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Review Article

Recent advancements in diagnostic tools in mitochondrial energy metabolism diseases

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ABSTRACT

The involvement of mitochondrial energy metabolism in human disease ranges from rare monogenic disease to common diseases and aging with a genetic and/or lifestyle/environmental cause. This wide ranging involvement is due to the central role played by mitochondrion in cellular metabolism, its role in cellular perception of threats and its role in effecting responses to these threats. Investigating mitochondrial function/dysfunction or mitochondria-associated cell-biological responses have thus become a common finding where the pathogenic processes are investigated. Although, such investigations are warranted, it is not always clear if mitochondria can indeed be associated with cause or merely playing a responsive role in disease pathology. As this key question is also essential to disease progression and therapy, it should be recognized in investigative design. We herewith, present an overview of the current approaches and technologies used and the practicalities around these technologies.

Contents

1. Introduction

The first report linking a deficiency (or "loose coupling") of the mitochondrial oxidative phosphorylation system (OXPHOS) to a human disease phenotype was by Luft [1]. This introduced mitochondrial medicine and the concept of mitochondrial disorders/disease as an inborn disorder of energy metabolism [2]. Since then, the classical view of mitochondrial disease is a rare monogenic nuclear (nDNA) or mitochondrial DNA (mtDNA) encoded deficiency of the OXPHOS system, which has led to the identification of more than 241 genes involved in mitochondrial disease [3] and regarded amongst the most frequently inherited metabolic disorders in newborns with any mode of inheritance possible [4]. This may not be surprising considering that, of the ~1000 proteins making up the mitochondrion (Mitocarta), the OXPHOS system consists of 89 subunits, of which 13 structural subunits are encoded by mtDNA (in addition to 22 tRNA and two

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rRNA's) and 76 are nuclear encoded [5,6]. In addition to these, a great number of proteins are involved in import, assembly and regulation of the OXPHOS system and its products. A further notable aspect of a deficiency of the OXPHOS system is varied and unpredictable biochemical consequences, which include alteration of respiration, coupling, ATP production, Reactive oxygen species (ROS) production and the structural and functional cellular effects that may result [7]. ROS production from mitochondria have been implicated in various forms of cell signaling processes including activation of eNOS [8], NF-κB [9], ERK, AKT, P38MAPK [10] and modulation of intracellular calcium [11]. Mitochondrial derived ROS serves as both; signaling agent as well as a major source of oxidative stress. Under mitochondrial stress, the Ca^{+2} level as well as ROS production increases, initiating apoptosis and necrosis. These factors, which are all key metabolites to cellular health sensing, contribute to the hallmark of mitochondrial disease, which is its clinical and biochemical heterogeneity and the wide-ranging approaches currently implemented or under consideration to treat disorders of energy metabolism [12]. It also created the awareness over time that the etiology of mitochondrial disease may not only be relevant to rare disease phenotypes, but also to other common diseases. As recently reviewed by Naviaux [13], cellular perception of threats (chemical, physical, microbial) are expressed in conserved but diverse metabolic signals in which mitochondria and mitochondrial-nuclear communication plays a central role. Consequently, with the elucidation of the molecular and cell biological fundamentals of human diseases, it is now clear that pathways directly or indirectly linked to mitochondrial energy metabolism is dysfunctional or deregulated in numerous rare and more common disease phenotypes. These include rare disease phenotypes such as Alpers disease, described already in 1931 [14], but only ~70 years later recognized as a mitochondrial disease resulting from *POLG* mutations [15]. The metabolic dysregulation of energy metabolism in cancerous cells described originally by Warburg in the late 1950s and 60s also helped to recognizing mitochondrial energy involvement in common disease phenotypes. Over recent times this involvement has been illustrated for several death-causing non-communicable and infectious diseases such as neurodegenerative diseases [16], cardiovascular diseases [17], type 2 diabetes mellitus [18], autoimmune disorders [19], cancers [20] and AIDS [21]. Moreover, a principle hypothesis for the aging process involves reduced mitochondrial function [22]. Not to be disregarded are the effects of modern environmental and other lifestyle factors (toxins, pollutants, diet, medication, etc.) which can impact directly or indirectly on mitochondrial function [23]. Much of these discoveries have come through the recent development of scientific methods and technologies directed at holistic and multidisciplinary investigations. With mitochondrial metabolism often playing a central role in disease pathology with various mitochondrial pathways involved and with varied cellular outcomes, such investigations are frequently warranted. We herewith present an overview of the current technologies used where mitochondrial function/dysfunction are investigated and the practicalities around these technologies.

