REVIEW

Mitochondrial epigenetics: an unnoticed phenomenon in the mitochondrial gene expression

Shalini Mani

Centre for Emerging Diseases, Department of Biotechnology, Jaypee Institute of Information Technology, Noida, UP, India

*Corresponding author e-mail: shalini.mani@jiit.ac.in

ABSTRACT

Epigenetics provides important information regarding maintenance of the gene expression profile. Extensive studies have shown that nuclear DNA nucleotides as well as histone proteins surrounding this DNA undergo different modifications and thus help in regulating the nuclear gene expression. Mitochondrial DNA is equally important like nuclear DNA for survival of the cell. However, whether mtDNA also undergoes similar epigenetic changes for the regulation of gene expression, is largely unknown. In the recent past, a number of studies have shown some covalent modifications, such as methylation or hydroxymethylation in the mtDNA, which is proposed to play a pivotal epigenetic role in regulating the replication and transcription of mtDNA. Post-translational modifications of mitochondrial transcription factor A (TFAM) are also suggested to be important for epigenetic modulations in mtDNA. These observations introduce a novel concept of mitochondrial epigenetics that can be defined as the study of changes in the mtDNA that can modify heritable phenotype in mitochondria itself without changing the mtDNA sequence. But the detailed mechanism through which mtDNA undergoes epigenetic modifications, as well as its regulation, is not much clear. Mitochondrial dysfunction is a well-known cause for different types of diseases and recently it has also been shown that mtDNA is differentially methylated in various neurological and metabolic diseases. In most of these diseases, this differential methylation was found to be associated with altered mitochondrial gene expression, but the exact mechanism through which mtDNA methylation controls the gene expression, remains elusive. In this review, the mitochondrial epigenetic modifications and their relationship with the regulation of mitochondrial gene expression has been summarized. The mitochondrial epigenetic modifications in different diseases and its significance in pathogenesis has also been highlighted.

KEYWORDS: *Mitochondria, Epigenetics, Methylation, mitochondrial DNA, TFAM,*

Citation: Mani S. Mitochondrial epigenetics: an unnoticed phenomenon in mitochondrial gene expression. Polymorphism 2020; 5: 53-67.

INTRODUCTION

Mitochondria are the key organelles in the cell and are known to perform various important functions crucial for viability of a cell. The primary functions of mitochondria include ATP synthesis, calcium homeostasis, apoptosis regulation, and cellular respiration (Kaniak-Golik and Skoneczna, 2015; Lee and Han, 2017). Tissues with very high energy demand, such as brain, heart, kidney and skeletal muscle are known to contain a large number of mitochondria for their proper functioning. Hence, any perturbation in the aerobic metabolism is predicted to primarily affect these high energy demanding organs. Mitochondria are unique in many different aspects from other cell organelles and the most important property is that they contain their own DNA within the mitochondrial matrix. The mitochondrial DNA (mtDNA) is prokaryotic in origin, hence the genomic architecture and complexity of mtDNA is significantly different from the nuclear DNA (nDNA; Yasukawa, 2018). The different subunits of respiratory chain enzymes are encoded both by mtDNA and nDNA. Hence, the rate of mitochondrial oxidative phosphorylation (OXPHOS) is controlled by both these genomes. Injury to the mitochondrial electron transport chain (ETC) or mutations of mtDNA are known to induce defects in the mitochondrial OXPHOS and thereby ATP synthesis. A large number of pathogenic mtDNA mutations leading to the mitochondrial dysfunction have also been suggested as important factors in the pathogenesis of different neurological disorders, aging, cancer, diabetes as well as a range of other human disorders (Tuppen et al., 2010; Golpich et al., 2017). Most of these pathogenic conditions arising due to mutations in mtDNA mutations are known to exhibit a very high level of genetic and phenotypic heterogeneity. Consequently, in spite of carrying a uniform genetic makeup, a great degree of phenotypic variation has been observed in different mitochondrial diseases. Such phenotypic variability is difficult to explain with the help of mutations of mtDNA as the primary pathogenic factor, implying that additional mechanisms must contribute to the phenotype, including epigenetic modifications. Thus, it is important to explore the role of epigenetic mechanisms in mtDNA disease, as epigenetic factors may not only serve to explain the observed phenotypic heterogeneity but also can explain the level of penetrance and the effect of environmental effects on this group of disorders. Epigenetics also provides the basis for differential gene expression in different cell types, despite having the common genetic makeup. Perturbed epigenetic mechanisms have been associated with a variety of human pathologies, but their role in the pathogenesis of mitochondrial diseases is mostly over-looked and only a limited but thought-provoking evidence are available in the literature. Hence, it is imperative to put all the evidence together to understand the most possible epigenetic mode of regulation of mtDNA gene expression. Hence, in this review article, we focus our discussion on the epigenetic regulation of mtDNA as well as highlight the possible impact of altered epigenetic mechanism on human health.

