# Chapter 2
# Biochemical and Molecular Methods for the Study of Mitochondrial Disorders

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## Introduction

Mitochondrial disorders are a group of genetically heterogeneous complex diseases [1–6]. Although mitochondrial structure and function involve two genomes, the biogenesis of mitochondrion and more than 99 % of its protein contents are encoded by the nuclear genome [7]. As a result, the majority of the mitochondrial disorders are caused by molecular defects in the nuclear genome [5, 6, 8–10]. Early studies of mitochondrial disorders focused on mutations in the 16.6 kb tiny mitochondrial DNA (mtDNA) because of its genomic simplicity and maternal inheritance [3, 11]. In the past decade, autosomal recessive mitochondrial disorders have been increasingly recognized [4, 5, 8, 10, 12–22]. Recent studies through integrated genomics and system biology identified approximately 1,500 nuclear-encoded proteins targeted to mitochondria [7], although mutations have only been identified in about 150 nuclear genes. The biogenesis of mtDNA requires nuclear-encoded genes, including DNA polymerase gamma (*POLG*) and DNA helicase (*TWINKLE*) for replication as well as purine/pyrimidine nucleoside kinases, *DGUOK* and *TK2*, for salvage synthesis and maintenance of deoxynucleotide (dNTP) pools. Defects in any of these genes cause mtDNA depletion [23, 24] or mtDNA multiple deletions [25, 26]. In addition, mtDNA transcription and mitochondrial protein biosynthesis require nuclear DNA (nDNA)-encoded factors [7, 27–29], including RNA polymerase, various transcription factors, small and large subunits (> 80) of mitochondrial ribosomal proteins, translation initiation and elongation factors, and all mitochondrial aminoacyl tRNA synthetases [22, 30–35]. The majority of mitochondrial respiratory chain complex subunits and all complex assembly factors are also nuclear encoded [28, 36–44]. Furthermore, the roles of mitochondria in autophagy, apoptosis, and reactive oxygen species (ROS) production have recently proven to be indispensible in the pathogenic mechanisms of neurodegenerative diseases (Parkinson, Alzheimer, Huntington, etc.)

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and cancer [45–47]. It is, therefore, conceivable that defects in any of the ~ 1,500 genes targeted to mitochondria are potentially detrimental to mitochondrial structure and function [3, 7, 28, 36–44, 47].

Due to the heterogeneity and complexity of mitochondrial disorders, it is crucial to have simple biochemical and molecular screening methods that will help pinpoint a specific group of candidate genes leading to the final diagnosis. This chapter will briefly review the currently available biochemical and molecular methods that can help with the diagnosis of mitochondrial disorders.

## Diagnostic Criteria and Diagnostic Algorithms

### *Criteria for the Diagnosis of Mitochondrial Respiratory Chain Disorders*

Based on the review of 118 patients referred for the diagnosis of mitochondrial respiratory chain disorders (MRCD) and comprehensive reevaluation of previously proposed diagnostic criteria [48], consensus general criteria have been developed [49]. The modified diagnostic criteria, separated into major and minor, for MRCD are listed in Table 2.1. If a patient's clinical, histological, enzymological, functional, molecular, and metabolic evaluations meet two major criteria or one major and two minor criteria, he or she is classified as having a definite diagnosis of MRCD (Table 2.2) [49]. If the patient meets one major and one minor, or three minor criteria, then he or she is classified as having a probable diagnosis (Table 2.2). These criteria are useful in assessing patients who have a diagnosis of MRCD. Using these criteria to evaluate 1,500 patients suspected of having mitochondrial syndromic hearing loss, 45 patients having both a definite diagnosis of MRCD and syndromic hearing loss were evaluated for their molecular defects [50]. Among this subset of patients, 18 harbored undisputed mtDNA mutations, and 11 had mtDNA multiple deletions and/or mtDNA copy number abnormalities [50].