2. Review

2.1. Overview of mitochondrion genomic architecture

Mitochondria are assumed to be originated from α-proteobacteria and found nearly in all eucaryotic organisms. Each mitochondrion contains circular double stranded DNA which is approximately 16.6 Kb in length and encodes for 37 genes that includes 22 tRNA, two ribosomal RNA genes used for RNA translation and thirteen structural subunits of oxidative phosphorylation (OXPHOS) of complex I (7 subunits), III (1 subunit), IV (3 subunit), and V (2 subunit) [5]. Inherited exclusively through the maternal line, the mtDNA has much higher rate of mutation as compared to nDNA [24]. It is believed that the absence of protective histones and its proximity toward OXPHOS system that produces ROS are major factors that contributed to mtDNA vulnerability. Although, the rate of mutation for nDNA was estimated to be 2.5×10^{-8} per base per generation, mtDNA mutation rate estimated to be 3×10^{-6}–2.7×10^{-5} per base per generation [25]. The high rate of mutation of mtDNA is not only responsible for the generation of deleterious mtDNA variants but also resulted in the accumulation of commonly occurring variants of mtDNA in various human populations that is why mtDNA is always a fascinating subject for evolutionary geneticist and population geneticist. Not only this, its property of having high number of copies in cells makes it a suitable target for forensic analysis. Interestingly, the presence of multiple copies of mtDNA per cell leaves upon the possibility that either all MtDNA in cells are not identical a termed known as heteroplasmy or the possibility of having identical mtDNA in all cells termed as homoplasmy [24]. Moreover, mtDNA in mammals are strictly maternally inherited with all homoplasmic DNA mutations transmitted to offspring [26], whereas, the transmission of heteroplasmic mtDNA mutations is complicated by both a selection process and a genetic bottleneck present during development of the female germline.

2.2. Molecular genetics approaches and diagnostic tools

Energy metabolism disorders are heterogeneous group of disorders that includes mitochondrial diseases caused by mtDNA and nDNA mutations. Since, these diseases are mainly diagnosed based on biochemical or molecular evaluation, both require extensive laboratory testing to determine whether the patient is affected with mitochondrial disease or not. Interestingly, molecular genetic testing is more important to solidify the diagnosis and have more inter-laboratory reproducibility [27] but also harbor disadvantage in presenting false positive result due to the presence of low heteroplasmy in peripheral blood samples. With the advancement in technologies, various molecular methodologies are used by different laboratories to overcome these diagnostic challenges.

2.3. Diagnostic tools for genetic investigations

In the past, diagnoses of mitochondrial diseases were carried out on the basis of family history, clinical, histological and biochemical studies. In addition to these studies, more testing tools had been utilized with the aim of better understanding of mitochondrial functionality such as screening of mutations in mtDNA/nDNA over the last several years. These techniques provided additional information on mitochondrial diseases that had not been detectable using traditional approaches. The major obstacle in the genetic investigation was to determine the mutations present in mitochondrial gene. Initially, the methods for screening of known or unknown mutations were indirect i.e. mutation was confirmed visualizing on gel. These can be easily done by the restriction fragment length polymorphisms (RFLP) [28], single strand conformation polymorphism (SCCP), temporal temperature gradient gel electrophoresis (TTGE), temperature gradient gel electrophoresis (TGGE) and denaturant gradient gel electrophoresis (DGGE). RFLP was considered as the first choice for genotyping. In this method, the PCR amplicons were generated to flank a polymorphic RE site and subjected to digestion and the site digested was determined using agarose gel electrophoresis and visualized under ultra violet (UV) radiation. The second method was SCCP that employs amplifying DNA fragments of 200–350 bp

