Mitochondrial genome variability: the effect on cellular functional activity

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Abstract: Mitochondria are the key players in cell metabolism, calcium homeostasis, and reactive oxygen species (ROS) production. Mitochondrial genome alterations are reported to be associated with numerous human disorders affecting nearly all tissues. In this review, we discuss the available information on the involvement of mitochondrial DNA (mtDNA) mutations in cell dysfunction.

Keywords: mitochondria, mutation, apoptosis, reactive oxygen species, ATP, electron transfer chain

Introduction
Mitochondria are often referred to as the “powerhouses of the cell” because they produce about 90% of cellular ATP.1 They also have some other vital functions participating in cell signaling, proliferation, and death. It is well known that mitochondrial dysfunction is implicated in a number of human diseases including, but not limited to, Leigh syndrome,2 Leber’s hereditary optic neuropathy,3 mitochondrial encephalomyopathy with lactic acidosis and stroke like episodes (MELAS),4 myoclonus epilepsy with ragged red fibers (MERFF),5 Alzheimer’s disease,6 Parkinson’s disease,7 and different forms of cancer.8 Mitochondrial diseases affect nearly all tissues, and thus, their clinical signs are extremely various, partly because the respiratory chain malfunction leads to aberrant cell signaling.9

Mitochondrial genome
The organization of human mitochondrial genome has been extensively studied and is currently relatively well known.10 It differs substantially from the nuclear genome in almost all aspects. Mitochondrial DNA (mtDNA) of mammals is a double-stranded circular molecule 16.6 kb long with heavy and light strands enriched by purines and pyrimidines correspondingly. mtDNA contains 37 genes with short non-coding sequences in between and one large triple-stranded non-coding region, D-loop, containing transcriptional promoters and at least one of the proposed replication origins. mtDNA is packaged into a nucleoid by mitochondrial transcription factor A (TFAM), which is suggested to condense DNA by inducing bending and then wrapping it into compact nucleoid structures.

Replication of mtDNA occurs even in post-mitotic cells and is independent from that of the nuclear DNA. Key replisome proteins include DNA polymerase γ, mtDNA helicase Twinkle, mitochondrial single-stranded DNA-binding protein, and mitochondrial RNA polymerase (which most likely functions as the mtDNA primase).11 Despite the relatively small size of mtDNA, its replication includes diverse mechanisms such as...
rolling circle, theta, strand-displacement, strand-coupled, and ribonucleotides incorporated throughout the lagging strand (RITOLS)/bootlace.\textsuperscript{12} The robust rolling cycle mechanism is used by plants, fungi, and nematodes. Theta model of replication was studied in \textit{Drosophila} and sea urchin. Mitochondrial genome in vertebrates is replicated via the following mechanisms: strand displacement, strand coupled, and RITOLS/bootlace. In the traditional strand displacement model, mtDNA replication is initiated in \textit{Oh} (origin of heavy strand). Continuous and unidirectional enlargement of this D-loop proceeds for a distance of about two thirds around the circular genome. When approximately two thirds of the genome length is replicated, \textit{Ol} (the initiation site for lagging strand synthesis) is exposed, and synthesis of the nascent L-strand proceeds in the opposite direction on the single-stranded template of the expanded D-loop.\textsuperscript{13} Holt et al\textsuperscript{14} proposed that more than one replication mechanism takes place in mammalian cells. In the proposed model named “bootlace”, pre-synthesized RNA fragments hybridize to the lagging DNA strand while leading DNA strand is being replicated. Strand-coupled model shares similarity with the theta model. Replication starts at a broad initiation zone containing the genes \textit{cytb}, \textit{nad5}, and \textit{nad6} and proceeds bidirectionally until the D-loop region. To date, a number of mitochondrial polymerases have been described besides DNA PolG, namely, PrimPol, PolIB, PolZ, PolH, and PolQ. However, their particular functions remain to be clarified.\textsuperscript{15}