The mitochondrial genome

Mitochondria are known to carry their own genome and interestingly it is the only non-chromosomal DNA in humans. Each cell contains a number of mitochondria, and each mitochondrion further contains several copies of the mitochondrial genome, so unlike nuclear genome, there are multiple copies of mtDNA in each cell, and more important the number of copies of mtDNA varies from cell to cell type and its energy requirement as well as origin. Though the mtDNA represents less than 1% of total cellular DNA, its mitochondrial gene products are essential for normal cellular function. Mitochondrial genome is a circular and double stranded genome where one strand called heavy strand (H-strand) is purine rich and on the other hand, the complementary light strand (L-strand), is rich in pyrimidines (Asin-Cayuela, 2007). The mitochondrial genome is comprised of 16,569 bp,

and encodes 13 polypeptides, that are integral components of the respiratory chain complexes and are important for the generation of ATP via OXPHOS. These polypeptides include ND1-ND6 including ND4L subunits of respiratory complex I, cytochrome B subunit of respiratory complex III, cytochrome c oxidase subunits I–III of respiratory complex IV, and ATP6 and ATP8 subunits of ATP Synthase. Apart from peptides, mtDNA also encodes for 22 tRNAs and 2 rRNAs (Calvo, 2010; Yasukawa, 2018). To regulate the expression of different mitochondrial genes, mtDNA also possesses a noncoding control region of ~1.1 kb length, called as D–loop. This region has important element to regulate the replication as well as two major transcription initiation sites, important for generating polycistronic transcripts from mtDNA (Shutt, 2006; Asin- Cayuela, 2007). The genomic organization of mtDNA with the details of coding and regulatory regions is described in Figure 1.

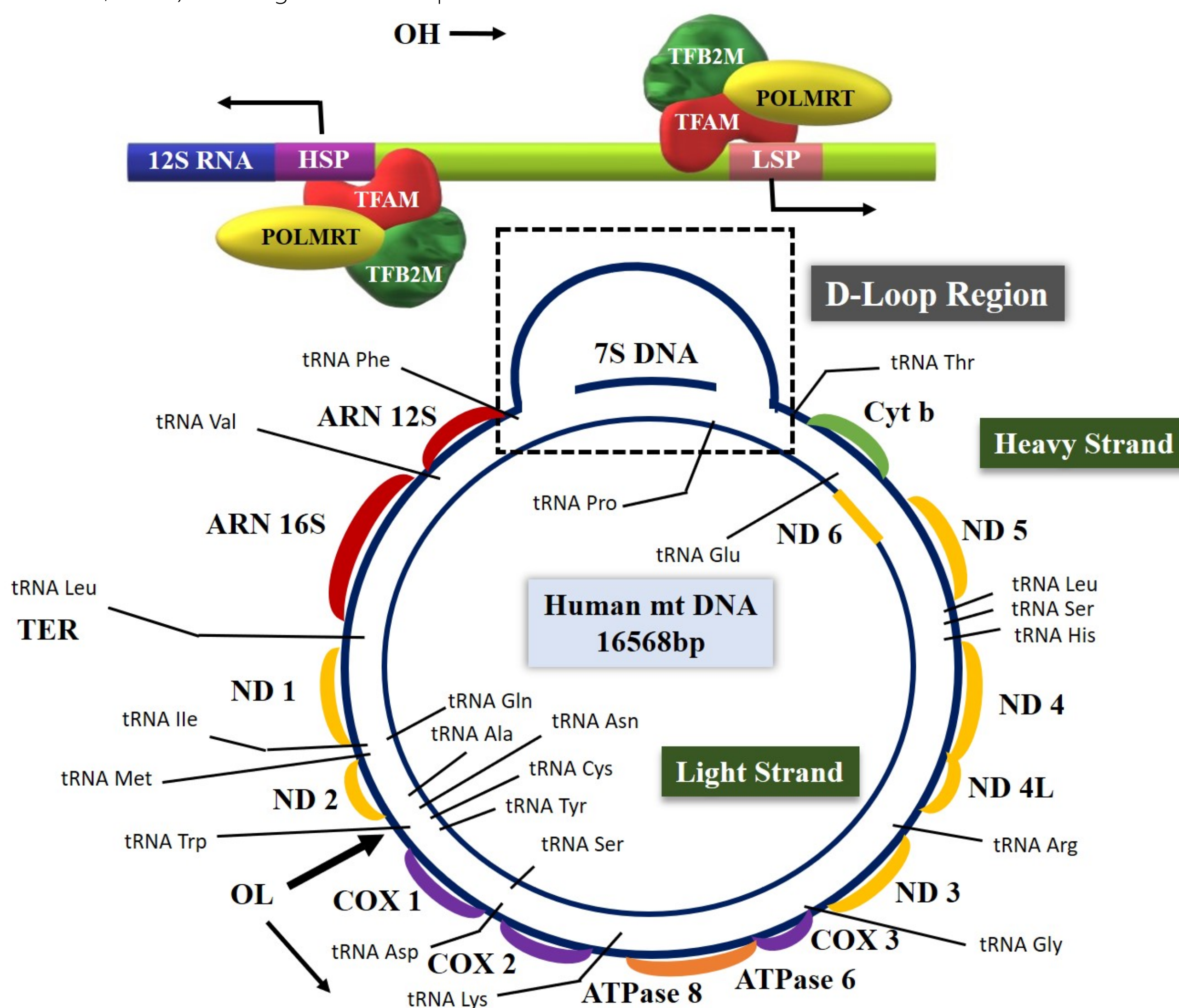

Figure 1: Schematic representation of different coding and regulatory regions in Human mtDNA

