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Minireview

Mitochondrial DNA methylation as a next-generation biomarker and diagnostic tool

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ABSTRACT

Recent expansion of our knowledge on epigenetic changes strongly suggests that not only nuclear DNA (nDNA), but also mitochondrial DNA (mtDNA) may be subjected to epigenetic modifications related to disease development, environmental exposure, drug treatment and aging. Thus, mtDNA methylation is attracting increasing attention as a potential biomarker for the detection and diagnosis of diseases and the understanding of cellular behavior in particular conditions.

In this paper we review the current advances in mtDNA methylation studies with particular attention to the evidences of mtDNA methylation changes in diseases and physiological conditions so far investigated. Technological advances for the analysis of epigenetic variations are promising tools to provide insights into methylation of mtDNA with similar resolution levels as those reached for nDNA. However, many aspects related to mtDNA methylation are still unclear. More studies are needed to understand whether and how changes in mtDNA methylation patterns, global and gene specific, are associated to diseases or risk factors.

Contents

Abbreviations: AD, Alzheimer disease; AIMs, amplification of inter-methylated sites; ALS, amyotrophic lateral sclerosis; ChIP, chromatin immunoprecipitation; CTH, cystathionine γ-lyase; CHARM, comprehensive high-throughput arrays for relative methylation; CBS, cystathionine β-synthase; DNMT, DNA methyltransferase; DS, Down's syndrome; Hcy, homocysteine; 5hmC, 5-hydroxymethylcytosine; MAT, methionine adenosyltransferase; MS, methionine synthase; MCA, methylated CpG island amplification; Ms-AP-PCR, methylation-sensitive arbitrarily primed PCR; MsCC, methylation-sensitive cut counting; 5mC, 5-methylcytosine; MTHFR, methylenetetrahydrofolate reductase; 5-MTHF, 5-methyl-tetrahydrofolate; MT, methyltransferase; MMAss, microarray-based methylation assessment of single samples; MBE, mitochondrial bifunctional enzyme; mtDNA, mitochondrial DNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; nDNA, nuclear DNA; PD, Parkinson disease; ROS, reactive oxygen species; SAH, S-adenosylhomocysteine; SAHH, SAH hydrolase; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; TET, ten–eleven translocation; THF, tetrahydrofolate; TFA, transcription factor A; TFB, transcription factor B; MeDIP and mDIP, methylated DNA immunoprecipitation; WGsBs, whole-genome shotgun bisulfite sequencing.

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1. Introduction

Among the epigenetic processes, DNA methylation is perhaps the best understood epigenetic adaption and the most common DNA modification [1]. Two methylated cytosine-derived bases, 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), have been detected in the DNA. 5mC is derived from incorporation of a methyl group at position 5 of cytosine (Fig. 1). 5hmC is produced from 5mC through a hydroxymethylation reaction catalyzed by TET (ten–eleven-translocation) (Fig. 1) [2]. While nDNA methylation is a well established feature, mtDNA methylation has been controversial and a matter of debate [3–9]. Therefore, very little attention has been devoted to mitochondrial epigenetics. The prevailing opinion was that mtDNA cannot be methylated for two main reasons: (i) methylase does not or cannot access mitochondria in vertebrates, and (ii) mtDNA is devoid of histones and is arranged in clusters, called nucleoids, which are bound to the mitochondrial membrane [10]. Only recently, methodological and functional approaches unequivocally have identified mtDNA methylation as part of mammalian mitochondria physiology. Using mass spectrometry, technological tool that overcomes the sensitivity problems of the bisulfite method, our laboratory demonstrated for the first time the presence of methylated bases (5-methyl-2′-deoxycytidine) in human mtDNA [11]. Then, Shock et al. [12] demonstrated the presence of a methyltransferase, DNMT1, inside mitochondria. Through a mitochondrial targeting pre-peptide DNMT1 translocates into mitochondria sequence, where it binds to mtDNA and modifies transcription of the mitochondrial genome. In addition to DNMT1 Chestnut et al. [13] found also the methyltransferase DNMT3a in mitochondrial fractions of mouse and human CNS.