using PCR in the presence of ^{32}P ATP. The fragment were denatured into single strands and subjected to non-denaturing PAGE. These fragments were folded into secondary structure and based on migratory patterns, the normal vs. mutant fragments were detected [29]. In contrary, TTGE was based on the difference in the sequence specific melting behavior of the fragments that increases gradually in a linear fashion over the duration of electrophoresis. Owing to the differential melting behavior, mutant and normal fragments were separated on gel as they denatured at different temperatures [30]. Similarly, in DGGE; instead of temperature, chemical was used as a denaturant [31]. In addition, TGGE method employs similar principle as that of TTGE but the difference is that instead of temperature gradient, constant temperature was applied [32]. These indirect methods have been replaced by more robust, highly sensitive direct method such as Sanger Sequencing [33] that was performed by amplifying overlapping fragments of whole mitochondrial genome using 24–36 primer pairs. Although, sequencing is robust and straightforward, it did not detect large deletions and degree of mitochondrial heteroplasmy. Due to its high polymorphic features, mtDNA fragments experienced calling bases that are not strictly representative of the mitochondrial sequences [34]. In order to overcome these obstacles, next generation sequencing (NGS) was performed on routinely basis. This technology not only detects mtDNA mutation and large deletions acquiring quantitative heteroplasmy, but also analyzed genes that were involved in causing disease or are suspected to cause mitochondrial dysfunction [35]. The main drawback in detecting mtDNA heteroplasmy is the presence of pseudogenes in the nuclear genome that are non-functional but share strong sequence similarity with mtDNA genes [36]. These pseudogenes result from the translocation of cytoplasmic mitochondrial sequences into the nuclear genome and are referred as "nuclear mitochondrial DNA transcripts" (Numt) [37]. Nowadays, high throughput expression profiling techniques such as Microarray and real time PCR (qPCR) have provided a wealth of information on the dynamics of mitochondrial disorders to study the changes in expression of mRNA in mitochondrial diseases. Despite these benefits, these expression profiles showed inconsistency of gene expression in many metabolic processes such as fatty acid metabolism, amino acid metabolism and also in the cell cycle regulation [38]. However, the principle drawback of these expression profiling techniques lies in their limited dynamic range, due to the poor quantification of weak signals. Moreover, mutation analysis based on nanotechnologies provides an early and non-invasive method [39]. Even though these techniques hold many advantages, they are expensive and time-consuming.