Unlike, nDNA, mtDNA does not contain any intron and is not covered by the histone proteins or protected by specific arrangement of nucleosome structures. Rather, it is organized into nucleoprotein complexes known as nucleoids. The major protein constituent of the mammalian nucleoid is the

mitochondrial transcription factor A (TFAM), although other proteins, such as transcription and replication factors, can associate with it temporarily. TFAM belongs to the high-mobility group domain proteins and induces a dramatic U-turn with an overall bend of 180° when bound to promoters or non-specific DNA. TFAM is a very abundant protein, present in about 1,000 molecules per mtDNA molecule or one TFAM protein molecule per 16 bp of mtDNA in the mammalian cells. TFAM binds randomly at single DNA duplex structures, occasionally inducing bending. Single TFAM molecule may also bridge neighboring DNA duplexes, resulting in cross-strand binding and looping out of mtDNA. TFAM is also involved in mtDNA transcription in addition to packaging mtDNA into nucleoids (Wang, 2006; Nicholls, 2018). Besides TFAM, a large number of mitochondrial proteins are enriched in nucleoid preparations as determined by proteomic analyses of native and cross-linked nucleoids. The nucleoid-associated proteins can be grouped into different functional classes. Different studies suggest that the key components of the mitochondrial transcription machinery include TFAM, mitochondrial RNA polymerase (POLRMT) and dimethyl adenosine transferase 2 mitochondrial protein (TFB2M). Similarly, the replication machinery is also suggested to be comprised of polymerase gamma (POLG), Twinkle, and mitochondrial single strand binding (mtSSB) proteins are prominently observed in nucleoids. Furthermore, RNA helicases, RNA-binding proteins, quality-control proteases, and mitochondrial RNA processing proteins as well as a subset of mitochondrial ribosomal proteins co-purify with the nucleoid.

Replication and transcription of mtDNA

Before discussing the possible mechanism of epigenetic changes in mtDNA and their influence on transcription and replication, it is important to get an overview about transcription and replication of mtDNA. The mitochondrial genome is highly efficient and has polycistronic mode of organization. mt DNA contains a transcription promoter and a replication origin as well. The nDNA, are known to have one individual promoter region per gene; however, being smaller in size the mtDNA contains only three promoter regions which are responsible for transcription of multiple genes to produce polycistronic transcripts. Out of these three promoter regions, two are located on the heavy strand and are named as HSP1 and HSP2. However, the promoter located on the light strand is named as LSP. The HSP1 is responsible for the transcription of 12S and 16S ribosomal RNAs while the HSP2 helps in transcription of the entire H-strand as a polycistronic transcript (Stewart, 2015). For transcription of the coding region of mtDNA, POLRMT, TFAM and TFB2M assemble at the promoters and thus initiate the synthesis of polycistronic RNAs. These RNAs are further processed into single mRNAs (Litonin, 2010; Holt, 2012). These promoters are located within or in the vicinity of the D-loop. In addition to the promoters, origin of replication at the H-strand is also present in the D loop only. However, the L strand origin of replication is known to lie outside the D-loop (Figure 1). The D-loop is known to exhibit a peculiar triple helix structure comprising of the light and heavy strand plus an additional 7S DNA primer, which forms the third nascent DNA strand (Nicholls, 2014). The 7S DNA is approximately 650 nucleotide strand and is located between the light and heavy strands. Though the detailed function of 7S DNA is not yet defined, it is hypothesized to participate in transcription and replication of mtDNA.

The replication of mtDNA is a complex phenomenon and till date no detailed mechanism has been deciphered. Based on the available evidence, three different models have been explained to propose the key steps involved in the replication of mtDNA (Holt, 2012; Nicholls, 2014). As per one model, 7S DNA is hypothesized to play a crucial role in mtDNA replication. As per this model, replication of H-strand initiates at the LSP and leads to the synthesis of 7S DNA (Nicholls, 2014, Bianchessi, 2016). Following it, with the help of mtDNA POLG enzyme, this newly-formed 7S DNA primes the synthesis of the H-strand (Nicholls, 2014,

Bianchessi, 2016). Although the functions of the D-loop are still doubtful, it is commonly accepted that this triple helical structure facilitates mtDNA replication by maintaining an open structure (Bianchessi, 2016). As D loop is predicted to carry different promoters and origin of replication, it can be considered as a likely candidate for epigenetic modifications, and thus can also have a major impact on the expression of mitochondrial genes. mtDNA transcription is still an active area of research as several unknown variables related to initiation, elongation, and termination remain to be elucidated.