Previous studies revealed that endoplasmic reticulum (ER) marks the sites of mitochondria fission. ER is wrapped around mitochondria causing its constriction.\textsuperscript{16} The initial constriction then enables the assembly of the ring made of highly conserved dynamin-related protein from the family of large GTPases (Drp1 in mammals, Dnm1 in yeast). This ring is supposed to provide the mechanochemical force that completes fission of the outer and the inner mitochondrial membranes. Recently, an important discovery regarding mtDNA replication was made by simultaneously imaging mitochondria, nucleoids, and the ER network at high spatial and temporal resolution using spinning disk confocal microscopy in U2OS and Cos7 cell lines.\textsuperscript{17} It appeared that mtDNA replication is tightly coupled with its division, and these processes take place in an area of ER–mitochondrion contact. The researchers also noted that the ER tubules are required for intracellular nucleoid distribution within the cell, which is of special importance for polarized cells.

**Mitochondrial genome alterations**

Mitochondrial genome has a much higher mutation rate than the nuclear genome,\textsuperscript{18} which varies across species.\textsuperscript{19} Somatic and inherited mutations in mtDNA are classified into deletions and single-nucleotide replacements that can be neutral or pathological. Inherited genome variations are transmitted via the maternal line because sperm mitochondria are subjected to degradation during fertilization. With progress in high-throughput sequencing methods, more pathological mitochondrial genome mutations associated with human diseases are being reported in the Mitomap database. It has been previously hypothesized that oxidative stress was a major reason for mtDNA variability.\textsuperscript{20} According to the modern point of view, most human mtDNA somatic mutations are due to the infidelity of mtDNA polymerase encoded by the nuclear gene \textit{POLG}.\textsuperscript{21} Most mtDNA deletions are rather big (>1 kb) and occur between two origins of replication. The exact mechanism of the deletion process remains unknown, but it can possibly be explained by polymerase stalling and mispairing during replication, as well as by topoisomerase II cleavage and other DNA double-strand breaks.\textsuperscript{22} Mitochondrial genome is polyploid, and it exists in numerous copies per cell. Therefore, a mitochondrial mutation can be homoplasmic when it is present in each DNA copy and heteroplasmic when it is present only in some copies of the mtDNA of an individual. The majority of pathogenic mutations are recessive. Studies showed that mutational load differs between tissues\textsuperscript{23} and that some mutations are tissue specific. There is a threshold (ratio of wild-type and mutant mtDNA copies) for the heteroplasmy level, above which pathological changes can be observed.\textsuperscript{24,25} The established values of this threshold are about 60%–90%, being lower in tissues more relying on oxidative phosphorylation.\textsuperscript{26} The threshold also varies according to the type of mutations, being approximately 50%–60% and 70%–90% for mtDNA deletion and point mutations, respectively.\textsuperscript{27}

**Mitochondrial protective pathways**

The fate of damaged mitochondria depends on two processes: mitophagy and repair. Mitophagy is dependent on receptors interacting with LC3-II on autophagosome via LC3-interacting region (LIR). Mitophagy receptors recognize molecules in the outer mitochondrial membrane (OMM), such as B-cell lymphoma 2 nineteen kilodalton interacting protein 3 (BNIP3), Nix, Bel2-like protein 13 (Bcl2-L-13) and FUN1, and in the inner membrane, such as cardiolipin. Several non-mitochondrial receptors recognizing both the target mitochondrion and the autophagosome include p62, NDP52, OPTN, NBR1, and TAX1BP1, which contain both LIR motif and ubiquitin-binding domain (UBD).\textsuperscript{28} Among different variants, mitophagy kinase PINK1/ubiquitin
Mitochondrial functions in the cell
Mitochondria and oxidative phosphorylation

