Chapter

Maneuvering Mitochondria for Better Understanding of Therapeutic Potential of mtDNA Mutation

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Abstract

Heterogeneity of mitochondrial diseases in terms of genetic etiology and clinical management makes their diagnosis challenging. Mitochondrial genome, basic mitochondrial genetics, common mutations, and their correlation with human diseases is well-established now and advances in sequencing is accelerating the molecular diagnostics of mitochondrial diseases. Major research focus now is on development of mtDNA intervention techniques like mtDNA gene editing, transfer of exogenous genes (sometimes even entire mtDNA) that would compensate for mtDNA mutations responsible for mitochondrial dysfunction. Although these genetic manipulation techniques have good potential for treatment of mtDNA diseases, research on such mitochondrial manipulation fosters ethical issues. The present chapter starts with an introduction to the factors that influence the clinical features of mitochondrial diseases. Advancement in treatments for mitochondrial diseases are then discussed followed by a note on methods for preventing transmission of these diseases.

Keywords: mitochondrial diseases, mtDNA intervention techniques, mitochondrial donation, genomics advancements, reproductive techniques

1. Introduction

Mitochondria are synonymized with energy thanks to their ability to produce most of the Adenosine Triphosphate (ATP) through the process of Oxidative Phosphorylation. In addition to ATP production, several metabolic processes like tricarboxylic acid cycle (TCA), fatty acid oxidation, ketogenesis, urea cycle (partly), heme and phospholipid synthesis take place in mitochondria [1, 2]. Role of mitochondria in cell death (apoptosis) is also well-established [3]. Recent research suggests new role of mitochondria in calcium homeostasis, iron and copper metabolism and inflammation and immunity [4]. Though oxidative phosphorylation puts aerobes at higher level in terms of efficiency of energy production, one unpleasant consequence of this important process is production of reactive oxygen species (ROS) also known as mitochondrial ROS (mtROS). The culprit for formation of these reactive species

is proton leak at the inner mitochondrial membrane. Formation of such species pose great threat to mitochondrial DNA (mtDNA) and may lead to mitochondrial dysfunction [5]. Once thought to be uncommon, mtDNA diseases are now known to be quite prevalent and their definition is no more restricted to defects in oxidative phosphorylation alone but also include defects in molecular processes like mitochondrial fission, fusion and translation [6–8].

The list of common mitochondrial diseases and syndromes is quite lengthy that include mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS syndrome), Leber's hereditary optic neuropathy (LHON), myoclinic epilepsy with ragged-red fibers (MERRF), Leigh syndrome and Pearson syndrome, Kearne-Sayre syndrome (KSS), chronic progressive external ophthalmoloplegia (CPEO) and neuropathy, ataxia and retinitis pigmentosa (NARP) [9–17]. Mutations in the mitochondrially-encoded genes are the most common cause of these diseases. Several mutations have been reported such as m.3243A > G, m.3271 T > C, m.1642G > A, m.9957 T > C, m.3272 T > C, m.1642G > A, m.1277A > G, m.13045A > C, m.13513G > A and m.13514A > G (all reported in MELAS [18–26]), m.8344A > G, m.8356G > A, m.3291 T > C, m.4279A > G (reported in MERRF [27–29]), G3460A, T14484C in LHON [30]. Recent review describes a comprehensive approach to study mitochondrial disorders caused by mutations through an example of m.3243 A > G [31].

Reviews on basic mitochondrial genetics, mutations and their correlation with human diseases are available [32–34]. Starting with unique features of mitochondria that decide the clinical presentation of mitochondrial diseases, this review focusses on advancement in mitochondrial DNA manipulation. Methods for preventing transmission of these diseases are discussed at the end.

2. Factors that govern clinical features of mitochondrial diseases

2.1 Heteroplasmy

Presence of several thousand copies of mitochondrial genome (mtGenome) per cell creates two conditions; homoplasmy and heteroplasmy. When all copies of mtGenome are identical, the scenario is described as homoplasmy. Heteroplasmy is a situation in which more than one mtDNA variants exist between the cells of an individual or within a same cell. Often this is due to de novo mutations either in germ line or in somatic cells. As a result, mitochondrial dysfunction can be seen only in specific cells, tissues, or organs. The rate at which the regions in the mtGenome evolves is much higher than that of nuclear genes. This reduces the possibility of all mtDNA molecules to be identical in an individual's cells. Considering the large copy number of mtGenome present, detection of mtDNA mutation is difficult until it is spread among enough mtDNA molecules in a given cell. Only when mutated mtDNA exceeds threshold levels, clinical consequences of such mutations are seen [35]. Absence of fixed functional threshold level makes the analysis of mtDNA results even more complicated. Variations in threshold frequencies have been reported for different types of tissues and mtDNA mutations.