Epigenetics and DNA methylation

Epigenetic changes are heritable in nature and result in altered gene expression without any modification to the original DNA sequence. The most common epigenetic modifications include covalent modifications on either DNA or proteins and the action of noncoding RNAs. Though, the DNA methylation always leads to gene silencing, histone modifications can either enhance or suppress gene transcription, depending on the nature of modifications. Histones and other proteins can be post-translationally modified via acetylation, phosphorylation, methylation, sumoylation, ubiquitination, and PARylation. All these modifications in histone proteins may influence the chromatin remodeling. This change in chromatin makes it more or less accessible for transcription factors and hence all these epigenetic mechanisms have significant roles in determining gene transcription. The epigenetic modification of DNA primarily includes its methylation. During DNA methylation, a methyl group is added on the cytosine base giving rise to 5-methylcytosine (5mC). In mammalian nDNA, CpG sites where cytosine base precedes a guanine base are considered as hot spot for methylation and 5mC frequently occurs on such di-nucleotides only. Hence these CpG clusters are of about 1 kb and scattered throughout the genome. These CpG rich regions are also called as CpG island (Wani, 2016), which are predominantly present within promoter regions. At the time of early embryonic development, CpG methylation patterns are formed by the de novo methyltransferases, such as DNMT3A and DNMT3B. The pattern of such de novo methylations are maintained with the help of maintenance methyltransferase, DNMT1. Apart from 5mC, the hydroxymethylated form of cytosine (hmC) was also identified as an epigenetic modification of DNA. Hydroxymethylation is thought to be an intermediate step in the demethylation of methylated base present in the nuclear genome. Methylation of DNA and CpG makes it less accessible by different transcription as well as regulatory protein factors and hence less expression of related gene. Hence, demethylation of DNA is also important to make its active for gene expression. Demethylation of DNA can occur by two different ways. First is passive demethylation which occurs during DNA replication when DNMT1 fails to maintain the existing methylation. Second method of demethylation involves the active participation and action of Ten-eleven translocation (TET) enzymes (Z.X Chen, 2011). Though TET-induced DNA demethylation process is not yet studied in great detail, however TET activity during demethylation is also reported for formation of 5hmC, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) in the nuclear genome (Z.X Chen, 2011). Briefly, different types of epigenetic modifications in nDNA and histone proteins are well known and these epigenetic modifications also help in explaining the differential gene expression in various cell types and tissues in an organism. Studies also suggest that these modifications can be affected by a number of factors such as environmental exposures, which result in alterations to various cellular processes that could eventually lead to a large number of abnormalities and health issues.

Regulation of mitochondrial gene expression by epigenetic modifications

A growing body of evidence also suggests that various environmental factors such as drugs, food additives and stress may contribute to

mitochondrial disease by causing epigenetic modifications to mtDNA.

One of the leading questions with respect to the regulation of mitochondrial gene expression is whether epigenetic-like modifications are present on the mtDNA and if such mechanisms certainly affect mitochondrial gene expression. Over the last decade, methylation of mtDNA has been extensively scrutinized and a large number of studies have identified this change in methylation pattern in both normal as well as disease conditions, in cell lines and tissue samples of mouse and human origin. This change in status of methylated DNA was also correlated with young versus aged mice. The different conditions like oxidative stress, nutrition, and environmental exposure were also found to affect the methylation status of DNA (Chestnut et al., 2011; Shock et al., 2011; Dzitoyeva et al., 2012; Bellizzi et al., 2013; Pirola et al., 2013; Wong et al., 2013; Ghosh et al., 2014; Baccarelli, 2015; Saini et al., 2017). As the DNA methylation and chemical modifications of proteins surrounding DNA, is the primary epigenetic modifications, hence the most important question is that if such modifications do exist in mtDNA or not? And the detailed mechanism for the epigenetic modifications of mtDNA is still not well studied, however based upon the predictions of various individual studies; the existence of epigenetic modification in mtDNA cannot be completely ignored.

Evidences for mtDNA methylation

mtDNA is naked DNA and different environmental factors may easily affect the function of this genome. However, the methodological limitations and the lack of in-depth knowledge on mitochondrial gene expression have prohibited the knowledge from reaching a clear level with respect to the epigenetic changes in mtDNA. A large number of recent studies on mtDNA methylation (Infantino et al., 2011; Shock et al., 2011; Bellizzi et al., 2013; Ghosh et al., 2014) and activation of mitochondrial methyltransferase (Chestnut et al., 2011; Shock et al., 2011) indicate towards the presence of mitochondrial epigenetic factors that have been possibly neglected till the recent past (Hong et al., 2013). However, emerging studies on changes in mtDNA methylation and localization of different methyltransferase, provide some interesting clues in this respect.

The methylation in the mtDNA was first reported almost five decades ago but since then it has always been a topic of controversy. During early years of 1970, Nass reported the presence of 5mC in mouse and hamster cell lines (Nass, 1973), which was later supported by studies conducted in bovine and rat liver mtDNA (Kirnos, 1976). Other older studies also reported that 2–5% of all mtDNA molecules fully methylated at specific CpG regions human fibroblast cultures and cell lines. These methylated statuses were analyzed using restriction digestion-based methods (Shmookler,1983). With the advancement of techniques such as pyrosequencing and bisulfite sequencing, the site-specific mtDNA methylation in human blood cells were also detected in the due course of time (Bellizzi et al., 2013; Baccarelli et al., 2015; Byun et al., 2016). In addition, methylation specific PCR techniques have detected substantial mtDNA methylation in liver tumor samples (Pirola et al., 2013). Overexpression of dnmt1 has been shown to translocate into mitochondria. Apart from methylated CpG, the overexpression of DNMT1 has been shown to translocate to mitochondria. These translocations were also found to be correlated with increased expression of some mitochondrial genes. This adds weight to the notion that the activity of DNMT1 may affect the functional capacity of mitochondria (Shock et al., 2011). Interestingly, apart from CpG methyltransferases, a more recent study further highlights the existence and significance of GpC methyltransferases and utilized it to target mtDNA. As a result, it was concluded that increased GpC methylation decreased the abundance of mitochondrial encoded transcripts (Van der Wijst et al., 2017). Though the mtDNA does not carry CpG island, but the extent of methylation within the D-loop and other parts of the genome is found to correlate with the expression of different mitochondrial genes (Pirola et al., 2013; Mishra,