A majority of proteins necessary for mitochondrial function are encoded by the nuclear genes and are transported from the cytoplasm. However, 13 mitochondrial genes encode proteins that are essential components of the respiratory chain: complexes (I–IV) and ATP synthase. Other mitochondrial genes are responsible for rRNA and tRNA synthesis, being used for their translation within the organelle. Proteins of the respiratory chain complexes are synthesized within the mitochondria and are located in the highly invaginated inner membrane. Oxidative phosphorylation is thoroughly explained elsewhere. In brief, pyruvate obtained from glycolysis is transferred from the cytoplasm into the mitochondrion, where it acquires acetyl groups from CoA. Acetyl groups are transferred to oxaloacetate and are oxidized to carbon dioxide and water in the tricarboxylic acid (TCA) cycle. Generated during glycolysis and the TCA cycle, electron-rich NADH and succinate intermediates of the TCA cycle are oxidized by the respiratory chain complexes I and II, respectively. The electrons then passed to ubiquinone, which diffuses to complex III in a reduced state in order to be re-oxidized. Respiratory chain complex III reduces cytochrome c, which in turn transfers the electrons to complex IV, where oxygen is reduced to water. This electron transfer leads to active pumping of hydrogen ions from the mitochondrial matrix into the intermembranous space. The resulting electrochemical gradient generated across the inner membrane facilitates proton translocation from the intermembranous space through the ATP synthase complex back into the matrix. This proton transfer is coupled with the reaction of ADP phosphorylation to generate ATP.

In addition to its importance for mitochondrial ATP synthesis, mitochondrial membrane potential (ΔΨ) is needed for protein import into the mitochondria, respiratory substrates uptake, and ion transport across the inner mitochondrial membrane. Therefore, its maintenance is crucial for proper functioning of the mitochondrion. It is known that mutations in the respiratory chain protein genes are accompanied with decreased mitochondrial membrane potential, whereas ATP synthase dysfunction is associated with an increase in ΔΨ.

Direct interrelation between mtDNA variations and reduced OXPHOS system activity and lower ΔΨ can be supported by studies discussed later. Cybrid technology allowed combining Leber hereditary optic neuropathy
Interestingly, proteins modulating this exchange are affected in many neurodegenerative diseases. There is an interrelation between the function of the electron transfer chain (ETC) and calcium ions exchange in the mitochondria. Calcium requires membrane potential for its influx, while increased concentration of calcium enhances ATP synthesis via the following enzymes: pyruvate, alpha-ketoglutarate and NAD-isocitrate dehydrogenases, and ATP synthase. Excess Ca\(^{2+}\) uptake can lead to the permeability transition pore (PTP) opening, release of cytochrome c, and cell death.

Pathogenic mtDNA mutations disturb the ΔΨ and calcium uptake and signaling consequently, which was supported by the results of several recent in vitro studies. It was demonstrated using mutant cybrids that homoplasmic mutation 13565C>T in the NADH gene (MT-ND5) leads to a significant reduction of NADH and mitochondrial membrane potential and is associated with a decrease in calcium uptake by the mitochondria. The authors suggested that in ND5 mutant ATP generated by glycolysis is required for both mitochondrial membrane potential maintenance and regulation of calcium homeostasis in the cell. With the help of induced pluripotent stem cells technology, a library of differentiated neural progenitor cells of patients carrying homoplasmic deleterious MT-ATP6 gene mutation (m.9185T>C) was generated. This variation is common in Leigh syndrome, neuropathy, ataxia, and retinitis pigmentosa (NARP). Patient-derived neural progenitor cells with m.9185T>C exhibited the downregulation of genes involved in mitochondrial calcium homeostasis in contrast to mutated fibroblasts or cybrids. These cells also demonstrated the reduction of calcium-induced calcium release from intracellular stores on glutamate stimulation.

In a yeast model, researchers investigated the relevance of two mutations in the MT-ATP6 gene (Atp6-P163S and Atp6-K90E corresponding to human MTATP6-P136S and MTATP6-K64E found in several cancer cell type) for cancer. Mutant cells had higher reactive oxygen species (ROS) and calcium ions level. The formation of the yeast permeability transition pore homolog (yeast permeability transition pore homolog and yeast mitochondria unspecific channel) after calcium induction was slower. The study demonstrated a link between ATP synthase function, ROS production, calcium homeostasis, and PTP formation when functioning of the porin complex in the mitochondrial outer membrane is disturbed by GFP tag fused to its component Om45p.

