Biochemical and molecular diagnosis of mitochondrial respiratory chain disorders

David R. Thorburn, Canny Sugiana, Renato Salemi, Denise M. Kirby, Lisa Worgan, Akira Ohtake, Michael T. Ryan

Abstract

Biochemical diagnosis of mitochondrial respiratory chain disorders requires caution to avoid misdiagnosis of secondary enzyme defects, and can be improved by the use of conservative diagnostic criteria. Pathogenic mutations causing mitochondrial disorders have now been identified in more than 30 mitochondrial DNA (mtDNA) genes encoding respiratory chain subunits, ribosomal- and t-RNAs. mtDNA mutations appear to be responsible for most adult patients with mitochondrial disease and approximately a quarter of paediatric patients. A family history suggesting maternal inheritance is the exception rather than the norm for children with mtDNA mutations, many of whom have de novo mutations. Prenatal diagnosis and pre-implantation genetic diagnosis can be offered to some women at risk of transmitting a mtDNA mutation, particularly those at lower recurrence risk. Mutations in more than 30 nuclear genes, including those encoding for respiratory chain subunits and assembly factors, have now been shown to cause mitochondrial disorders, creating difficulties in prioritising which genes should be studied by mutation analysis in individual patients. A number of approaches offer promise to guide the choice of candidate genes, including Blue Native-PAGE immunoblotting and microarray expression analysis.

Keywords: Mitochondrial disease; Respiratory chain; Mutation; mtDNA; Assembly; Microarray

1. Introduction

Genetic disorders of the mitochondrial respiratory chain are probably the most common group of inborn errors of metabolism, affecting at least 1 in 5000 individuals [1]. Investigation and management of these disorders pose problems at multiple levels. There is an extraordinary diversity of clinical presentations, affecting most organ systems, alone or in combination, and with almost any age of onset. Some patients present with symptoms that are strongly suggestive of a mitochondrial cytopathy, but many have nonspecific symptoms that overlap with other diagnoses. When a mitochondrial aetiology is suspected, the first decision is how to investigate the patient in the most efficient, least invasive and least costly manner. Direct analysis for mitochondrial DNA (mtDNA) rearrangements or common point mutations in blood or urine sediment is worth attempting in patients suspected of classic conditions such as LHON, MELAS, MERRF, CPEO, Kearns–Sayre, Pearson and Leigh syndromes. However, in other patients,
the diagnostic yield of such analysis can become vanishingly small. Mutations in more than 30 nuclear genes are known to cause mitochondrial dysfunction [2], but clinical suspicion alone is usually not enough to warrant mutation analysis of specific genes.

In many patients, particularly children, diagnosis relies heavily on identifying deficient activity of one or more of the mitochondrial respiratory chain enzymes. An enzyme diagnosis is often the starting point for further investigation of the underlying molecular aetiology. We will review recent advances in interpretation of enzyme investigations, some evolving concepts about mtDNA mutations and approaches to identifying the genetic basis of respiratory chain disorders.

2. Respiratory chain enzymes and diagnostic criteria

If the residual activity of one or more respiratory chain enzymes is markedly deficient in a tissue biopsy or cultured cell line then the results may be regarded as definitively abnormal. In many cases though, residual enzyme activity may be only moderately decreased or some other factor may make interpretation difficult. Problems in the biochemical diagnosis of respiratory chain enzyme defects have been reviewed previously [3–7]. Briefly, these include tissue specificity, difficulty in defining realistic normal ranges, inability of enzyme assays to detect some functional defects, variation in assay protocols and lack of widely accepted diagnostic criteria and a quality assurance scheme.

Perhaps the major current problem in diagnosis of mitochondrial disorders is distinguishing between a primary respiratory chain enzyme defect and a secondary defect caused by factors such as tissue pathology or problems in sample processing. The difficulty in defining normal ranges is illustrated by comparing the normal ranges for complex I activity in skeletal muscle quoted by different centres. The quoted lower limit of the normal range varies from 24% to 28% [8–10], 36% to 51% [11–14] to as high as 79% [15] of the control mean. Admittedly, these ranges are defined using different methods to express complex I (relative to protein amount, citrate synthase or other respiratory chain enzyme activities) but opinions clearly differ on how low the activity needs to be to be regarded as deficient.

