	37	Confirmed
	14482C→A	Hom/Het	M→I	LHON	10	Provisional
	14482C→G	Hom/Het	M→I	LHON	17	Confirmed
	14484T→C	Hom/Het	M→V	LHON	37	Confirmed
	14487T→C	Het	M→V	Leigh/dystonia	39	Confirmed
	14495A→G	Het	L→S	LHON	17	Provisional
	14498T→C	Hom/Het	Y→C	LHON	17	Provisional
	14568C→T	Hom	G→S	LHON	7	Provisional
	14596A→T	Hom	I→M	LHON	19	Provisional

Fifty *MTND* gene mutations listed on the MITOMAP database⁹ or recently reported¹⁰ were given a pathogenicity score (maximum score of 40) according to the criteria described in the Methods and in table 1. The acronyms and abbreviations used to describe the clinical and disease presentations not used in the text are as given on the MITOMAP database.

*Status refers to whether a mutation is described as heteroplasmic (Het) or homoplasmic (Hom) on MITOMAP.

AD, Alzheimer's disease; CPEO, chronic progressive external ophthalmoplegia; DM, diabetes mellitus; ESOC, epilepsy, stroke-like episodes, optic atrophy and cognitive decline; LDYT, LHON and dystonia; LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; NIDDM, non-insulin dependent diabetes mellitus; PD, Parkinson's disease.

Of the 50 mutations, 22 (44%) were heteroplasmic, 18 (36%) were homoplasmic, and 10 (20%) have been reported as both heteroplasmic and homoplasmic. Furthermore, these 50 mutations analysed were reported in association with a wide variety of diseases. The most common disease to be associated with mutations in the *MTND* genes was LHON, with 28 of the 50 mutations (56%) being reported in association with this mitochondrial disease. The mean score for these 28 mutations was 14, with a maximum score of 39 and a minimum score of 0. Eleven mutations, including the three recently published ones, were reported to be associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke like episodes (MELAS). The mean score achieved by these mutations was 25, with a maximum of 39 and a minimum of 5. The third most common disease

associated with mutations in the *MTND* genes was Leigh disease, which was associated with seven of the 50 mutations. The mean score for this group was 34, with a maximum score of 39 and a minimum score of 26.

The LHON mutations are subclassified into four groups in the published literature: those that are accepted as primary (directly causative) mutations, of which there are three (11778G→A, 14484T→C and 3460G→A); those that are novel mutations reported to be primary because they are not found in association with a known primary mutation; those that are secondary (found in association with disease but only when a primary mutation is present); and those mutations that are reported in overlap syndromes where LHON is only one clinical feature (for example, with Leigh's disease or MELAS). The mean score for the three primary

Figure 1 Graph showing the distribution of pathogenicity scores attained by mutations reported to be associated with LHON on the MITOMAP database.² White rectangles represent mutations classed as secondary LHON mutations; grey rectangles represent rare mutations reported as primary mutations; hatched rectangles represent mutations associated with LHON-overlap syndromes; black rectangles represent the three well characterised primary LHON mutations (3460G→A, 11778G→A, and 14484T→C). LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes.

LHON mutations was 37 (maximum 39, minimum 34), whereas the mean for novel "primary" mutations was 13 (maximum 31, minimum 0). The mean for secondary LHON mutations was 5 (maximum 9, minimum 0) and the mean score for overlap mutations was 31 (maximum 37, minimum 25) (fig 1).

DISCUSSION

The investigation of patients with suspected mitochondrial disorders now commonly includes sequence analysis to detect mtDNA mutations. Sequence variants arise frequently in the mtDNA, and it is important to distinguish between those changes that are neutral and those that are pathogenic. The canonical criteria of DiMauro and Schon⁷ are currently used to assess the pathogenic potential of novel sequence variants. However, these criteria have various limitations which suggest that additional criteria are needed to assess the likely pathogenic nature of individual sequence changes.

Defects of complex I are the most common biochemical abnormalities in patients with mitochondrial respiratory chain disorders. The seven *MTND* genes span just over 6000 bases of the mitochondrial genome, comprising 38% of the total mtDNA. The first-ever mtDNA point mutation was described in an *MTND* gene, and over the past 17 years, almost 100 pathogenic point mutations in the mitochondrial genome have been reported.

Devising a system to determine reliably the pathogenic nature of mtDNA mutations is important. We must be as confident as possible about the pathogenic nature of any sequence change, because there are profound implications for the patient and the family. Our scoring system was "woven" from three separate strands: the canonical criteria of DiMauro and Schon, functional evidence of pathogenicity, and multiple reports of pathogenicity of a mutation from independent groups. The numerical values assigned to each criterion within the scoring system were carefully balanced to allow for some evidence to be weighted more heavily.

To define the boundaries of neutral sequence variants, the status of mutations on MITOMAP was critically evaluated. Eight mutations included on the list of pathogenic mutations were also listed among the large group of neutral polymorphisms in complex I genes. The mean score for these mutations was 5 and all changes with scores of 10 and below had little evidence to suggest that they were pathogenic. Thus we defined any changes with scores of 10 and less as "polymorphisms"

MITOMAP status was considered when setting the boundaries to define mutations as "pathogenic". The mean

score achieved by those mutations listed as "confirmed" on MITOMAP was 29, with a maximum score of 39 and a minimum of 0. Within this group, two secondary LHON mutations scored especially poorly: the 4917A→G mutation scored 0 and the 4216T→C mutation scored 5. It is now recognised that these sequence changes are haplogroup markers¹⁶ and a direct pathogenic role is unlikely. If these two mutations are excluded, a more realistic mean score of 34 is achieved. Therefore, those candidate mutations scoring 30 or more advance to the category of "pathogenic".