Studies performed in the last two years indicate that epigenetic modification of cytosine in mtDNA is much more frequent than previously believed. Furthermore, mitochondrial epigenetic can modulate nDNA and nDNA epigenetic may affect mtDNA. Thus, epigenetic events regarding mitochondria are now frequently indicated as “mitoepigenetics” [14]. Similar to nDNA methylation [15–18], occurrence of the abnormal mtDNA methylation is often depending on different factors, such as diseases, environment, drugs, and food. Thus, abnormal mtDNA methylation is attracting increasing attention as potential biomarker.

In this paper we will address recent developments in mitoepigenetic studies, particularly those aimed to investigate mtDNA methylation changes associated with diseases and other conditions, such as environmental pollution exposure, aging, drug treatment, and oxidative stress. In addition, the most recent technologies used to detect DNA methylation are reviewed.

2. The one-carbon cycle and mitochondria

The methylation of cytosine in nDNA and mtDNA is required for the maintenance of the epigenetic code and regulation of gene expression, stabilizing chromatin structure in the nDNA [19]. The universal methyl donor is S-adenosylmethionine (SAM), which is produced in the metabolic cycle of methionine (Fig. 2). Methionine is activated to SAM by methionine adenosyltransferase (MAT) transferring adenosine from ATP. Methyl transfer from SAM is catalyzed by methyltransferases (MTs) producing S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to homocysteine (Hcy) by SAH hydrolase (SAHH). In the trans-sulfuration pathway, homocysteine is irreversibly degraded to cystathionine by the vitamin B6-dependent enzyme cystathionine β-synthase (CBS) and then further catalyzed to cysteine, a precursor of glutathione, by the vitamin B6-dependent enzyme cystathionine γ-lyase (CTH). In the remethylation pathway (folate cycle), the methyl group from 5-methyl-tetrahydrofolate (5-MTHF) is transferred to homocysteine by the vitamin B12-dependent enzyme methionine synthase (MS), producing methionine and tetrahydrofolate (THF) (Fig. 2).

Mitochondria metabolism regulates production of SAM through synthesis of ATP and folate. Folate cycle reactions are duplicated in cytosol and mitochondria [20]; they are linked through exchange of serine and glycine, which are interconverted by the mitochondrial and cytosolic serine hydroxymethyltransferase (SHMT) through methylene-tetrahydrofolate (Fig. 2). Mitochondria regulate the switch between SAM and nucleotide synthesis by the action of the mitochondrial bifunctional enzyme (MBE), which is active in embryonic and cancer cells (promoting purine and pyrimidine synthesis) and is turned off in adult cells (favoring SAM synthesis and DNA methylation) [21,22]. Cytosolically synthetized SAM is transported into mitochondria by means of the specific mitochondrial carrier SAMC, where it is used for all mitochondrial methylation processes [23] (Fig. 2).

Based on the central role of one-carbon pathway in methylation reactions and nucleotide synthesis, it is not surprising that disruption of these pathways, either due to nutritional deficiencies (folate and B-vitamins) or genetic factors, has been linked to different human diseases, such as

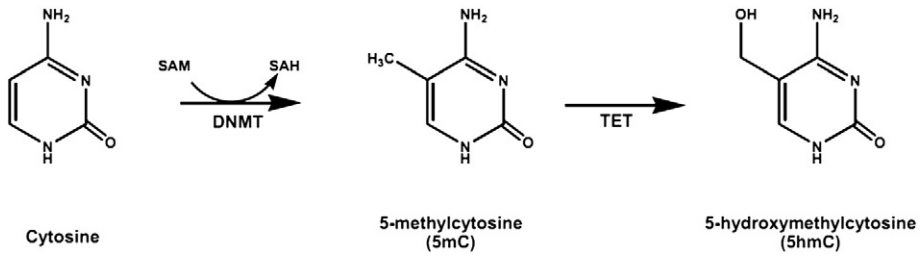

Fig. 1. Reaction of cytosine methylation and hydroxymethylation. Abbreviations: DNMT, DNA methyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TET, ten–eleven translocation.