2.2 Mitochondrial DNA bottleneck

Mitochondrial genome, unlike its nuclear counterpart, shows uniparental transmission. Considering a single-parent origin, theoretically, mitochondrial DNA of a

mother and her progeny should not show any variations. But, in reality, extensive variations have been reported in humans [36, 37]. Accumulation and enrichment of mutant mitochondria thus suggests presence of mitochondrial bottleneck; a concept that describes why mtDNA of an embryo may differ significantly from that of its mother [38].

3. Manipulation of mitochondrial DNA

Diagnosis and monitoring clinical progression of mtDNA diseases is difficult due to multi-copy nature of mtGenome. Fortunately, many harmful mtDNA mutations are heteroplasmic and this paves the way for curing these disorders. If mutated copies of mtDNAmolecules can be removed selectively from the pool of wild type molecules, heteroplasmy can be reduced and cellular biochemical defects can be cured. However, manipulating heteroplasmy has been challenging due to several barriers. Some of these barriers and attempts to overcome them are discussed in this section.

3.1 First barrier: difficulty in mitochondrial transfection

Mitochondria have two lipid bilayers that includes outer and inner membranes. While outer membrane allows easy transport of small molecules like ATP, proteins less than 10 KDaand ions, the inner mebrane brings selectivity barrier. Hydrophilic molecules cannot cross this barrier due to presence of cardiolipin; a hallmark mitochondrial lipid with four alkyl tails. It is this impermeability of inner membrane to the hydrophilic molecules that makes the passage of DNA through mitochondrial membranes difficult.

3.2 Strategies to overcome mitochondrial membrane barrier

One of the effective ways to treat mitochondrial diseases is to introduce wild type genes into the mitochondria. The approaches for introducing genes can be broadly classified into three categories namely physical, chemical, and biological methods. Physical methods are relatively simple and straightforward. Methods like microinjection, particle bombardment, electroporation and sonication have been used for delivering exogenous genes into the mitochondria [39, 40]. Separate carrier molecules are not required in these methods which eliminates the toxicity problems of such molecules. However, drawbacks of these methods include random distribution of DNA in mitochondrial matrix and the risk of damage of target cell during cell membrane penetration [40].

Many chemical-based methods have been reported for mitochondrial gene delivery. Considering hydrophobicity and presence of negative charges on mitochondrial membrane, cationic and amphiphilic carrier molecules have been used to enclose the negatively charged DNA [41]. Plasmid DNA was introduced to mitochondria using rhodamine-pDNA-nanoparticle complex [42] where the dye facilitated movement of nanoparticles across the plasma membrane and mitochondrial membrane. Mitochondria-specific liposomes were used for successful release of plasmid DNA in mitochondrial matrix [43], however, certain limitations like cytotoxicity and low transfection efficiency were noted. Improved version of liposome-based nanocarrier came in the form of MITO-Porter [44, 45]. Current research focuses on improving

the mitochondrial targeting and reducing the toxicity to target cells. New ligands are being explored and linked to chemically synthesized carrier molecules that target the mitochondrial receptors.

Understanding of mitochondrial targeting signal peptide (MTS)-mediated translocation has provided a new biological approach for specific mitochondrial gene delivery. Carrier molecules having DNA-binding ability were conjugated to MTS. DNA oligomer peptide nucleic acid (PNA) that has polyamide bond rather than usual sugar-phosphate backbone, was conjugated to MTS and this MTS-mediated PNA could successfully enter the mitochondrial matrix through the translocase of outer membrane (TOM) and that of inner membrane (TIM) [46, 47]. Though this approach has some shortcomings like low mitochondrial targeting (as PNA tends to be localized in nucleus) and the restricted size of genes-to be-transferred, this is a clear indication that MTS can be successfully applied in mitogene delivery in near future. Use of viral vectors, especially adeno-associated virus (AAV), have been tested for mitochondrial gene delivery [48]. The wild type human mitochondrial genes were added to MTS-AAV complex to compensate mutated and defective NADH ubiquinone oxidoreductase subunit 4 (ND4) gene which is the culprit for LHON [49]. In addition to these physical, chemical, and biological methods, there are several combinatorial approaches that have been tested. A recent review [50] gives details of these methods and also discusses the need for new approaches.