**Mitochondria as calcium depots**

Besides being the energy producing units of the cell, mitochondria also act as local calcium (Ca\(^{2+}\)) depots to maintain the intracellular calcium homeostasis, which is based on two continuous and opposite processes: mitochondrial calcium efflux and influx. In case of calcium overload, the mitochondrion acts as a sink for the excess calcium ions via voltage-dependent anion channel (VDAC) in the outer membrane and highly selective calcium uniporter (MCU) in the inner membrane. The uniporter is a mitochondrial membrane potential-dependent calcium selective channel located in the inner mitochondrial membrane. Calcium efflux is carried out by two mechanisms: H\(^+\)/Ca\(^{2+}\) exchange and Na\(^+\)/Ca\(^{2+}\) exchange. It is known that mitochondria operate in close conjunction with the ER in distinct sites called mitochondria-associated membranes (MAMs) that perform calcium exchange between the two compartments.

Interestingly, proteins modulating this exchange are affected in many neurodegenerative diseases. There is an interrelation between the function of the electron transfer chain (ETC) and calcium ions exchange in the mitochondria. Calcium requires membrane potential for its influx, while increased concentration of calcium enhances ATP synthesis via the following enzymes: pyruvate, alpha-ketoglutarate and NAD-isocitrate dehydrogenases, and ATP synthase. Excess Ca\(^{2+}\) uptake can lead to the permeability transition pore (PTP) opening, release of cytochrome c, and cell death.

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**Mitochondria and oxidative stress**

Hosting the processes of oxidative phosphorylation, mitochondria are also cellular sites of ROS production. In the respiratory chain, electrons derived from NADH or succinate...
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tion may leak and react with O_2 or other electron acceptors generating free radicals like the superoxide anion (O_2^−), which can be further reduced to the hydroxyl radical (OH·) and hydrogen peroxide (H_2O_2). Respiratory chain complexes I and III are predicted to be the major sites for ROS production. ROS can damage lipids, proteins, and DNA, and several defense mechanisms against ROS exist within the cell. Superoxide is converted initially to H_2O_2 by two intracellular superoxide dismutases (SODs): Cu−Zn SOD (SOD1) and MnSOD (SOD2), and H_2O_2 in turn can be transformed into H_2O by catalase or glutathione peroxidase. There exist a number of non-enzymatic cellular ROS scavengers, for example, ascorbate, flavonoids, carotenoids, and glutathione. Thus, oxidative stress results from an imbalance between ROS generation and disposal and thereby creates oxidative damage.48

A connection between increased ROS production and mtDNA mutations has been demonstrated in numerous studies. In an early study using human cybrids generated from the fusion of enucleated MELAS or MERRF patients cells (containing the following mtDNA mutations: A3243G in the tRNAleu(UUR) and A8344G in the tRNAlys) with human osteosarcoma cells devoid of mtDNA (143B), elevated ROS level was observed.49 In another study on HeLa cells transfected with vectors expressing mutants of MT-ND2 gene (namely G4831A and A4605G), ROS production was markedly increased in comparison with wild-type and vector-alone controls.50 A study performed on a mouse model expressing a proofreading-deficient version (D257A) of the mtDNA polymerase γ demonstrated increased spontaneous mtDNA mutation rates. Although muscle mitochondria in D257A mice had impaired bioenergetics associated with compromised respiration, lower ATP content, and decreased membrane potential, these effects were not accompanied by an increase in mitochondrial ROS production or oxidative damage.51

Within the cell, ROS serve as important signal transducers and actively participate in the inflammation, cancer development, and aging.52 The low levels of ROS promote proliferation.53 whereas high levels of ROS lead to apoptosis. Therefore, it was suggested that an “ROS window” is required for normal/cancer cell functions: above this level, cell death is activated, and below it, proliferation is blocked. Modifying ROS production in tumor cells is among attractive strategies of cancer treatment.54

It is still unclear whether the connection between mtDNA mutations and human aging is causative or merely correlative. A widely known free radical theory of aging has been proposed by Harman.55 Modern studies attempted to explain the links between mtDNA mutations and aging in more detail. For instance, a recent study explored the effects of C150T polymorphism in the D-loop of mtDNA, which was detected earlier in the Italian centenarians. It turned out that cybrids with haplogroups positive for polymorphism C150T have lower ROS production rate comparing to haplogroup-matched cybrids without that transition. The authors concluded that lower ROS production rate may affect the longevity.56