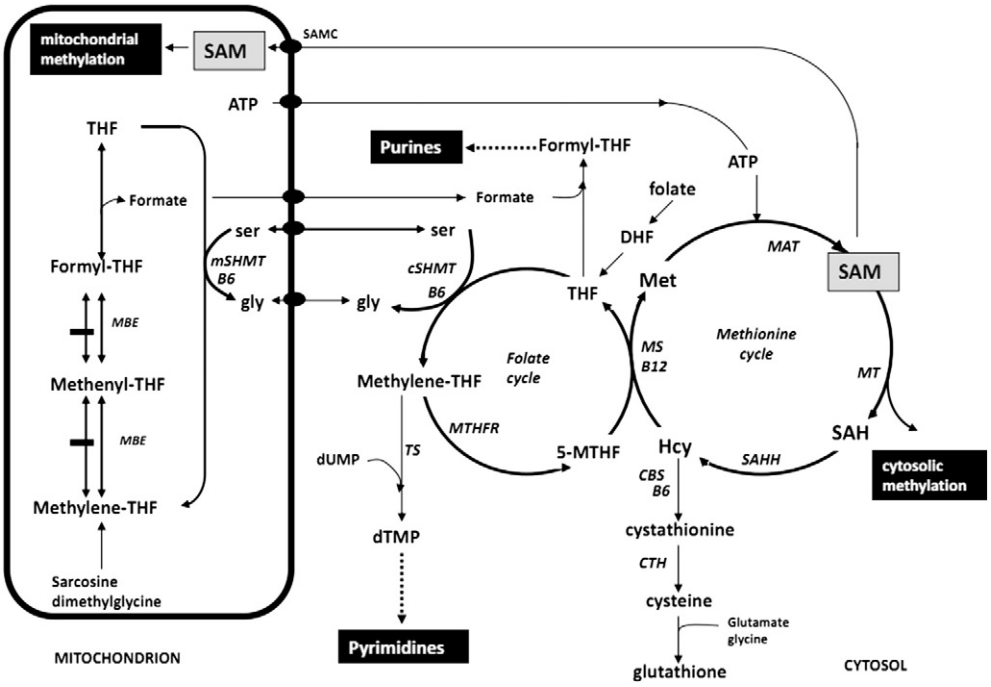

Fig. 2. Complete one-carbon cycle showing the connection between mitochondrial and cytosolic folate metabolism and methionine cycle. The switch between SAM synthesis and nucleotide synthesis depending on MBE activity is also shown. Abbreviations: CTH, cystathionine γ-lyase; CBS, cystathionine β-synthase; DHF, dihydrofolate; Hcy, homocysteine; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; 5-MTHF, 5-methyl-tetrahydrofolate; MT, methyltransferase; MBE, mitochondrial bifunctional enzyme; SAH, S-adenosylhomocysteine; SAHH, SAH hydrolase; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; B6, vitamin B6; B12, vitamin B12.

Alzheimer and Parkinson diseases, psychiatric disorders and dementia [24–33]. Thus, it is likely that impairment of global one-carbon pathways might affect both nDNA and mtDNA methylation. For example, we have demonstrated that impairment of cellular methyl cycle in DS subjects, probably due to the location of CBS and other methyl cycle genes on chromosome 21, limits cellular availability of SAM and its mitochondrial uptake resulting in reduced methylating power levels in DS mitochondria compared to controls [11].