3.3 Barrier 2: eliminating mutant mtDNA molecules

Elimination of mutant mtDNA molecules can reduce the threshold of mutant molecule load. Total elimination of mutant mtDNA is not required because a small reduction in mutant mtDNA load just below the threshold can improve the clinical scenario of a diseased person.

3.4 Strategies to selectively target mutant molecules

Construction and characterization of mitoApaLI; one of the several mitochondria-targeted restriction endonucleases developed so far, and its significant role in shifting heteroplasmy towards one of the two mtDNA haplotypes is explained in detail in a recent book chapter [51]. The prerequisite (also a limiting factor) of using mitoREs is that the target mutation should result in a unique restriction site to avoid breaking of wild type mtDNA. Different methods of mitochondrial transfection and strategies to deal with heteroplasmy are summerized in **Figure 1.**

Two recent gene editing systems namely mitochondria-targeted transcription activator-like effector nucleases (mitoTALENs) and mitochondria-targeted zinc finger nucleases (mtZFN) can selectively target single nucleotide mutations and can degrade them. Minczuk and Gammage laboratories have extensively used mtZFN to shift heteroplasmy [52, 53]. The mitoTALENs have been used to target specific mutations from animal and human-derived cells [54]. Although these gene therapy approaches are quite promising, we need to be careful because of the risk involved in this approach. mtDNA copy number may go down significantly and there may be undesirable off-target effects while attempting elimination of mutated copies. Crisper-Cas 9 cannot be used for this purpose because it needs single-guide RNA for gene editing and RNA import in mitochondria is restricted [55].