Our experience is that tissue pathology (e.g., caused by other inborn errors of metabolism or non-metabolic causes impacting on mitochondrial numbers or function) can widen the normal ranges for respiratory chain enzymes even when expressed as enzyme ratios. One needs to be especially wary of using just citrate synthase ratios, which may be prone to misdiagnosis if used without considering the whole profile of enzyme activities and tissue pathology [7]. Such issues led us to develop a conservative diagnostic classification scheme based on the rationale that a definite diagnosis must have support from at least two types of evidence. The system is analogous to the revised Jones criteria for rheumatic fever [16] and defines objective diagnostic criteria as major or minor criteria for clinical, pathological, enzymatic, functional, molecular and metabolic parameters [17]. Diagnostic certainty is defined as definite, probable or possible depending on the number of major and minor criteria. A similar approach has been proposed elsewhere [18], and such schemes offer the potential to minimise misdiagnoses and act as a basis for development of consensus diagnostic criteria.

3. Evolving concepts about mtDNA mutations

In the 16 years since they were first identified, it has become apparent that mtDNA mutations are a common cause of mitochondrial disease in adult patients, but are less common than nuclear defects in children with mitochondrial disease. However, there appear to be some common misconceptions that may potentially hinder the optimal approach to investigation and management of patients, particularly children. Several of these misconceptions are discussed below.

mtDNA mutations are not a negligible cause of mitochondrial disease in children but account for probably a quarter of diagnosed patients. We have identified pathogenic mtDNA mutations in 61 of ~300 children diagnosed with an OXPHOS disorder in our centre [2]. However, relatively few patients have had full mtDNA sequencing so this figure represents an underestimate of the proportion of patients with mtDNA mutations. For children with the most common respiratory chain enzyme defect, complex I deficiency, it used to be thought that mtDNA mutations were quite rare (i.e., <5% of cases) but it now appears that they cause at least 20% of cases [19,20]. To date, eight mutations in the MTND3, MTND4, MTND5 and MTND6 genes have each been identified in multiple centres and appear to be recurrent mutations (Table 1). Virtually all the mutations identified to date in nuclear-encoded complex I subunit genes appear to be family-specific (see references listed in Table 2), so the simplest way to obtain at least some molecular diagnoses in complex I-deficient patients is to test for these eight recurrent mtDNA mutations.

A family history suggesting maternal inheritance of disease can be an important clue in initiating investigation for a mtDNA mutation. However, this does not mean that such family histories are common in patients with mtDNA mutations. We have studied 50 families in which the proband was a child with a pathogenic mtDNA mutation. Only nine had a history that was strongly suggestive of maternal inheritance, for example a maternal aunt, uncle or cousin having symptoms consistent with mitochondrial disease. Ten had a possible maternal history (e.g., symptoms such as migraine, diabetes, exercise intolerance) but in >60% of the families there was no suspicion of maternal inheritance. A possible or strong suspicion of maternal inheritance appeared to be more common in patients with a
tRNA mutation (10 of 15 probands) than those with a subunit mutation (9 of 25) or deletion (0 of 10). It is now becoming apparent that many children with mtDNA mutations have what appear to be de novo mutations that are not detectable in the mother’s blood but have amplified up to a high mutant load in a single generation. This has been observed for many years with mtDNA deletions, but we noted some years ago that up to a quarter of patients with the MTATP6 T8993G mutation appeared to have de novo mutations [21], and the same has been found more recently for mutations in the MTND subunit genes [19,20].

Some mtDNA mutations (e.g., the common MELAS A3243G mutation and mtDNA deletions) can disappear from cultured cell lines, at least when grown in the absence of uridine and pyruvate[22]. This has led to the suggestion that patients who have an enzyme defect expressed in both muscle and fibroblasts are unlikely to have a mtDNA mutation[23]. In our experience this is clearly not the case.

For 22 of 23 patients with mtDNA subunit mutations (in the MTATP6, MTND1, MTND3, MTND5 or MTND6 genes) where we could test fibroblasts, the mutant load was retained at a level high enough to cause an enzyme defect or clinical phenotype[2].