### *Diagnostic Algorithms*

Since mitochondrial disorders are complex and a large number of genes are involved, disease diagnosis is often challenging, requiring the incorporation of clinical, biochemical, and histochemical evaluations, followed by final confirmation of the diagnosis by the identification of deleterious mutations in causative genes. Wong et al. [5, 6, 8–10] proposed an algorithm that could be used to help diagnose mitochondrial disease. Briefly, a patient is first brought to the clinician's attention of having a mitochondrial disorder because of family history and/or clinical presentation. Clinical evaluation includes recognizable syndromes caused by common mtDNA mutations or nuclear gene defects [5, 6]. Minimally invasive assessment may include imaging,

**Table 2.1** Modified diagnostic criteria based on Walker et al. [48], Bernier et al. [49], and Scaglia et al. [50]

| | Major criteria | Minor criteria |
|---|---|---|
| Clinical | Clinically complete RC encephalomyopathy, *recognizable mitochondrial syndromes*[a], or a mitochondrial cytopathy fulfilling three conditions[b] | Symptoms compatible with an RC defect[c] |
| Histology | Greater than 2 % ragged red fibers (RRF) in skeletal muscle | Smaller numbers or RRF, SSAM, or widespread electron microscopy abnormalities of mitochondria |
| Enzymology | Cytochrome *c* oxidase negative fibers or residual activity of an RC complex < 20 % in a tissue; < 30 % in a cell line, or < 30 % in two or more tissues | Antibody-based demonstration of an RC defect or residual activity of an RC complex 20–30 % in a tissue, 30–40 % in a cell line, or 30–40 % in two or more tissues |
| Functional | Fibroblast ATP synthesis rates > 3 SD below mean | Fibroblast ATP synthesis 2–3 SD below mean, or fibroblasts unable to grow in galactose media |
| Molecular | Nuclear or mtDNA mutation of undisputed pathogenicity[d] | Nuclear or mtDNA mutation of probable pathogenicity[d] |
| Metabolic | | One or more metabolic indicators of impaired metabolic function |

The details of this modified diagnostic criteria are described by Bernier et al. [49]
*ATP* adenosine triphosphate, *SSAM* subsarcolemmal accumulation of mitochondria
[a]Leigh disease, Alpers disease, Lethal infantile mitochondrial disease, *mtDNA deletion syndromes*, including Pearson syndrome and Kearns–Sayre syndrome, MELAS, MERRF, NARP, MNGIE, LHON, mtDNA depletion syndrome, *mtDNA multiple deletion syndromes* including progressive external ophthalmoplegia (PEO) and myopathy. Italicized parts are modifications from Bernier et al. [49]
[b](1) Multisystemic symptoms typical for an RC disorder; (2) a progressive clinical course with episodes of exacerbation due to infection or drug toxicity, or *a family history*; and (3) other possible metabolic or nonmetabolic disorders have been excluded by appropriate testing
[c]Added pediatric features: stillbirth associated with a paucity of intrauterine movement, neonatal death or collapse, movement disorder, severe failure to thrive, neonatal hypotonia, and neonatal hypertonia as minor clinical criteria
[d]See Table 2.2 for the definition of definite and probably mutations

**Table 2.2** Classification of diagnosis

| Diagnosis | Definition by number of criteria met |
|---|---|
| Definite | Two major criteria or one major plus two minor |
| Probable | One major plus one minor or three minor |
| Possible | One major or two minor, one of which must be clinical |

and blood and urine chemistry (Biochemical Assessment Section). Molecular sequence analysis of genes responsible for recognizable syndromes can be performed on blood DNA. With the development of next-generation sequencing, it is possible to analyze the entire mitochondrial genome comprehensively or the analysis of specific panels of nuclear genes, all mitochondrial-targeted nuclear genes, the whole exome, or the whole genome directly using the DNA extracted from blood samples [51–53].

Invasive investigation, on the other hand, may include biochemical, histochemical, and molecular studies of liver, skin, or muscle biopsy. The type of test to be performed depends on the type of tissue available from the patient. The results from invasive tissue studies may support the diagnosis and/or point to a group of genes responsible for the disease and direct the sequence analysis of a specific group of genes.