2015). In addition to CpG methylation, mtDNA is also shown to carry a typical non-CpG methylation pattern (CpC, CpA and CpT) as well (Campbell et al., 1999; Bellizzi et al., 2013; Bianchessi et al., 2016; Blanch et al., 2016; Koh et al., 2018). This non-CpG methylation pattern is characteristic of prokaryotic genomes (and because of being prokaryotic in nature, the presence of such methylation patterns can be easily justified in mitochondria). Thus, based on the reports of these selected studies, methylation in mtDNA can be suggested to exist in different forms such as 5mC and 5hmC, CpG, CpC, CpA and CpT as well.

Till date, most of the studies have aimed at identifying methylation at CpG sites of mtDNA; however, non-CpG methylation and adenine methylation within mtDNA have also been observed (Bellizzi et al., 2013). In the year 2018, the presence of 6mA in the human mitochondrial genome was reported with the help of 6mA-cross-linking-exonucleases equencing. After detailed comparative analysis, it was observed that the mtDNA carry >8,000 times higher 6mA than nDNA. In case of nDNA, the 6mA nucleotides were usually present within a "6mAT" dinucleotide motif; however, the distribution of 6mA was relatively uniform throughout mtDNA and no clustering was observed. Thus, endosymbiotic theory of mitochondrial origin together with the presence of approximately 8,000-fold higher 6mA than nDNA further suggests that mtDNA methylation may predominantly occur on adenine. Studies have also observed that the presence of 6mA was well correlated with meting of mtDNA followed by recruitment of mtSSB to mtDNA (Koh et al., 2018).

Though the preliminary evidence of DNMT activity in mitochondria was observed in vertebrates, the expression of various DNMTs seems to be controversial in different cell types. Rather, the expression of these DNMTs appears to be preferential depending upon the cell and tissue type being studied (Saini et al., 2017). Additional experiments with downregulation of DNMT1, DNMT3a and 3b in mice suggest that the knock-outs of these enzymes completely abolished the CpG methylation from mtDNA, but the non CpG methylation remains unaffected. Apart from methylation, the demethylation also seems to be an important step in mtDNA. As Bellizi et al and Shock et al have identified characterized and predicted its localization in mitochondria (Bellizzi et al., 2013; Shock et al., 2011). In their studies, they identified TET1 and TET2 proteins in the mitochondrial function. Another support to this observation came from the study of Manev et al., 2012 who reported the localization of the TET enzymes in the mitochondria of mouse neuronal tissues. These results further suggest the role of TET enzyme in demethylation of the mtDNA.

Thus, the methylation pattern and its regulation in mtDNA seem to be significantly different from nDNA. Unlike methylation at CpG island in nDNA, the mt DNA is methylated at many non CpG sites such as CpC, CpA and CpT. Various studies also confirmed the presence of important enzymes involved in methylation and demethylation of mtDNA, such as DNMT1, DNMT3a, DNMT3b, and two TET enzymes (TET1 and TET2) within mitochondria (Chestnut et al., 2011 Shock et al., 2011; Chen et al., 2012; Dzitoyeva et al., 2012; Bellizzi et al., 2013; Wong et al., 2013; Maresca et al., 2015). But the methyl transferase enzyme which is responsible for methylation of mtDNA at adenine has not yet been identified. As the D loop is an important region which harbors HSP and LSP elements and is easily accessible to proteins during mtDNA replication and transcription, it will be of great advantage if the methylation within the mitochondrial D-loop can be measured (van der Wijst et al., 2017).

Modifications of TFAM and the modulation of mitochondrial gene expression

For nDNA, histone modifications are important epigenetic modifications that affect the gene expression. However, in case of mitochondria, mtDNA is not associated with histones and hence mtDNA is unable to undergo histone-mediated gene expression regulation (Choi et al., 2011). But it