A final misconception that warrants mention is the idea that donor egg in vitro fertilisation is the only reproductive option that can be offered to women at risk of transmitting a mtDNA mutation. Two other options can be considered, at least for women who have a relatively low recurrence risk. These are prenatal diagnosis by testing of mutant load in chorionic villus (or amniocytes) and pre-implantation genetic diagnosis.

### Table 1
Mutations in mtDNA-encoded complex I subunit genes causing mitochondrial encephalopathies reported by diagnostic centres in different countries

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Australia</th>
<th>France</th>
<th>Italy</th>
<th>UK</th>
<th>Japan</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTND1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3460A</td>
<td>1^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3697A</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3946A</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3949C</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTND3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T10158C</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T10191C</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTND4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C11777A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>G11778A</td>
<td></td>
<td>1^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTND5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T12706C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>A12770G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A13045C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A13084T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G13513A</td>
<td>4^b</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>6</td>
<td>1 (USA)</td>
</tr>
<tr>
<td>A13514G</td>
<td></td>
<td>1^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTND6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G14459A</td>
<td>2</td>
<td></td>
<td>3 (USA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T14484C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T14487C</td>
<td>1</td>
<td>1</td>
<td></td>
<td>1 (Dutch)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References [20,38,39,44] [19,45–47] [48–50] [51–55] [56,57] [40,58–60]

^a These mutations are usually associated with Leber’s hereditary optic neuropathy but the patients listed here had childhood-onset encephalopathies.

^b Including one patient not previously reported.

### Table 2
Classification of mutations in nuclear-encoded complex I subunit genes causing mitochondrial encephalopathies

<table>
<thead>
<tr>
<th>Gene</th>
<th>M/M</th>
<th>M/X</th>
<th>X/X</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDUFS1</td>
<td>1</td>
<td>2</td>
<td>–</td>
<td>[61]</td>
</tr>
<tr>
<td>NDUFS2</td>
<td>3</td>
<td>2</td>
<td>–</td>
<td>[62] (Worgan et al., unpublished)</td>
</tr>
<tr>
<td>NDUFS3</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>[63]</td>
</tr>
<tr>
<td>NDUFS7</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>[64]</td>
</tr>
<tr>
<td>NDUFS8</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>[65,66]</td>
</tr>
<tr>
<td>NDUFY1</td>
<td>5</td>
<td>3</td>
<td>–</td>
<td>[61,67,68] (Worgan et al., unpublished)</td>
</tr>
<tr>
<td>NDUFY2</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>[69]</td>
</tr>
<tr>
<td>NDUFS4</td>
<td>–</td>
<td>–</td>
<td>7</td>
<td>[29,36,70–73] (Worgan et al., unpublished)</td>
</tr>
<tr>
<td>NDUFS6</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>[35]</td>
</tr>
</tbody>
</table>

The numbers listed represent only the proband in each family. Mutations are classified as (M) missense or (X) splice site, stop codon or frameshift mutations expected to result in unstable mRNA species. Thus, patients designated “M/M” have two alleles encoding missense mutations, “M/X” patients have one missense and one RNA destabilising mutation, and “X/X” patients have two RNA destabilising mutations.
genetic diagnosis. There are limitations with these approaches, particularly regarding prediction of clinical outcome based on a measured mutant load, and these need to be made clear to prospective parents. Nevertheless, there is now wide agreement that prenatal diagnosis can and should be offered to some women [24]. Increasing evidence suggests that pre-implantation genetic diagnosis (i.e., embryo biopsy) is also a logical option for women at risk of transmitting a mtDNA mutation. Pre-implantation genetic diagnosis allows the preferential selection of unaffected embryos for transfer in order to establish a pregnancy, thus avoiding the need to terminate an already established pregnancy. An attractive feature of pre-implantation genetic diagnosis for mtDNA mutations is that it may provide valuable information on the distribution of mutant loads in individual oocytes even if a successful unaffected conception is not achieved. This information could be used to guide subsequent reproductive choices. More detailed descriptions of the suitability of such approaches for women with different mtDNA mutations are given elsewhere [25–28].