## Biochemical Assessment

### *Lactic Acid and Pyruvate*

Elevation of lactic acid in blood or CSF, although nonspecific, is a common phenomenon in patients with MRCD, particularly patients with severe autosomal recessive type [16, 18, 21, 32, 35, 54–58]. Severe infantile lactic acidosis has been reported in patients with mutations in numerous genes including *MPV17* [21], *SUCLA2* [54, 59], *SUCLG1* [18], *BCS1L* [30], *DARS2* [22], *PUS1* [30], *TK2* [60, 61]. The blood lactate/pyruvate ratios indirectly reflect the NADH/NAD+ cytoplasmic redox state [62], therefore, an accurate measurement of pyruvate is also necessary. Elevated plasma alanine is an indication of the accumulation of pyruvate. Defects in pyruvate metabolism such as pyruvate dehydrogenase complex deficiency, pyruvate carboxylase deficiency, or biotinidase deficiency will cause pyruvate elevation in blood and/or CSF.

### *Plasma/CSF Amino Acids*

Amino acid analysis is usually performed using ion-exchange chromatography followed by postcolumn derivetization in an automated amino acid analyzer. Recently, tandem mass spectrometry (MS/MS) is also being used. Elevated alanine is defined as having an alanine:lysine ratio > 3:1 and alanine:phenylalanine+tyrosine ratio > 4:1, or an absolute elevation in alanine above 450 μ(mu)M [63]. Elevation of tyrosine and/or phenylalanine without the presence of succinylacetone may be an indication of the hepatocerebral form of mtDNA depletion syndrome [8]. Generalized aminoaciduria with renal tubular acidosis and glycosuria similar to Fanconi syndrome has also been observed in patients with mtDNA deletions [64, 65].

### *Urine Organic Acid*

Urine organic acids reflect the catabolites of amino acids, carbohydrates, and fatty acids. Analysis is usually performed by gas chromatography followed by mass spectrometry. Lactic aciduria is not a good discriminator for mitochondrial disorders.

Elevations in TCA cycle intermediates, ethylmalonic acid, and 3-methyl glutaconic acid are common but rarely diagnostic of a specific mitochondrial disease [66–68]. Nevertheless, elevations of 3-methyl glutaconic acid and 3-methyl glutaric acid have been reported in multiple patients with mutations in *TMEM70*, a chaperone protein needed for proper assembly of complex V, ATP synthase [69, 70]. Dicarboxylic aciduria is another common finding in mitochondrial disorders due to impairment of fatty acid β-oxidation. In particular, moderate elevations in methylmalonic acid (MMA) have been observed in patients with mutations in the *SUCLA2* and *SUCLG1* genes, which cause mtDNA depletion [16, 18, 54, 71].

## *Carnitine and Acylcarnitine*

Carnitine, β-hydroxy-γ-trimethylammonium butyrate, is a positively charged molecule required for the transfer of long-chain fatty acids from the cytoplasm to the mitochondrial matrix for β-oxidation. A deficiency in any step of β-oxidation results in abnormal levels of total and free carnitine in plasma, and an abnormal acyl-carnitine profile suggesting a specific defect of fatty acid oxidation. Carnitine deficiency and fatty acid oxidation defects may be secondary to MRCD. Quantitative analysis of acyl-carnitine is usually performed by tandem mass spectrometry (MS/MS) or HPLC followed by electrospray ionization (ESI)-MS/MS.

## *Histochemistry and Immunohistochemistry*

Mitochondrial proliferation in skeletal myofiber is suggestive of a respiratory chain disorder, which is revealed as ragged red fibers (RRF) on modified Gomori trichrome staining as red granular deposits of mitochondrial in the subsarcolemmal space [72]. The RRFs are shown as ragged blue fibers (RBFs) on histochemical staining of succinate dehydrogenase (SDH) [72]. RRFs or RBFs can also appear in myopathic forms of mtDNA depletion syndromes [25, 26, 73]. SDH histochemistry is also useful for the diagnosis of complex II deficiency. Another useful histochemical evaluation is the staining for cytochrome c oxidase (COX). Muscle fibers with normal COX activity appear brown, while negative fibers stain poorly. However, sequential staining with SDH causes the COX deficient fibers to stain dark blue. Immunohistochemical staining uses antibodies against specific protein subunits of the respiratory chain complexes to reveal the defective genes. For example, a muscle biopsy from a patient with Kearns–Sayre syndrome (KSS) may show marked reduction of COXII (mtDNA encoded) but normal COXIV (nDNA encoded) immunohistochemical staining. Anti-DNA antibodies can be used to identify muscle fibers with abnormal mtDNA content by showing normal nDNA staining but reduced mtDNA staining suggesting mtDNA depletion [72].