3. Mitochondrial DNA

MtDNA represents less than 1% of total cellular DNA. However, its mitochondrial gene products are essential for normal cellular function. The human mtDNA, maternally inherited, is made up of 16.569 bp with 435 CpG sites and 4747 cytosine residues at non-CpG sites. Both cytosines at CpG sites and cytosines at non-CpG sites can be methylated although with different frequency. It encodes two rRNAs, 22 tRNAs and 13 ORFs (open reading frames), all of which translate into polypeptides that are essential components of the multi-subunit complexes driving oxidative phosphorylation (Fig. 3). The mtDNA also contains a short non-coding region with control elements, including an origin of replication and transcriptional promoters [34]. Transcription starts from three promoters, each of which generates a specific polycistronic RNA unit [35]. The heavy strand promoter and the light strand promoter generate proteins that essentially span the entire genome, except for the major non-coding region. mtDNA is packed into aggregates called nucleoids or mitochromosomes. The most abundant component of nucleoids is the transcription factor A (TFAM) which significantly contributes to compact and pack mtDNA, similar to the action of histones [36,37]. Alterations of TFAM content determine a change in mitochromosome and expose mtDNA to the action of DNMTs. It was found that DNMT has different accessibility to different sites on mtDNA depending on the level of protein occupancy [38]. Interestingly, TFAM is down-regulated in cultured amniocytes from Down's syndrome with respect to normal fetuses [39].

A large number and wide variety of mtDNA mutations have been identified, and 200 disorders have been associated with specific point mutations, single and multiple deletions, or depletion of mtDNA [40]. Pathogenic mutations can affect a varying proportion of mtDNA (heteroplasmy). The proportion of heteroplasmy can vary from person

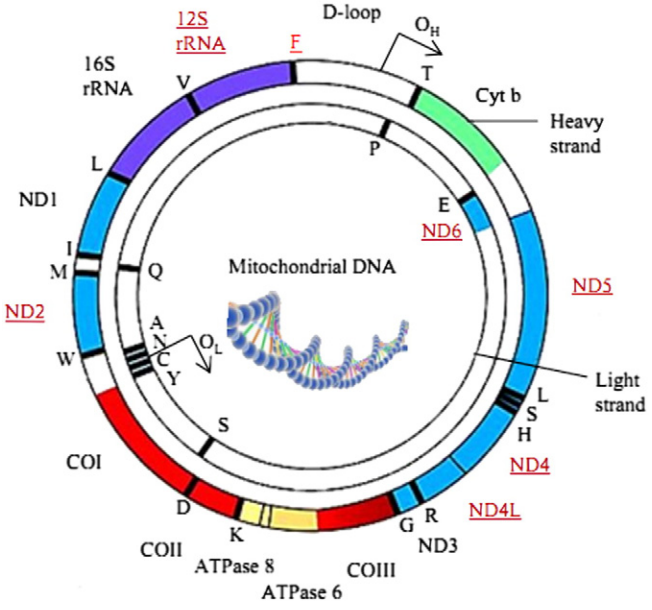

Fig. 3. Mitochondrial DNA diagram. Positions of each of the 13 mitochondrial genes encoding polypeptides, tRNAs, rRNAs, origins of heavy-strand and light-strand replication (O_H and O_L, respectively) are shown. Outer and inner rings correspond to heavy strand and light strand, respectively. Genes reported in this review that are differently methylated are in red and underlined.

to person and among adjacent cells within the same person [41]. Since over thousands of copies of mtDNA are present within the cell, usually a threshold of heteroplasmy should be reached before the onset of a mitochondrial pathology. This threshold is tissue-specific and depends on energetic and other mitochondrial functions.