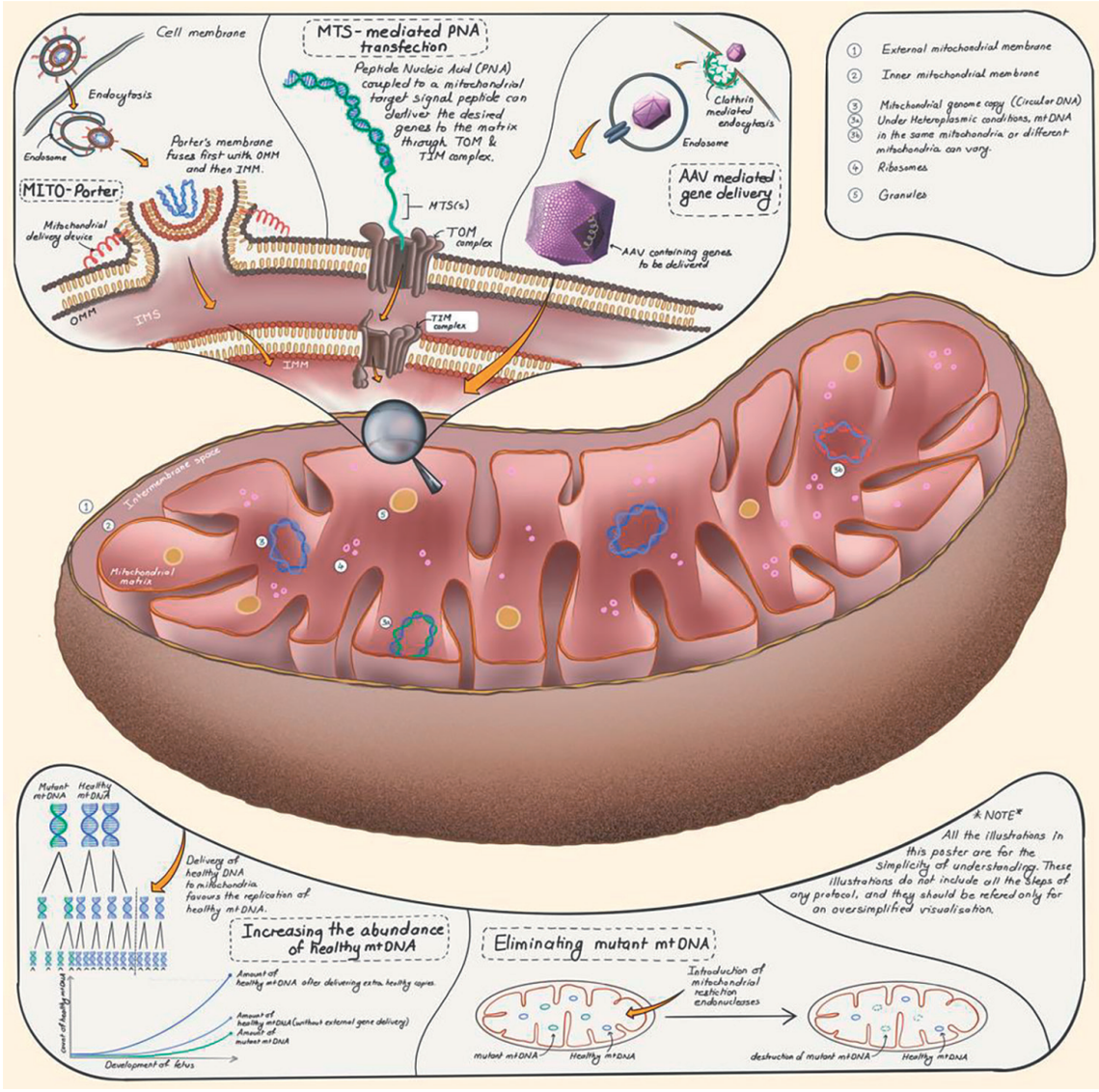

Figure 1.
The figure is a schematic representation of different methods of mitochondrial transfection and strategies to deal with heteroplasmy. Abbreviations used in the figure are: Outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM), mitochondrial target signal peptide (MTS), translocase of outer membrane (TOM), translocase of inner membrane (TIM), adeno-associated virus (AAV) and mitochondrial DNA (mtDNA).

4. Decrease in NAD^+ levels

Nicotinamide adenine dinucleotide oxidized (NAD^+) is a coenzyme required for action of many enzymes like polyADP ribose polymerase (PARP) and sirtuin deacetylases. Substantial decrease in NAD^+concentration and the ratio of NAD^+/NADH was reported in the cells having defective mitochondria [56]. Defective respiratory chain cannot reoxidize NADH to oxygen. This results in reduction of pyruvate to lactate by lactate dehydrogenase generating NAD^+. Transport of excess lactate outside the cell leads to lactate acidemia, which is a common feature of mitochondrial diseases. Increasing the cellular levels of NAD^+ either through supplementation or through bringing changes in enzymes involved in its synthesis have been reported [57].

A recent approach tested in mice was to reoxidize extracellular lactate to pyruvate and bring it back to the cell for its re-reduction by lactate dehydrogenase thus increasing NAD^+/NADH ratio [58].

5. Prevention of transmission of mitochondrial diseases

5.1 Options to prevent transmission

Mitochondrial DNA is maternally inherited and genetic bottleneck makes it even more peculiar. Therefore, options different from those with nuclear genetic defects must be considered. It is important to know which mutation a woman carries and its level; especially in those cases who harbor heteroplasmic mtDNA mutations. Genetic diagnosis and expert counseling is invaluable for such cases. Post-counseling options include voluntary childlessness and adoption. Prenatal testing and preimplantation genetic diagnosis (PGD) are recently available alternatives. PGD includes in vitro fertilization (IVF) and embryo development to blastocyst stage. Because of inherent issues with IVF, PGD has limited chance to succeed.

5.2 Mitochondrial replacement therapy (MRT) or mitochondrial donation

MRT is probably the only way available to those couples who are suffering from mitochondrial disease and wish to have a healthy child. In such cases, nucleus is taken from a mother carrying defective mitochondria and transferred to an enucleated oocyte or egg of a woman with healthy mitochondria. Embryo formed after this procedure (also called as three parent embryo) will have nuclear DNA from both parents but mitochondrial DNA from another mother. Ideally such embryo should be free from defective mitochondria. Using this technique in human oocytes, good quality embryos could be formed as reported by several workers [59, 60]. Though potentially this is a great advancement, mitochondrial donation may raise ethical issues [61]. Also some workers observed that the nucleus which was transferred to enucleated oocyte/egg showed presence of contaminating defective mitochondria. Enrichment of such contaminating mitochondria may cause mitochondrial disease in individuals generated through MRT. This issue becomes more sensitive when female embryos are generated after MRT because they will be passing on their defective mitochondria to the next generation. MRT females may show same mitochondrial disease and infertility as their mothers. In future, better understanding of maternal inheritance of mitochondria will improve the efficacy of this therapeutic method and make it a sustainable approach for betterment of individuals across the generations. Another issue that may hamper the progress of mitochondrial donation is availability of oocyte donors because this involves hormonal treatment.

6. Conclusions

Advances in DNA sequencing are accelerating the diagnosis of mitochondrial diseases and helping in assessment of heteroplasmy levels. Although molecular diagnosis is crucial, it can only identify the problem but cannot solve it. Input from reproductive biologists are equally important for comprehensive analysis and personal care of diseased individuals. Development of new treatments through further advancements in gene therapy holds great promise for the sufferers of mitochondrial disease.

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