### Electron Microscopy (EM)

EM studies can reveal abnormal ultrastructural changes of mitochondria including mitochondrial number, shape, size, absence of cristae, and paracrystalline inclusions. However, these ultrastructural changes are not specific for mitochondrial disorders.

### Assay of Electron Transport Chain Activities (ETC)

Mitochondrial respiratory chain activities are studied by spectrophotometric assay of the ETC enzyme complexes: complex I (NADH-ubiquione oxidoreductase), complex II (succinate-ubiquinone oxidoreductase), complex III (decylubiquinone-cytochrome *c* reductase), complex IV (cytochrome *c* oxidase), complex I+III (NADH-cytochrome *c* reductase), or complex II+III (succinate-cytochrome *c* reductase). The measurement of ETC complex activities is based on the absorbance change of the substrate, either NADH or cytochrome *c*, depending on the complex being assayed [74, 75]. Mutations in specific complex protein subunits or complex assembly factors cause an isolated complex deficiency, while mutations in nuclear genes responsible for mtDNA biosynthesis and maintenance of mtDNA integrity result in multicomplex deficiencies [8, 21]. Mutations in enzymes involved in coenzyme $Q_{10}$ ($CoQ_{10}$) biosynthesis result in $CoQ_{10}$ deficiencies and exhibit reduced activities of combined complexes I+III and II+III [17, 76, 77]. The spectrophotometric assay of complex V (F1-ATPase) in tissue homogenate measures in the reverse direction by linking the hydrolysis of ATP to the oxidation of NADH using pyruvate kinase (PK) and lactate dehydrogenase (LDH) [74, 75]. The rate of NADH oxidation is monitored by measuring the absorbance decrease at 340 nm. Assays of complex V may also be linked to complex I [74, 75].

### Oxygen Consumption Rate (OCR)

Live cells and isolated mitochondria from fresh muscle tissue offer the advantage of assaying integrated mitochondrial function such as oxygen consumption and oxidative ATP synthesis rates [78]. By using complex I substrates (glutamate, malate, and pyruvate) or complex II substrate (succinate), ATP synthesis rate in the presence (state III, ADP-stimulating) or absence (state IV, ADP-limiting) of ADP is measured. The ratio of state III rate to state IV rate is a good indicator of the integrity of the inner membrane of the isolated mitochondria. The ratio of the amount of ADP usage and oxygen consumption directly reflects the efficiency of oxidative phosphorylation and can indicate abnormalities of ATP synthase or coupling [79, 80].

### Analysis of Respiratory Chain Protein Complexes using Blue Native Gel

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a powerful diagnostic tool for the detection of assembly defects in the enzyme complexes of oxidative phosphorylation [81–84]. This method separates all five complexes on the gel, which

is followed by immunoblotting with commercially available antibodies [85]. If there is an assembly defect, the complex with the defect will be absent. Using established in-gel activity staining [86, 87], residual enzymatic activity of the oxidative phosphorylation complexes can also be measured. Mitochondria contain their own protein translation machinery. Defects in mitochondrial protein translation result in decreased mitochondrial protein synthesis. The molecular defects may be in the mitochondrial genome, such as mutations in mitochondrial tRNA, rRNA, or mRNA. Mutations in nuclear genes encoding mitochondrial aminoacyl tRNA synthetases; posttranscription RNA-modification enzymes; translation initiation, elongation, or termination factors, or mitochondrial ribosomal protein subunits; can all potentially cause impaired mitochondrial protein translation. Therefore, specific radioactive labeling of the mitochondrial translation protein products in cultured cells followed by the analysis of the proteins on blue native gel is useful in the study of disease-causing mutations in both the mitochondrial and the nuclear genomes [82, 84, 88].

#### Coenzyme $Q_{10}$ ($CoQ_{10}$)

Coenzyme $Q_{10}$ serves as a mobile electron carrier to shuttle electrons from complex I or II to complex III. Mutations in genes involved in $CoQ_{10}$ biosynthesis cause primary $CoQ_{10}$ deficiency. Conversely, secondary $CoQ_{10}$ deficiency in muscle may be due to defects in mtDNA or nuclear genes such as electron-transferring flavoprotein dehydrogenase (*ETFDH*). Intracellular levels of coenzyme Q and its redox status are important markers of oxidative stress associated with MRCD [89–92]. The measurement of coenzyme Q concentrations in biological specimens including plasma, white blood cells, or tissues is performed by high-performance liquid chromatography (HPLC) methods with electrochemical (EC) detection [93] or liquid chromatography-tandem mass spectrometry [94, 95].