is important to point out here that despite lacking histone proteins, the mtDNA is not naked. Rather the mtDNA is clustered in protein-DNA complexed structure called nucleoids. The nucleoid is the main component which completely covers the mtDNA (Bogenhagen, 2012; Ngo, 2014). TFAM is an important nuclear encoded mitochondrial localized protein. It is primarily responsible for the packaging and organization of the protein-mtDNA complex (Fisher et al., 1992; Bogenhagen, 2012; Ngo, 2014) and thus have comparable functional significance like histone proteins. In addition to the packaging of the mtDNA as nucleoid, TFAM is also found to promote replication, transcription and general maintenance of mtDNA (Marina, 2015). It binds to the mtDNA and initiates the transcription at the LSP and HSP1 promoters. On the other hand, HSP2 transcription seems to be independent of TFAM, but depends on POLRMT and TFB2M (Zollo et al., 2012). However, TFAM can perform a dual function on HSP2 whereby it can activate or repress the transcription at HSP2, depending on the TFAM: TFB2M/POLRMT ratio (Zollo et al., 2012; Lodeiro et al., 2012). TFAM appears to competitively repress HSP2, but its activity is diminished when the concentrations of TFB2M and POLRMT are high (Lodeiro et al., 2012). Thus, it has been observed that accessibility of different proteins and regulatory factors to mtDNA depends upon the occupancy level of TFAM. Thus, the mtDNA regions, which are highly occupied with TFAM, are comparatively less accessible by DNMTs and difficult to methylate (Rebelo et al., 2009). It indicates towards the fact that TFAM activity plays a crucial role in determining the methylation pattern of mtDNA. However, it is possible that the PTMs of TFAM may alter its biding ability to mtDNA and thus, indirectly influence the methylation of mtDNA. The most common PTMs of histone proteins which change the chromatin state and gene expression in the respective regions are acetylation and methylation. Interestingly, TFAM has also been reported to undergo PTMs like acetylation, phosphorylation and ubiquitination (Dinardo et al., 2003; Lu et al., 2013; Santos et al., 2014). Out of these three different PTMs, the phosphorylation is found to impair the binding affinity of TFAM to mtDNA. However, the detailed mechanism of PTMs in TFAM and their effect on TFAM activity and translation to gene expression modulation remains inconclusive. Apart from TFAM, other important proteins which are the components of nucleoid and associated with mtDNA include mtDNA helicase, mtSSB, Twinkle and POLG. As part of the nucleoid, POLG, Twinkle and mtSSB are thought to be only involved in mtDNA replication, but TFAM is known to be involved in both transcription and replication of mtDNA (Spelbrink, 2010; Garrido et al., 2003; Clayton, 2003). Thus due to its versatile functions and tight regulation over the expression of mtDNA, TFAM appears to be the most important protein whose activity may greatly influence epigenetic regulation of mitochondrial gene expression.

Mitochondrial epigenetics and spectrum of diseases

As per studies conducted in the past few decades, a rapidly increasing number of disease conditions are found to be associated with mitochondrial defects. Recent studies report an increasing number of disease conditions to be associated with changes in mtDNA methylation at various loci. This brings about an important question whether the changes in mtDNA methylation are the primary cause of those diseases or just a consequence of the disease phenotype. Due to a strong nuclear mitochondrial cross-talk, the change in mitochondrial epigenetics can modulate nDNA and nDNA epigenetic may affect mtDNA too. Thus, a number of diseases may possibly be associated with changes in the mitochondrial epigenetics. Interestingly, the epigenetic regulation of mtDNA has received increasing attention in the last few years due to its implication in clinically relevant diseases and based upon some preliminary studies, the significance of mtDNA methylation has been proposed to be responsible for pathogenesis of different diseases.

Neurological disorders

Mitochondrial dysfunction in neuronal tissue is

associated with neurodegenerative diseases (NDDs); hence, factors affecting the mitochondrial metabolism and its activity may play a pivotal role in the initiation and progression of such diseases. As per various recent reports, the changes in mtDNA methylation are predicted to affect the mitochondrial function and hence the change in methylation of mtDNA may further act as a potential candidate in the etiology and therapy of NDDs. Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease and it is caused due to the death of motor neurons (brain, brainstem, spinal cord), eventually leading to paralysis and death. In year 2011, Chestnut and coworkers determined the global 5mC content and DNMT protein levels in mitochondria and nucleus isolated from both brain and spinal cord motor neurons of mice, as well as in cortical motor neurons from 12 ALS patients. As per the outcome of their study, it was revealed that the high level of DNA methylation in both nuclei and mitochondria of motor neurons may be responsible for driving apoptosis in these cells (Chestnut et al, 2011). Following this study, they further revealed the abnormality in methylation patterns of mtDNA as well as DNMT3A levels in mitochondria of skeletal muscles and spinal cord of pre-symptomatic ALS mice carrying mutations in the SOD1 gene (Wong et al., 2013). In a recent study, blood DNA samples from 114 individuals, including ALS patients, pre-symptomatic carriers of ALS-causative mutations, and non-carrier family members were collected and a comprehensive study was performed. As a result, it was observed that there was a significant decrease in the methylation of the D-loop region of mtDNA in carriers of ALS-linked SOD1 mutations. In addition, a significant negative correlation was also observed between the D-loop methylation levels and the mtDNA copy number in these patients (Stoccoro et al., 2018). As SOD1 is one of the major antioxidant enzymes, the observed D-loop hypomethylation in SOD1 carriers could represent a defensive modification to counteract the increased burden of oxidative damage by increasing the mtDNA replication (Stoccoro et al., 2018). These observations further indicate towards the impairment of mtDNA methylation in ALS tissues. This defect in mtDNA methylation may be exhibited as early event before the onset of ALS symptoms in carriers of SOD1 mutations.