Setting the boundaries for the other two groups was more difficult. It was decided that, because many of the mutations associated with MELAS and Leigh disease with good evidence of pathogenicity scored between 21 and 29, these would be the boundary scores for the "probably pathogenic" group. The scores defining the "possibly pathogenic" group were set, by the process of elimination, at 10 to 20.

Using this grouping system, 21 of the 50 complex I mutations (42%) fell into the group of neutral sequence variants, 10 were classified as possibly pathogenic, three as probably pathogenic, and 16 (32%) as pathogenic. We recognise that these groupings are somewhat arbitrary, but they are designed to act as guides and each mutation should be evaluated on the evidence provided. We also note that almost half of this set of mutations was probably misclassified as pathogenic, and that the value of a more evidence based scoring system will benefit this area.

The 50 point mutations analysed using the scoring system were distributed throughout the seven *MTND* genes. If all sequence variants listed on MITOMAP, both polymorphic and pathogenic, are considered, the *MTND5* gene contains the most sequence changes with 141. The *MTND4*, *MTND2*, *MTND1*, and *MTND6* genes contain 91, 74, 85, and 56, respectively. The fewest mutations arose in the *MTND3* and *MTND4L* genes with 37 and 23, respectively. If only those mutations scoring above 20, specifically those that are probably or almost certainly pathogenic, are considered, five arose in *MTND5*, four in *MTND1*, four in *MTND4*, three in *MTND6*, two in *MTND3*, one in *MTND4L*, and none in the *MTND2* gene.

These differences in the number of mutations in the different *MTND* genes can be simply explained by normalising the number of substitutions to the sizes of the genes. Thus the *MTND5* gene is the largest of the complex I genes, spanning 1811 bases, and it contains the most sequence variants (141). The second, third, fourth, and fifth largest genes are *MTND4* (1377 bases), *MTND2* (1041 bases), *MTND1* (955 bases), and *MTND6* (524 bases), respectively. The

MTND3 and *MTND4L* genes are the smallest, spanning 345 and 296 bases, and these genes had the fewest sequence variants with 37 and 23, respectively. Each gene therefore has a comparable proportion of variant sites of between 6.6% and 10.7%.

The number of point mutations scoring 20 or above also roughly corresponds to the size of the gene with *MTND5* containing the most (five). The percentage of pathogenic sites within each gene is also comparable, ranging from 0 to 5.4%. However, no point mutations scoring 20 or above were reported in *MTND2*, despite being the third largest of the complex I genes. This may simply indicate that this gene is not commonly screened for mutations, and a small, 2 bp deletion in *MTND2* has been shown to be pathogenic.¹⁷ Alternatively, this gene might have a higher "tolerance" for mutation, or a much lower tolerance (that is, mutations might tend to produce embryonic lethal phenotypes).

The mutations associated with LHON highlight many of the difficulties seen with defining pathogenicity of complex I mutations. The mean score for the primary LHON mutations (11778G→A, 14484T→C and 3460G→A) was 37 (maximum 39, minimum 34). However, the mean score for novel "primary" mutations was much lower, at just 13 (maximum = 26, minimum = 1). These values suggest that some of these mutations are unlikely to be pathogenic, or—at the very least—that the mutation warrants further investigation. We would suggest that, in patients with LHON-like conditions who carry a candidate mutation that is not one of the well established primary mutations, complete sequence of the mitochondrial encoded complex I genes and functional studies should be determined in an effort to put the assignment of pathogenicity on a firmer footing. Confirming pathogenicity to a particular sequence variant is often difficult because of the limited family information and biochemical evidence available. However, if incorrect, the implications of wrong assignments for the patient and the rest of the family are considerable.

The mean score for the secondary LHON mutations, such as the 13708G→A mutation that often occurs in conjunction with the 14484T→C primary mutation,¹⁸ was just 5 (maximum 9, minimum 0). Similar low values are obtained for other secondary mutations and we suggest that these are most likely to be neutral sequence variants. It is recognised that mtDNA of haplogroup J influences the clinical course of LHON in patients with the 14484T→C mutation, but no specific haplogroup associated mutation has been identified unequivocally.¹⁹

Conclusions

Molecular genetic techniques have improved greatly over the last decade, and mtDNA sequencing is now commonplace in the diagnostic laboratory. The challenge for investigators lies not in detecting mutations, but in determining which of them are pathogenic. The complex I pathogenicity scoring system provides researchers with guidance as to which features confer pathogenicity to a mutation. The scoring system can be used to assess the pathogenicity of novel mutations detected in the *MTND* genes of a patient with suspected mitochondrial disease and it can be used to evaluate published evidence implicating an *MTND* gene mutation in disease. The scoring system is easy to apply and it improves upon the canonical criteria by balancing both canonical evidence of pathogenicity and functional evidence of pathogenicity. The pathogenicity groupings provide a reasonable guideline as to the pathogenicity of any given *MTND* gene mutation.

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The supplementary table can be viewed on the journal website: www.jmedgenet.com/supplemental

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