Mitochondria and cell proliferation and death

Mitochondria play a key role in cell death and proliferation. ETC dysfunction is often followed by impaired cell proliferation. Cybrids with the 3634A>G mutation in ND1 were unable to proliferate on galactose culture medium in contrast to glucose medium. This was indicative of negative effect of the mutation on cell viability, when cells were forced to obtain ATP from the OXPHOS system. Activity of complex I and complex I + III of ETC was significantly lower in mutated cybrids relative to the wild-type controls.57 In the in vitro study using cybrids carrying the heteroplasmic mutation 8839G>C in MT-ATP6 gene associated with NARP syndrome, it was demonstrated that these cybrids were characterized by a lower cell proliferation and decreased mitochondrial membrane potential compared to isogenic wild-type cybrids.57 The exact mechanism of ETC dysfunction influencing cell proliferation remains unclear, but several explanations have been offered. A recent study using clustered regularly interspaced short palindromic repeats-based genetic screen proposed that the key function of the ETC required for cell proliferation is to enable the synthesis of aspartate, a precursor in purine and pyrimidine synthesis.58 Results of another in vitro study performed on cells with depleted mtDNA showed that genetic reconstitution of the mitochondrial membrane potential restored ROS level and aspartate synthesis necessary for cell proliferation. Therefore, mitochondrial membrane potential is essential for cell proliferation.59

According to the current understanding, mitochondria play a central role in activating apoptotic cell death in response to cellular dysfunction.60 In vitro, high levels of Ca^{2+}, oxidative stress, and low ATP induce the mitochondrial PTP opening, which allows the diffusion of low-molecular-weight solutes across the inner membrane of the mitochondrion. Although the components of the pore are not entirely defined, ATP synthase was proposed to form its core.61 This leads to mitochondrial swelling and rupture of the OMM with the subsequent release of pro-apoptotic proteins, such
as cytochrome c, SMAC/Diablo, apoptosis-inducing factor, and endonuclease G, into the cytoplasm, where they activate downstream pathways of cell death.62 As mentioned earlier, ROS can also play the role of cell death triggers. It was shown that cybrids with m.9035T>C in the ATP6 gene responsible for a defective F0-ATPase had less than half of the steady-state content of ATP along with an eightfold higher ROS basal level, which led to cell death. Since these effects were compensated by the addition of antioxidants (CoQ10 and vitamin E), it was suggested that cell death was induced by abnormally high levels of ROS.63

Resistance of cancer cells to chemotherapeutics can provide a good example of interconnection between the mitochondrial genome alterations and apoptosis. Several investigations showed that mtDNA mutations could help tumor cells evading cell death in response to chemotherapy. In vitro, T6124C mutation in the cytochrome oxidase subunit 1 gene could affect the resistance of prostate cancer cells to chemotherapeutics (simvastatin). According to the analysis of caspase 3 and multiple proteins involved in cellular apoptosis, mutant cells were more resistant to simvastatin than wild-type control cells.64 The resistance of cybrids generated from human pancreatic cancer cell lines CFPAC-1 and CFPAC-2 to cancer therapeutics has been studied. The first line had four mutations (T10970C in the ND4 gene, T8696C, T9070G in the ATP-6 gene, and A2905G in the 16S rRNA gene), the second line harbored two mutations (G6267A in the COI gene and G10176A in the ND3 gene). Cybrids from healthy subjects’ mitochondria and EB8 cells were used as controls. On exposure to staurosporine, 5-fluorouracil, and cisplatin, mutant cybrids exhibited potential anti-apoptotic activity. This effect was further observed in vivo in mice bearing tumors generated from the above-mentioned cybrids.65

Sarcopenia, age-induced loss of skeletal muscle mass and function resulting from fiber atrophy and loss of muscular fibers, is another example of cell death associated with mitochondrial ETC dysfunction. Studies on rat models demonstrated that regions of muscular fibers with deletions in genes of ETC proteins were positive for markers of cell death.66,67

Mitochondria-associated diseases and conditions

Mitochondrial ETC malfunctions have dramatic consequences for the cell, including ATP and calcium uptake decrease, as well as increased ROS production. These effects can potentially lead to cell dysfunction or death. The list of mitochondria-associated disorders is quite long: from rare syndromes to cancer and neurodegenerative diseases (according to the United Mitochondrial Disease Foundation [UMDF], http://www.umdf.org). For some of these conditions, the exact mechanisms of pathogenesis remain to be elucidated, although it is known that most of them are characterized by insufficient energy supply, calcium dysostosis, or ROS accumulation.