## Molecular Evaluation

Various methods are used for the detection of molecular defects. Depending on the purposes, these methods can be used alone or in combination. These methods will be discussed in two categories: the detection of mutations in the mitochondrial genome and in the nuclear genes.

### *Analysis of Alterations in the Mitochondrial Genome*

#### Detection of Recurrent Common Mutations

The recurrent common mtDNA point mutations are usually screened by the PCR/RFLP or PCR/ASO (allele-specific oligonucleotide) method [96–99]. The former is performed by testing each known mutation individually, while the latter is

carried out with multiplex PCR of the regions containing the common point mutations followed by dot-blot ASO analysis [96–99]. Other mutation detection methods including TaqMan allele discrimination assays or Sanger sequencing of the specific region of the mitochondrial genome can also be used [100–102].

### Detection of Rare or Unknown Point Mutations in the Mitochondrial Genome

Historically, there are several mutation detection methods for screening unknown mutations including single-strand conformation polymorphism (SSCP) [103], temporal temperature gradient gel electrophoresis (TTGE) [104, 105], temperature gradient gel electrophoresis (TGGE) [106], denaturant gradient gel electrophoresis (DGGE) [107, 108] and denaturing high-performance liquid chromatography (cHPLC) [109]. Since Sanger sequencing is required to confirm the exact mutations resulting from the detected changes, these indirect mutation detection methods have been replaced by direct Sanger sequencing of the target regions using specific primers [100–102, 110]. Sanger sequencing analysis of the whole mitochondrial genome is usually performed by PCR amplification using 24–36 pairs of primers amplifying overlapping fragments covering the entire mitochondrial genome followed by sequencing using specific primers [110]. Although this method is straightforward, it does not, however, detect large deletions or quantify degree of mutation heteroplasmy. In addition, due to the extensive polymorphic features of the mtDNA, the high frequency of single nucleotide polymorphisms (SNPs) in the multiple primer sites, and the interference of nuclear mtDNA homology sequences, the true mtDNA fragments may not be efficiently amplified or not amplified at all leading to calling bases not truly representative of the mtDNA sequence. Yet, these obstacles can be resolved by long-range PCR of the entire mitochondrial genome followed by massively parallel sequencing, which has now become routinely performed [111]. The advantages of this newly developed approach is the simultaneous detection of mtDNA point mutations and large deletions while also acquiring quantitative heteroplasmy information for every single nucleotide of the entire 16.569 kb genome, deletion breakpoints, and deletion heteroplasmy (Comprehensive one-step analysis of the mitochondrial genome section) [111].

### Quantification of the Point Mutation Heteroplasmy

Knowing the degree of mutation heteroplasmy and its tissue distribution is crucial for correlating clinical phenotype with test results of patients with mtDNA disorders [5, 6, 11, 96, 112]. Traditionally, this is performed by the addition of $\gamma$-$^{32}$P-ATP to the PCR mixture at the last cycle followed by restriction fragment length polymorphism (RFLP) analysis and quantification of DNA bands by PhosphorImager [96]. However, due to the disadvantages of using radioactive material and time-consuming RFLP procedures, this method is gradually being replaced by the

development of a nonradioactive, more accurate and rapid real-time amplification refractory mutation systems quantitative PCR (ARMS-qPCR) method, which provides simultaneous detection and quantification of heteroplasmic mtDNA point mutations [113–116].

### Detection of Large mtDNA Deletions

Large single deletions of mtDNA are usually detected by Southern blot analysis [11, 96, 99, 117–119]. However, procedures for quantification of deletion heteroplasmy and determination of the breakpoints are tedious and may involve the usage of radioactive material similar to the procedures described in Sect. 4.1.3 except that the restriction fragments are hybridized with nick-translated $\alpha$-$^{32}$P-dCTP or nonradioactively labeled probes such as digoxygenine [11, 96, 117–119]. The recent development of custom oligonucleotide array comparative genome hybridization (aCGH), MitoMet© allows reliable detection of mtDNA deletions with elucidation of deletion breakpoints and the percentage of deletion heteroplasmy [118, 120–122].