2.4. Classical approaches to modern approaches

Energy metabolism disorder became an increasingly recognized clinical entity till the twentieth century. In order to address a definite diagnosis of mitochondrial diseases, several iterations of clinical diagnostic criteria were proposed by expert clinicians categorizing individuals with suspected mitochondrial disease as "Probable", "Possible", "Unlikely", or "Definite", based on clinical and biochemical findings that are characteristics of known mitochondrial disease syndromes [13]. The category of "Probable" mitochondrial disease described, if biochemical evidence of impaired respiratory chain or enzyme activity was present, whereas, "Possible" described, if biochemical evidence of impaired respiratory chain was not performed or normal. Similarly, "Unlikely" described as having too few symptoms to be suspicious of classical mitochondrial disease and "Definite" mitochondrial disease had the genetic basis for their disorder clearly determined by an individualized, time-labor, cost-intensive, genetic diagnostic approach. Remarkably, the first mtDNA mutations were not identified until 1988, since then, significant efforts were done to categorize array of genetic variants of these disorders [19]. Despite these gains in understanding the genetic basis of mitochondrial diseases, clinical diagnostic methods evaluating a patient for all potentially pathogenic mtDNA mutations were found to be limited. In fact, genetic testing was limited in most clinical diagnostic cases and were restricted to testing for a panel of common MtDNA point mutations of well-recognized mitochondrial diseases such as mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy and ragged red fibers (MERRF), neurogenic ataxia and retinitis pigmentosa (NARP) and several MtDNA deletions resulting in Pearson syndrome, progressive external ophthalmoplegia (PEO), or Kearns–Sayre Syndrome. For such common mitochondrial mutations, the clinically-based genetic diagnostic evaluation for a specific molecular etiology was often halted. Indeed, whole MtDNA genome analysis was successful over PCR-based Sanger sequencing which was not widely available on a clinical diagnostic basis until the mid-2000s [40]. However, genetic testing is expensive, costing on an average several dollars to sequence a single gene [41]. Since sequencing was introduced to clinical testing, clinicians select one or few nuclear genes to test based upon the symptomatic or biochemical specific findings in their patients. Apart from mitochondrial genes, nuclear genes play a vital role in mitochondrial diseases and estimated to cause approximately three quarter of pediatric and one-third of adult mitochondrial diseases. These nuclear genes causing mitochondrial diseases were detected by means of karyotype (nuclear chromosome analysis) used to evaluate large aneuploidies. In addition, targeted fluorescence in situ hybridization assays used to investigate identifiable microdeletion syndromes, such as DiGeorge syndrome or Williams syndrome. Very few individual nuclear genes were available to be sequenced for mitochondrial diseases in a clinical diagnostic laboratory. Furthermore, linkage analysis was explored to identify a candidate gene region that might harbor a disease-causing nuclear gene mutation(s). Hence, it was among the main methods used for identification of the nuclear genes causative of mitochondrial diseases. A significant improvement in the ability to diagnose maternally-inherited mitochondrial disease came with the widespread clinical diagnostic availability of modern technique such as whole mtDNA genome sequencing [42]. Rather than evaluating dozen of common mutations, whole mtDNA genome sequencing permitted identification of all known and potentially novel disease-causing mutations in a single platform. Several methodologies were utilized for mtDNA genome analysis by different clinical diagnostic laboratories in twenty first century, ranging from the gold-standard Sanger based method to surveyor-based heteroduplex analysis to chip-based array analysis and nanotechnology [42]. These methodologies differed significantly in their ability to detect low-level heteroplasmy and substantial clinician energy was required to assure the proper tissue was assayed and testing methodology was employed in a given case. Identification of mtDNA depletion or multiple deletion(s) was suggestive of a primary nuclear genetic disorder due to mutations in any of nuclear genes involved in mitochondrial nucleotide metabolism [42]. Detailed information on the biochemical phenotype prior to high throughput genetic testing will be of benefit to the candidate genes. Moreover, gene analysis became clinically available over time, as exemplified by a targeted "MitoMet Array" that could be used to identify a deletion in a specific gene, as when only a single pathogenic mutation was identified in a patient for a gene in which disease was known to result only in a recessive fashion. Furthermore, developments in genetic testing will change the role of biochemical diagnosis of mitochondrial dysfunction. It can be expected that there will be a greater demand for more detailed functional studies to confirm the pathogenicity of new molecular

genetic variants. Therefore, there is no doubt that high throughput molecular genetics method such as Bioenergetic Health Index (BHI) Profiling serves as a "canary in a coalmine" and therefore increases the possibilities for diagnostic testing of mitochondrial disorders. BHI act as a biomarker of an individual's overall capacity to produce sufficient energy to fight diseases associated with mitochondrial dysfunction and is accomplished by O_2 dependent quenching of porphyrin based phosphors such as XF extracellular flux analyser. This system allows measurements of oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR), which can be correlated with OXPHOS and glycolysis respectively [43]. In general, a high BHI of an individual signifies healthy cells, whereas, low BHI of an individual signifies defects in the electron transport chain associated with the mitochondrial dysfunction. BHI has already been successful in many studies such as diabetes [44], Alzheimer [45], septic shock [46] and fibromyalgia [47]. In future, studies combining BHI with genomics or various cellular indicators would be a step toward personalized based cell measurements.