A mechanistic study using induced pluripotent stem cells was performed to clarify the molecular pathological mechanism of action of mutation 5541C>T associated with MELAS. By using iPSC-based strategy, namely, disease-relevant iPSCs derived from myoblasts of the patient, it was demonstrated that m.5541C>T inhibited complex IV formation. That in turn impaired mitochondrial biogenesis and bioenergetics leading to decreased ATP level, increased oxidative stress level, and damaged membrane potential.68

It was suggested that some cell types in mtDNA patients should function relatively normally until there is an increased ATP demand, but highly energy-demanding cells, such as myocytes and neurons, can be severely affected. Mitochondrial myopathy (MM) can affect literally any muscle; therefore, it has various clinical features: from myalgia to spontaneous abortion. Subsarcolemmal accumulation of mitochondria to compensate the lack of ATP production is a classic feature of MM, which can be detected using histochemistry methods (so-called ragged blue fibers) or the Gomori trichrome staining (so-called ragged red fibers [RRF]).69 Decreased energy production resulting from ETC proteins mutations can have fatal consequences in cardiac myofibers, highly depending on energy for contraction. It was estimated that the heart consumes approximately 8% of total body energy. Cardiomyopathy is one of the causes of heart failure, which, despite the enormous cost of medical healthcare, still remains the leading cause of mortality among the elderly.70

ATP production and Ca2+ homeostasis are vital for adequate neuron function. ATP is required for the maintenance of the Na+/K+ gradient crucial for impulse transmission. Moreover, ATP functions as an extracellular signaling molecule. The lack of energy in the brain due to mutations in the ETC genes is often manifested by seizures, which are a clinical sign of mitochondrial epilepsy.71 Calcium fluxes play critical roles in the regulation of neurite outgrowth and synaptogenesis, synaptic transmission and plasticity, and neurons survival. According to the so-called calcium hypothesis of neurodegenerative diseases, atrophic and degenerative processes in the neurons in Alzheimer’s disease, Parkinson’s
disease, and amyotrophic lateral sclerosis are accompanied by alterations in calcium homeostasis.\textsuperscript{72} Neurological disorders are also characterized by higher levels of ROS.

The Mitomap database contains various pathological mutations in the mitochondrial genome associated with neurological disorders like Alzheimer’s disease, LHON, MELAS, MERRF, NARP, Leigh syndrome, and Kearns-Sayre syndrome. Each disorder is presented with several mtDNA alterations. For example, LHON typically manifested as bilateral, painless, subacute visual failure, with 90\% of cases being due to one of the three mutations in NADH dehydrogenase complex subunit genes: m.3460G>A, m.11778G>A, and m.14484T>C. The first pathogenic variant is associated with the most severe phenotype.\textsuperscript{73}

In addition to the disorders described above, accumulating experimental data indicate the involvement of mitochondrial dysfunction and mutations in aging and cancer. It is known that mtDNA mutations accumulate with aging and contribute to cell death in older patients. Impaired OXPHOS function promotes invasion, metastases, and apoptosis resistance of tumor cells. Detailed description of mitochondria participation in aging and tumor development and progression is beyond the scope of the present review and can be found elsewhere.\textsuperscript{74,75}

**Conclusion**

Mitochondria play several important roles in cell functions, and mitochondrial dysfunction affects cell activity, proliferation, and death. Even though it is obvious that alterations in mitochondrial genes are involved in human diseases, there is currently a lack of understanding of the exact underlying pathological mechanisms. Further mechanistic studies that can clarify exact molecular steps in pathogenesis of mitochondria-associated disorders are required.

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