4. Mitochondrial DNA methylation evaluation in biological specimens

The measurement of biomarkers in tissues and blood specimens has become an integral component of many epidemiologic studies. There is widespread interest in exploring the relationship between genomic 5mC and 5hmC content and clinical outcomes, as well as environmental, lifestyle and dietary exposures. Nuclear DNA methylation profile in tissues and biological fluids is already used for early diagnosis of cancer but also for staging cancers [42,43]. Cell-free circulating DNA in blood has also been successfully used for detection and diagnosis of cancer, and monitoring of treatment efficacy [44,45]. Most studies of DNA methylation in blood have been carried out using total white blood cells isolated from the simple centrifugation of blood and collection of the "buffy coat" [46]. Centrifugation of blood over a density gradient allows separation of the more abundant granulocytes (about 85% of total white blood cells) from mononuclear cells. Isolation of pure lymphocytes from specific lymphocytes subpopulation requires flow cytometric separation using cell surface receptor antibody binding. Similar routine methods have not yet been defined for the mtDNA for many reasons. First, only recently methylation of mtDNA has been demonstrated in man. Additionally, mtDNA analysis raises many challenges, among which extraction from blood circulating cells, procedure that needs to be accurately experimented. This problem has been circumvented in cultured cells and tissues, for which effective procedures for mitochondrial extraction are available, each leading acceptable yields with minimal damage to integrity. MtDNA isolation methods are also available tools for directly isolating mtDNA from a variety of cells and tissues in high yield and purity, without contaminations from genomic DNA. The purified mtDNA can be used for a variety of studies. Starting from 20×10^6 lymphoblastoid cells from DS subjects and control cells we have evaluated mtDNA methylation and some mitochondrial metabolites with LC–MS/MS [11]. Mitochondrial global and specific gene methylation levels have been successfully determined in different cellular conditions.

4.1. Global mtDNA methylation as biomarker

Emerging evidences highlight the existence of a different mtDNA epigenetic regulation in normal and pathological conditions, particularly in neurodegeneration and aging. Thanks to more direct and accurate methods now it is possible to determine the global 5mC and 5hmC content into mitochondria as indication of the mtDNA methylation status. In the next section we will review the pathological and physiological conditions in which global methylation levels, as 5mC and 5hmC, have been determined.

4.1.1. Global 5mC and 5hmC in neurodegenerative diseases

Specific methylation patterns have been identified in nDNA from different neurodegenerative diseases and chromosomal aneuploidies. On this basis specific non-invasive methods have been developed to identify chromosomal aneuploidy [47]. On the contrary, mtDNA methylation sites have not yet been analyzed in neurodegenerative diseases or chromosomal aneuploidy, with the exception of the finding [11] that mtDNA is hypomethylated in DS mitochondria despite the increase of mtDNA content in DS [48]. These results contribute to the understanding of the mechanism of instability of DS mtDNA by suggesting a relationship between the abnormal methyl metabolism and the mtDNA mutations found in DS subjects [49]. Since mtDNA damage has been indicated as a relevant event for DS etiology through oxidative stress and ATP depletion [50], it cannot be excluded that changes in mtDNA methylation could modify mtDNA sensitivity to damage, leading to adverse effects regulating aneuploidy.

Recently, changes in the level of 5mC and DNMTs, DNMT1 and DNMT3a, have been detected in neuronal mitochondria from patients with amyotrophic lateral sclerosis (ALS) suggesting that motor neurons can engage epigenetic mechanisms involving DNMT upregulation and increased DNA methylation to drive apoptosis. These cellular mechanisms could be relevant to human ALS pathobiology and treatment [13]. Although mtDNA methylation has not yet been investigated in other neurodegenerative diseases, the suggested central role for mtDNA integrity and mitochondrial function in the etiology of AD, PD and dementia [51] indicates that mtDNA methylation could play a role in these disorders.

Finally, using bisulfite sequencing and methylated DNA immunoprecipitation in peripheral blood, the presence of 5mC and 5hmC was demonstrated in the human D-loop mtDNA from cell cultures and tissues [52].