### Measurement of mtDNA Copy Number

Alteration in mtDNA copy number is an indication of mitochondrial disorder. Increased mtDNA content suggests a compensatory mechanism due to deficient mitochondrial function [123], while reduced mtDNA content implies defects in mtDNA biosynthesis, usually secondary to mutations in nuclear genes [8–10, 16, 18, 21, 59, 112, 124–126]. Measurement of mtDNA copy number is performed by real-time quantitative PCR using an mtDNA probe and a unique nuclear gene reference [112, 116, 127]. The copy number ratio of mtDNA/nDNA is a measure of mtDNA content [112, 127]. Since mtDNA content varies among different tissues and in some tissues it also changes with age, the mtDNA copy number of an individual is compared with the mean value of tissue and age matched controls [8, 21, 112]. The mtDNA content in muscle tissue from patients with encephalomyopathic mtDNA depletion syndrome is < 50 % of that of tissue and age-matched control mean, while the mtDNA content in the liver specimen from patients with hepatocerebral or infantile hepatic form of mtDNA depletion syndrome is < 20 % of that of the matched control [8, 21, 112]. Measurement of mtDNA content in an affected tissue may assist with narrowing the cause of the disease to a group of nuclear genes for sequence analysis [5, 6]. Therefore, assessment of mtDNA copy number in affected tissues such as muscle and liver is a screening method for the identification of mtDNA depletion syndromes or mtDNA compensatory overamplification [25, 73, 123].

### Comprehensive One-Step Analysis of the Mitochondrial Genome

As described in the above analysis of alterations in the mitochondrial genome sections, it is clear that comprehensive analysis of the mitochondrial genome requires the

application of multiple techniques to detect point mutations, deletions, mutation heteroplasmy, deletion breakpoints, and mtDNA depletion [5, 96, 123]. This step-wise approach complicates the molecular diagnosis of the disease. Recent advancement of next-generation sequencing enables the analysis of the entire mitochondrial genome comprehensively by massively parallel sequencing [52, 111]. A recent report by Cui et al. [111] demonstrates that massively parallel sequencing strategy can provide complete qualitative and quantitative information on mtDNA point mutations, single and multiple deletions, degree of mutation heteroplasmy, deletion breakpoints, and heteroplasmy in one step [111, 128, 129].

## Analysis of Nuclear Genes Causing Mitochondrial Disorders

### Sanger Sequencing

Sanger sequencing has been used as the gold standard to detect mutations in candidate genes by using specific primers to amplify the coding exons and ~ 50 nucleotides of the flanking intronic regions of the gene of interest followed by sequencing of the PCR fragments. Selection of candidate genes for sequencing is a challenge because mitochondrial disorders are genetically and clinically heterogeneous as briefly mentioned in "Diagnostic Criteria and Diagnostic Algorithms" [5]. An estimated 1,500 nuclear-encoded proteins are associated with mitochondrial biogenesis, structure, and function (Table 2.3) [7]; however, mutations in only about 200 have been reported to cause disease [5]. The selection of candidate genes for diagnostic sequencing analysis is generally based on the patient's clinical presentation and family history along with the molecular, imaging, or biochemical characteristics [5, 6]. For example, if the mtDNA content in the affected tissue, such as muscle or liver, is showing depletion by the qPCR method [112], then a group of genes responsible for the hepatocerebral form of mtDNA depletion syndrome may be sequenced [5, 6, 8–10, 21, 112]. If there is an indication of myopathy and/or progressive external ophthalmoplegia, and multiple mtDNA deletions, then the genes responsible for these phenotypes may be sequenced [10, 12, 25, 26, 130].