4. How do we decide what gene(s) to investigate?

More than 30 mtDNA genes and more than 30 nuclear genes have now been shown to have mutations causing human disease [2], and it is likely that many more nuclear gene defects await discovery. The extreme genetic diversity of mitochondrial disorders makes it difficult to prioritise which gene(s) should be investigated in patients with known or suspected mitochondrial disorders. In some cases the clinical, pathological and biochemical results for a patient may suggest a limited number of genes to investigate. In consanguineous families or other families with multiple affected siblings, it is possible to identify candidate chromosomal regions using a “whole genome scan” of several hundred polymorphic STS markers. It is also possible to prioritise genes for mutation analysis in such families based on the analysis of a limited number of polymorphic STS markers that flank relevant candidate genes [29].

In patients where a cultured cell line expresses a biochemical defect, it may be possible to prioritise candidate genes by attempting phenotypic rescue of the enzyme defect using a panel of retroviral vectors expressing the relevant candidate genes [30,31]. More laborious methods can be used to identify the chromosomal location of some defects using techniques such as microcell-mediated chromosome transfer [32–34], but identifying the chromosomal locus does not necessarily result in the rapid identification of a candidate gene, so this approach is not suitable for routine candidate gene analysis.

Even when patients have a specific biochemical diagnosis, there may still be a large number of potential candidate genes. The most common respiratory chain enzyme defect, complex I deficiency, illustrates the difficulty of deciding which genes to investigate. Human complex I consists of at least 46 subunits, 14 of which are conserved even in prokaryotes. These 14 core subunits comprise seven encoded by mtDNA genes and seven by nuclear genes. Pathogenic mutations causing human disease have been described in all 14 core subunit genes [35], and one other gene, NDUFS4, encoding a “supernumerary” subunit that appears to play an important role in regulating complex I activity by reversible phosphorylation [36]. We recently identified pathogenic mutations in a second supernumerary subunit gene, NDUFS6, in three children with lethal neonatal complex I defects [35]. Mutations in these 16 mtDNA and nuclear genes appear to account for less than half of all patients with complex I deficiency, so many other causative genes await discovery. There is not a strong genotype/phenotype relationship for the 16 known complex I genes, so how can we decide on the most efficient way to investigate a molecular diagnosis in complex I deficient patients?

One approach to prioritising candidate genes causing complex I deficiency would be to determine if mutations in each gene tended to result in reproducible profiles of change in specific protein or RNA species. For example, do mutations in some complex I subunit genes lead to a specific increase or decrease in the amount of certain subunits, stress response proteins, anti-oxidant enzymes or a specific effect on complex I assembly? If this was the case then it may be feasible to identify the most likely causative gene by immunoblotting with a panel of antibodies or by analysis of RNA expression using a microarray. Such approaches are likely to be much more efficient than having to perform mutation analysis on 16 or more candidate genes. As yet, we have only a partial answer to the feasibility of this approach due to the relatively small numbers of cell lines studied in different centres with mutations in the various genes. However, there are several promising leads worth further investigation.

Manganese superoxide dismutase levels appear to be upregulated only by some causes of complex I deficiency [37]. SDS-PAGE immunoblotting of complex I subunits shows some differences between cell lines with different subunit mutations, but in our experience, Blue Native-PAGE immunoblotting is a more powerful approach. Unlike SDS-PAGE, which denatures enzyme complexes into individual subunits, Blue Native-PAGE retains enzyme complexes in their intact and enzymatically active form. Both the amount of the fully assembled complex, and the presence of any smaller stalled assembly intermediates, can then be determined by immunoblotting using one or more subunit antibodies. Our results and those of others suggest that commercially available antibodies can be used to classify complex I patient cell lines into different assembly categories. For example, cell lines with mutations in the MTND1, MTND6, NDUFS8, NDUFS2 and some unidentified nuclear gene defects cause gross diminution in the amount of assembled complex I, while mutations in the
MTND3, MTND5, NDUFS7 and other unidentified nuclear gene defects cause only modest loss of assembled complex I [20,35,38–41]. Mutations in the NDUFS4 and NDUFS6 genes cause accumulation of a complex I assembly intermediate approximately 200 kDa smaller than wild-type complex I [35,41]. Additional antibodies can be used to identify a broader range of distinct assembly intermediates [42].