2.5. Involvement of Genome Wide Association Studies (GWAS)

Since human genome research has provided researchers with new tool and technologies to identify various genetic variations of common diseases such as Heart attack, diabetes and cancers, Genome Wide Association Studies (GWAS) emerged as a comprehensive and biologically agnostic approach in searching for unknown disease variants and has been found to be very successful in identifying novel genetic loci for various human complex traits [48]. The principle aim is to identify novel genetic variants to elucidate the disease biological pathways and eventually lead to identification of new molecular markers for diagnostic application, or drug targets for therapeutic intervention [49]. In addition, these methods provide new insights into the molecular pathways of complex diseases even when the most of the disease causing variants remain discerned from the correlated markers [50]. Thus, the primary outputs of the GWAS were merely association signals, since it was very difficult to find out how much each type of variant (rare vs. common) had contributed in the prognosis of disease. Specifically, GWAS provides information about association between variants and disease and the task of moving association signal from casual variants to a molecular and cellular mechanisms involved in generating phenotypic effects have emerged as a major roadblock in translation research [51]. Thus, these associations of variants with phenotypic traits are useful information for researchers to develop better ways to detect, treat and prevent the disease, but such translation takes time and is mainly dependent on the bridging gap from association signal to mechanism. Moreover, GWAS are laying the foundation toward 'Personalized medicine', where one-size-fits-all approach to medical care will be transformed to more individualized and customized approach [52].

2.6. Role of metabolomics in mitochondrial disease

Functional genomics has been in the focus since many years to unravel the functional and biological mechanisms. Recently, a new field of omics has emerged in a diagnostic arena named as "Metabolomics". Metabolomics is the comprehensive profiling of metabolites and other small molecules that has certain advantages over other relative youth omics such as genomics, proteomics or transcriptomics. It measures chemical phenotypes that are resultant of genomic, transcriptomic and proteomic variability, thus providing more integrated profile of biological state and also costs three to four magnitude lower than the transcriptomic and proteomic. Detection of metabolites is carried out in cells, tissues or biofluids by either nuclear magnetic resonance (NMR) or mass spectroscopy (MS) [53]. NMR is based on the change in the behavior of molecules when placed in a magnetic field allowing the identification of different nuclei based on their resonant frequency. Contrary to this, both gas chromatography (GC–MS) and liquid chromatography (LC–MS) analyses in mass spectroscopy determine the composition of particles based on the mass-to-charge ratio in charged particles. Out of these two, NMR holds an advantage that it is a highly quantitative technique, as each metabolite peak will have an integral value which is directly proportional to the metabolite concentration. However, mass spectroscopy does have a higher sensitivity of metabolite detection than NMR (picomolar compared to micromolar) therefore more compounds can be identified and analyzed [54]. Despite that, there will be an increasing demand for a more detailed functional analysis of individual genetic variants, in order to confirm the diagnosis in individual patients. The combination of a sophisticated bioinformatical approach with detailed functional analysis of individual proteins and pathways will be required to unravel the mechanisms behind protein malfunction and pathogenesis of mitochondrial disorders [55]. In addition, detailed information on the biochemical phenotype prior to high throughput genetic testing will be of benefit to the selection of groups of candidate genes and to bioinformatical evaluation of genetic variants. For example, in the case of a proven complex I deficiency, the high-throughput genetic examination could focus on complex I genes. Therefore, the biochemical evaluation of mitochondrial functional state will play an important role in the diagnostic examination of mitochondrial patients in the foreseeable future.

3. Conclusion and future outlook

Mitochondrial disorders are among one of the rapidly increasing diseases worldwide. Traditional diagnostic tools for mitochondria are often invasive, costly and in many cases are not properly diagnostic. However, advent of high throughput technology has revolutionized mitochondrial medicine providing less invasive and more classic diagnostic options for the evaluation of suspected mitochondrial patients. This method will broaden the testing availability to a wider range of patients leading to more accurate diagnosis and treatment of affected individuals.

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Conflict of interests

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