The increase in the methylation of mtDNA D loop in entorhinal cortex of patients with Alzheimer disease (AD)-related pathology was also observed. It was also noticed that the extent of D loop methylation was different as per the stage of the disease (Jang et al., 2017). Interestingly, the methylation level in the D loop was higher in early disease stages than in late stages, and this dynamic pattern of methylation of this region was also observed in transgenic AD mice (APP/PS1 mice) along with disease progression (Blanch et al., 2016). In the same study, D-loop methylation levels were also found to be decreased in post-mortem substantia nigra sample of ten patients with Parkinson's disease (PD) (Blanch, 2016). From these two contrasting observations, it appears that mtDNA is differentially methylated depending upon the nature of the disease. Methylation levels of the mtDNA D-loop region were further determined in blood DNA samples collected from a total 133 late-onset AD patients and 130 matched controls. As a result, a significant reduction in the methylation level was observed in AD patients (Stoccoro et al., 2017).

The pattern of DNA methylation is also disturbed in Down syndrome (DS) patients and global hypermethylation is observed in the nDNA, whereas the mtDNA is reported to be hypomethylated in these patients (Pogribna et al., 2001; Chango et al., 2006; Infantino et al., 2011). It is important to note that most of the DS patients have also been shown to harbor a higher risk of developing early onset AD. These data collectively suggest that the degree of methylation of this region might help in characterizing the different stages of the neurodegenerative process.

Cardiac disease

Though not many studies are available indicating the association between mtDNA methylation and

cardiovascular disease (CVD), a study conducted by Baccarelli et al showed that platelet-derived mtDNA is hypermethylated in CVD patients. This methylation status was compared with healthy controls and it was found to be high, regardless of their age, race or BMI (Baccarelli et al., 2015). Unlike other studies where researchers mostly focus on the state of the D-loop methylation, this study looked at the methylation patterns within mtDNA genes. As a result, it was observed that MT-COI, MT-COII, MT-COIII, and MT-TL1 genes were hypermethylated in the CVD patients. This change in methylation level in different genes of mtDNA suggests the potential mechanism for the regulation of gene expression in mtDNA of these patients.

As per literature, a strong association between obesity and CVD is proven, but the reason behind all the adults with obesity not developing CVD is poorly understood. Hence, in an attempt to explore the reason for such an observation, the platelet mtDNA methylation was studied using a nested case-control study of 200 adults with overweight or obesity who were CVD-free at the baseline. 84 out of these 200 patients developed CVD within 5 years, while 116 remained CVD-free. As a result, MT-COI, MT-COIII, and MT-TL1 were found to be hypermethylated in those patients who developed CVD. Since methylation of these mtDNA genes was independent of conventional CVD risk factors, this may represent a novel intrinsic predictor of CVD risk in adults with overweight and obesity (Corsi et al., 2020).

Cancer

The cancer cells are highly proliferating in nature and require a lot of energy for their high metabolic activities. Mitochondria being the power house of the cell, the copy number of mtDNA are strictly regulated during cellular differentiation in cancer cells. In most of the cancer-based studies, this change in copy number of mtDNA has been found to be associated with their methylation status. For instance, in breast cancer, 8 aberrant mtDNA methylation sites were found to be tightly dysregulated in the D loop (Han et al., 2017). In case of colorectal cancer also, the expression of ND2 gene and mtDNA copy number was higher than that of the corresponding non-cancerous tissue (Gao et al., 2015). Methylation level was also found to be variable as per the different stages of cancer. Interestingly, the methylation on the D-loop region in stage III/IV colorectal cancer tissues was also significantly decreased, compared with that in stages I/II colorectal cancer tissues. As the treatment of Caco-2 cells with DNA methylation inhibitor 5-aza-deoxycytidine results in an increase of the mtDNA copy number and ND-2 expression, this de-methylated status of D loop may be strongly correlated with a high mtDNA copy number and MT-ND2 gene expression in these patients. Similar study by Feng et al., 2012 also reported the differential mtDNA methylation in colorectal cancer and this change in methylation was also in correlation with an increase in the mitochondrial MT-ND2 expression. Another study of Tong et al., 2017 also supported the predictions and revealed that demethylation of the 4th and the 6th/7th CpG islands of D-loop was directly correlated with an increase in the mtDNA copy number in colorectal cancer, thereby triggering cell proliferation, cell cycle progression and reducing apoptosis. These results can further be extrapolated to confirm that hypermethylation of mtDNA may be negatively correlated with copy number of mtDNA number as well as tumor progression.

Apart from D loop, the role of differential methylation in other regions of mtDNA has also been studied in different cancer types. For instance, Maekawa et al., 2004 observed the absence of mtDNA methylation in the MT-RNR2, MT-COI and MT-COII loci in 15 cancer cell lines as well as malignant and healthy tissues collected from 32 patients with gastric cancer and 25 patients with colorectal cancer. It is important to note that during this study, the methylation within the D-loop was not assessed nor the mitochondrial gene expression levels were measured. Their study also revealed that the CpG sites in mtDNA of different cancer cell lines

and tissues of gastric and colorectal cancer remain unmethylated and methylation of mtDNA seems to be a rare phenomenon in them. Recently, Morris et al., 2018 also observed that hypomethylation of mtDNA at certain sites were not associated with the expression of different mitochondrial genes. These observations leave important unanswered questions with regard to whether the changes in gene expression are a direct or indirect effect of D-loop methylation. Thus, the relationship between mtDNA methylation and cancer should be studied further.