### Oligonucleotide Array Comparative Genomic Hybridization (aCGH)

Although sequencing analysis detects point mutations and small insertion/deletions, it does not detect large intragenic or whole gene deletions. A custom-designed oligonucleotide array-based comparative genomic hybridization (aCGH) method has been developed to provide both tiled coverage of the entire 16.6 kb mitochondrial genome and high-density coverage of nuclear genes involved in mitochondrial biogenesis, structure, and function [120]. Several studies have demonstrated that this design is able to detect large deletions in both nuclear and mitochondrial genomes [9, 120, 121, 131–134]. Most importantly, the breakpoints and degree of heteroplasmy of large mtDNA deletions can be estimated [120, 121, 135]. In addition,

**Table 2.3** Categories of nuclear-encoded proteins targeting to mitochondria

| Nuclear genes targeted to mitochondria | | | | | | | |
|---|---|---|---|---|---|---|---|
| Category | Number | Category | Number | Category | Number | Category | Number |
| Apoptosis | 32 | Metabolism[a] | 356[a] | Proteases | 24 | aa-tRNA Synthase | 18 |
| Chaperone | 11 | Mito dynamics | 12 | Nucleaes | 2 | Signal trans duction | 34 |
| Cytochromes | 12 | Replication | 4 | Translation[b] | 34 | Protein folding and modification | 21 |
| Cytoskeletal | 11 | Transcription | 15 | MRPLs | 47 | Protein import: TIMMs and TOMMs | 28 |
| DNA repair | 11 | Rregulation | 7 | MRPSs | 29 | RC complex and assembly | 107 |
| Fe–S cluster | 4 | Porins | 3 | Transporters | 80 | RNA modification and processing | 7 |
| Immune | 3 | ROS | 16 | Others | 31 | Unknown | 113 |

[a] Metabolic pathways include amino acids, carbohydrates, creatine, iron, ketones, Kreb cycle, lipids, nucleotide, phospholipid, porphyrin, steroid, tetrahydrofolate, tRNA, CoQs, pyruvate, etc.
[b] *Translation* initiation, elongation, termination proteins, and factors.
[c] *MRPLs* mitochondrial ribosomal proteins large subunits, *MRPSs* mitochondrial ribosomal proteins small subunits.
[d] *Transporters* ATP-binding cassette, iron, nucleotides, solutes, and uncouplers.

for patients with mtDNA depletion syndromes, this array can detect copy number changes in both nuclear genes and mtDNA (reduced mtDNA copy number). A good example is that of a baby boy who developed liver failure during infancy. A liver biopsy revealed severe mtDNA depletion (only 6 % of tissue matched control). His older brother also died from liver failure during infancy. Sequence analysis found a heterozygous mutation, p.E227K, in the *DGUOK* gene. Since the hepatocerebral form of mtDNA depletion syndrome is an autosomal recessive disorder, the family history and the mtDNA content in liver suggested that a second mutant allele must be present. The aCGH analysis revealed a heterozygous deletion of exon 4 in the proband and the carrier mother [133], and the mtDNA profile showed an approximate 97 % reduction in copy number, consistent with mtDNA depletion [133]. Several other examples showing large deletions in genes, including POLG, DGK, TK2, TP, and MPV17, responsible for mtDNA depletion, have also been reported [9, 120, 121, 131–134].

### Next-Generation Massively Parallel Sequencing

Step-wise sequence analysis of several individual candidate genes (Table 2.3) by Sanger sequencing is tedious, time consuming, and costly. However, the newly developed massively parallel sequencing (MPS) allows simultaneous sequencing of multiple genes at high coverage and low cost. The small 16.6 kb mtDNA genome

can be amplified with overlapping primers followed by MPS [52]. This method can reliably detect mtDNA heteroplasmy at a 5 % level, but it, unfortunately, does not detect large deletions. Conversely, the method described by Cui et al. detects both point mutations and large deletions with heteroplasmy and exact deletion breakpoints in a one-step comprehensive approach (comprehensive one-step analysis of the mitochondrial genome section) [111]. As for nuclear genes, a proof-of-concept study was conducted by Vasta et al. [53] by using microarrays to capture the mtDNA genome along with 362 nuclear genes related to mitochondrial disorders, followed by MPS. However, this study did not report the identification of causative mutations in unknown patients with mitochondrial disorders. More recently, Calvo et al [51] performed "MitoExome" MPS analysis of mtDNA and coding exon sequences of $\sim$ 1,000 nuclear genes encoding mitochondrial-targeted proteins. These investigators were able to identify reported mutations in ten patients and novel mutations that require confirmation of pathogenicy in 13 others [51]. The MPS approach is promising, but in order to apply it to a clinical diagnostic laboratory, many obstacles remain, including false negative, false positive, variant interpretation, and functional confirmation of pathogenicity. Also, even by capture of $\sim$ 1,000 genes with MPS, about 50–75 % of patients with mitochondrial disorders remain undiagnosed [51]. The utilization of whole exome or whole human genome sequencing analysis may be necessary in order to find the molecular cause of the disease in these situations [136–138]. Table 2.3 lists the categories of $\sim$ 1,500 genes with mitochondrial involvement. Due to the extreme clinical and genetic heterogeneity of the mitochondrial disorders, exome and/or whole genome sequence analyses may be the ultimate solution.