If mutations in specific genes result in typical RNA expression profiles in patient cell lines or tissues, then microarrays could potentially be used to prioritise candidate genes for mutation analysis. Three approaches have been used, namely generic cDNA arrays, customised cDNA arrays and generic oligonucleotide arrays. Most attention has focused on customised cDNA arrays, or so-called “mitochips” comprising cDNAs representing hundreds of proteins with mitochondrial localisations. Although several groups are using such arrays, as yet only one report has been published. This showed some promise in that complex I defects caused by mutations in different genes showed differential expression of some genes in all cell lines and others only in specific cell lines [43].

We have recently begun using generic oligonucleotide microarrays to study RNA expression in complex I-deficient patient cell lines. These microarrays contain 60-mer oligonucleotides representing 17,260 genes from the Compugen 19k oligonucleotide library and are several-fold cheaper than equivalent cDNA microarrays. Our initial studies focused on two cell lines from unrelated families, which had been shown by complementation analysis to have complex I defects in the same unknown autosomal gene. RNA from the first patient cell line and a control cell line underwent competitive hybridisation to four arrays. RNA from the other patient cell line was compared to control RNA in the same way in separate experiments. The 50 most differentially expressed genes were determined for each patient cell line and 10 of these were in common. Analysis of the arrays from both patient cell lines showed that one of the six most under-expressed genes in each patient cell line was the NDUFS6 gene, encoding one of the supernumerary subunits of complex I. The two patients were subsequently shown to have homozygous mutations, a splicing defect in one family and a deletion of two exons in the other, resulting in almost complete loss of NDUFS6 mRNA [35].

Further work needs to be done to determine whether RNA profiling in this manner may give a profile predictive of the underlying gene defect. An attraction of this approach is that in some patients, RNA profiling may directly detect under-expression of a candidate gene. As with the NDUFS6 patients, this could include genes not previously known to cause a mitochondrial disorder. It is unclear if such success is likely to be a reasonably common or extremely rare occurrence, and there are several reasons why this approach could fail. Available microarrays will lack some, or perhaps many, potentially pathogenic genes. For example, the Compugen 19k library we used lacks 4 of 38 known nuclear-encoded complex I subunit genes (NDUFS7, NDUVF3, NDUFA5, NP17.3) and 1 of 23 other known genes (listed elsewhere [2]) causing RC defects (ANTI). Given the variability inherent to such analyses, microarray studies would probably not be expected to reliably detect cell lines with only one null allele. It may be necessary to have two mutations causing mRNA instability for direct identification of the causative gene. The expected proportion of such mutations is unknown but it is instructive to analyse the mutations reported to date in complex I subunit genes. All 21 probands reported to date with mutations in the seven nuclear genes encoding core subunits have had either two missense mutations or one missense mutation and one mutation expected to cause mRNA instability (Table 2). In contrast, all nine probands with mutations in the supernumerary subunit genes (NDUFS4 and NDUFS6) have had homozygous mutations expected to cause mRNA instability. Two points can be made about this striking observation. Firstly, since patients with two null mutations of the core subunit genes have not been identified, it is likely that complete loss of any of these subunits would be incompatible with foetal survival. In contrast, since all mutations in the supernumerary subunits may be null mutations, it is likely that virtually complete loss of function of these subunits may be needed to cause disease. If that is the case for other subunits, then microarray expression analysis of complex I-deficient cell lines may prove a useful tool to guide choice of genes for mutation analysis.

In summary, at present, there is no simple way to prioritise candidate genes for mutation analysis in patients with complex I deficiency or other respiratory chain defects. Testing a limited number of recurrent mtDNA mutations may achieve a diagnosis in 10–20% of complex I patients. Analysis of polymorphic STS markers that flank relevant candidate genes can be useful in the minority of cases where the family is consanguineous or has multiple affected siblings. If one has access to a panel of retroviral vectors expressing the relevant candidate genes then this could be an efficient screening procedure. Blue Native-PAGE immunoblotting shows considerable promise as a means of identifying assembly profiles that may be predictive of the underlying gene defect. Further analysis of this approach and of microarray expression analysis is required to validate their usefulness.

Acknowledgements

This work was supported by a Senior Research Fellowship (DT) and grants from the National Health and Medical Research Council (NHMRC) of Australia, the Australian Research Council, the Muscular Dystrophy Association (MDA) of USA and the United Mitochondrial Disease Foundation.
References


D.R. Thorburn et al. / Biochimica et Biophysica Acta 1659 (2004) 121–128