Metabolic diseases

The literature supports the association of diabetes with qualitative and quantitative (including mtDNA methylation) variations in the mitochondrial genome (Zheng et al., 2016). The mtDNA methylation is reported to be associated with early insulin sensitivity as well as BMI. A study showed that insulin signaling influences mtDNA methylation in obese human subjects (Zheng et al., 2015). Further, the hypermethylated status of mtDNA D loop were also predicted to be strongly associated with obesity and insulin resistance, this increased level of methylation at the D-loop region was correlated with a decrease in the mtDNA copy number. The decrease in copy number of mtDNA may affect the overall gene expression of mtDNA and hence cause mitochondrial dysfunction. As insulin secretion, glucose uptake and insulin sensitivity are energy driven process, mitochondrial dysfunction and its relationship with diabetes mellitus is much appreciated. Altered S-adenosylmethionine (SAM) is also known to be associated with mitochondrial dysfunction and insulin resistance. This is also demonstrated that SAM is associated with mitochondrial density and insulin sensitivity in skeletal muscle cells (Engin, 2017). The level of SAM is also reported to influence the density of mtDNA in the skeletal muscle, following improvement in the insulin sensitivity as well. The function of mitochondria may further be correlated with age, which easily develops insulin resistance to reduce the effect of insulin on glucose (Petersen et al., 2003).

In addition to the insulin resistance, obese individuals are also prone to liver diseases, such as NASH (Yu et al., 2013; Ande et al., 2016). NASH is characterized by triglyceride accumulation, hepatocellular damage and inflammation. Mitochondrial dysfunction may behave as the major culprit in the development of NASH. Defective mitochondrial functions may lead to disruption of lipid metabolism in mitochondria and hence favor the development of NASH. In case of NASH, apart from D loop, the other regions of mtDNA are also known to be hypermethylated. For instance, a study conducted by Pirola and colleagues showed that the MT-ND6 region was around 20% more methylated in these patients and evidently this upsurge in methylation was well associated with a decrease (>50%) in mRNA and protein expression of MT-ND6 gene (Pirola et al., 2013). The ND6 protein is a subunit of the mitochondrial complex I, which is a vital component of the electron OXPHOS for ATP generation. Thus, any change in the MT-ND6 expression may negatively impact the function of mitochondrial function, which includes lipid metabolism, insulin secretion and insulin signaling, thus contributing to the pathogenesis of this disease. Interestingly, this increase in methylation of MT-ND6 gene was also found to be associated with the progression of the disease condition. Thus, this study presents an interesting observation with respect to methylation of mtDNA regions other than D loop and its relation with NASH pathogenesis.

CONCLUSION

In conclusion, though there are different independent studies which support the existence of epigenetic modifications in mtDNA, the detailed mechanism for such modifications as well as their regulation is not yet completely understood. Similarly, the role of mtDNA methylation in pathogenesis of different diseases also remains an interesting biological paradox. The published data are just enough to justify the association between mtDNA methylation and gene expression but are

unable to explain the detailed mechanism through which methylated mtDNA help in the regulation of mitochondrial gene expression.

Based upon all the studies related to epigenetic modifications of mtDNA, it may be hypothesized that modifications in the mtDNA D loop and consequently compromised ability of TFAM and POLG to bind to mtDNA may play significant roles in determining the expression of mtDNA. As mitochondria are very important organelles for all the cellular activities, any change in the expression of mtDNA may affect the cellular function and cause different diseases in case of unfavorable conditions. Thus, it can be further emphasized that studies aiming to explore the detailed mechanism and effect of mtDNA methylation and TFAM phosphorylation and acetylation may provide new insights in the field of mitochondrial epigenetics, which could further help in targeted drug designing for treating the wide variety of diseases, known to be associated with dysfunctional mitochondria. Thus, the future research must address some key aspects in the mitochondrial epigenetics. Primarily, it is important to explore the role of different enzymatic activities such as DNMT1 and TETs in the regulation of mitochondrial epigenetics. The next important aspect to be explored should understand the mitochondrial expression and regulation of these enzymes as well as their distribution in different tissues in normal and diseased conditions. Lastly, the research is also required to establish specific mtDNA methylation profiling in case of different diseases such as cancer, metabolic disease, cardiovascular diseases and neurodegenerative disorders. Such studies may lay down a foundation for the discovery of new biomarkers based upon the methylation pattern of mtDNA. Thus, altogether there is an urgent need to study the detailed mechanism of mitochondrial epigenetics and its association with different diseases. The outcome of such studies will fill the gap of our knowledge which has been created due to overlooking of this important process of our cellular system.

Acknowledgements

The author acknowledges Ms Aarushi Singh for preparing the figure included in the manuscript. The author also acknowledges Ms Sakshi Tyagi for helping in preparing the reference section of the manuscript.

Conflict of interest statement

The author declares to have no conflict of interest.

Authors’ contributions

Shalini Mani is the sole author, who conceived the idea and prepared the manuscript.

Source of Funding

No specific funding was received for this work.

Declaration of originality

The author declares that the work/review presented in this manuscript is original and has not been copied from elsewhere without appropriate citations.

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