### Interpretation of Sequence Variants

With the improvement in next-generation sequencing, a large number of novel or rare sequence variants have been rapidly discovered. To understand the clinical and/or functional significance of these variants, they should be properly classified based on family history, inheritance, clinical correlation, functional studies, and literature reports [139, 140]. The classification and guidelines published by ABMG in 2008 for the interpretation of novel variants using ACMG's Standards and Guidelines should be followed [141]. Detailed procedures have also been published in *Methods in Molecular Biology* [139, 140]. Several publically available databases and softwares that are accessible on the internet include Human Genome Mutation Database (HGMD), Single Nucleotide Polymorphism Database dbSNP, PubMed, Online Mendelian Inheritance in Man (OMIM), Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotypng (PolyPhen), ESE, NetGene, and BDGP (Table 2.4) [139, 140]. Although all these databases and in silico analytical algorithms help in the interpretation of novel and rare variants, the most challenging task is to provide experimental functional evidences to support the pathogenicity of the sequence variants. Hopefully, advances in functional genomics will greatly facilitate the interpretation of sequence variants.

**Table 2.4** Internet accessible databases and analytical software

| Name of databases | Abbreviation | Web address |
|---|---|---|
| *Databases* | | |
| Human Genome Mutation Databases | HGMD | http://www.hgmd.cf.ac.uk |
| Single Nucleotide Polymorphism Database | dbSNP | http://www.ncbi.nlm.nih.gov/projects/SNP |
| NCBI MEDLINE database of life sciences | PubMed | http://www.ncbi.nlm.nih.gov/PubMed |
| Online Mendelian Inheritance in Man | OMIM | http://www.ncbi.nlm.nih.gov/omim |
| *Mutation database for specific nuclear genes* | | |
| mtDNA polymerase gamma | POLG | http://tools.niehs.nih.gov/polg |
| Optic atrophy gene 1 | OPA1 | http://lbbma.univ-angers.fr/lbbma.php?id=9 |
| *Databases specific for the mitochondrial genome* | | |
| | MitoMap | http://www.mitomap.org/MITOMAP |
| | mtDB | http://www.mtdb.igp.uu.se/ |
| | tRNA | http://mamit-trna.u-strasbg.fr/Summary.asp |
| *Computational algorithms for the prediction of pathogenicity of missense variants* | | |
| Sorting Intolerant From Tolerant | SIFT | http://sift.jcvi.org |
| Polymorphism Phenotyping-2 | PolyPhen2 | http://genetics.bwh.harvard.edu/pph2 |
| Prediction of pathogenicity | PONP | http://bioinf.uta.fi/PON-P/Pathogenic_or_not_prediction_methods.shtml |
| *Computational algorithms for the prediction of pathogenicity of splice site alterations* | | |
| ESE Finder 2.0 | ESE2 | http://rulai.cshl.edu/tools/ESE2 |
| NetGene2 | | http://www.cbs.dtu.dk/services/NetGene2 |
| BDGP splice site predictor | | http://www.fruitfly.org/seq_tools/other.html |

## Conclusion

Diagnosis of the complex dual genome mitochondrial disorders requires application of multiple biochemical and molecular approaches. The methods mentioned in this chapter likely will continue to be used in diagnostic laboratories for several years until a more reliable comprehensive whole genome molecular strategy is developed. Although whole exome and whole genome approaches are currently available, these methods have their own limitations. Overall, the quality of MPS tests varies tremendously among different laboratories, and so does the cost of these tests. In addition, it is important that clinicians understand the quality control and quality assurance procedures and compliance to the regulatory agents.

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