4.1.2. Global 5hmC in drug treatment

There are a number of methylation-specific drugs that have been developed to directly affect nuclear DNA methylation [53,54]. For instance, valproic acid, a histone deacetylase inhibitor, is used as pharmacological tool for neuroepigenetic research. Recently, its effect on mtDNA was demonstrated by evaluating mtDNA 5mC and 5hmC levels [55]. Mouse 3T3-L1 cells treated for 1 and 3 days with valproic acid showed a reduced 5hmC but not 5mC content in mtDNA after the prolonged treatment. Interestingly, the prolonged valproic acid treatment also decreased mitochondrial TET1 expression both at mRNA and protein levels. The reduction of mtDNA 5hmC is probably dependent on nuclear histone deacetylase inhibition causing the initial increase of TET1 transcription, which is followed by a delayed compensatory decrease of TET1 protein in mitochondria. The observed effects of valproic acid on mitochondrial epigenetics may have implications for a better understanding of both therapeutic and unwanted effects of this drug and possibly other histone deacetylase inhibitors. Moreover, since mitochondria do not contain histones, it is likely that the mtDNA methylation/hydroxymethylation ratio rather than histone modification is important for mitochondrial epigenetics.

4.2. Specific mtDNA gene methylation as biomarker

Beyond determination of global mtDNA methylation, specific mtDNA encoded genes have been found to be differently methylated in particular cellular conditions or in different cell lines. Two of them, ND6 and 12S rRNA, display different methylation levels in oxidative stress following oxidative insult and exposure to environmental pollution, respectively. Identification of mtDNA encoded genes differently methylated in murine aging brain suggests the possibility that mtDNA methylation is targeted during aging. In the next sections we will review physiological and pathological conditions in which specific mtDNA encoded gene methylation has been evaluated.

4.2.1. ND6 (complex I subunit 6) in oxidative stress and NASH

Under conditions of oxidative stress, such as hypoxia and ethanol, DNMT1 is upregulated through the expression of PGC1α and NRF-1 [12,56]. Increase of DNMT1 expression suppresses the expression of ND6 through methylation of its gene [12], whereas ND1 is upregulated. Although the meaning of opposite ND1 and ND6 regulation is not understood, a proposed mechanism involves an interaction of MTERF1 (mitochondrial terminator factor 1) with 5-methylcytosine in the CpG dinucleotides and/or its interaction with mtDNA-bound mtDNMT1. Since mitochondria are the main source and immediate target of ROS, it is conceivable that a rapid increase of mtDNMT1 protein levels is vital for the adaption to oxidative stress. Under uncontrolled ROS production oxidative stress can lead to apoptosis as a consequence of impairment of the electron transport, proton translocation and ATP synthesis [57].

ND6 gene is target of differential methylation also in the nonalcoholic fatty liver disease (NAFLD) [58]. On this point, Pirola et al. [59] reported that the encoded ND6 mitochondrial gene is highly methylated in the liver of NASH (nonalcoholic steatohepatitis) compared to simple steatosis (SS) patients and its methylation status significantly impacts on ND6 transcriptional regulation. Indeed, liver ND6 is significantly decreased both at mRNA and protein levels in NASH patients versus SS patients, suggesting that the expression of this mitochondrial gene, which is regulated by an epigenetic modification, may play an important role in the pathogenesis and disease progression. This effect is probably dependent on the enhanced expression of the DNMT1 in the liver of NASH patients.

4.2.2. 12S rRNA in aging and environmental factor exposure

Several studies in humans have established an association between DNA methylation and different environmental factors [60]. An interesting epidemiological study performed by Terry et al. [46] on nDNA methylation in white blood cells demonstrated a difference in DNA methylation by selected risk factors including demographic (age, gender, race), environmental exposure (benzene, persistent organic pollutants, lead, arsenic and air pollution) and other risk factors (cigarette smoke, alcohol drink, and diet). Although data are still limited, emerging evidences now support the view that also mtDNA is influenced by environmental exposures. On this point an interesting study has been performed by Byun et al. [61] on mtDNA methylation from workers highly exposed to airborne pollutants (i.e. steel workers exposed to metal-rich particulate matter (PM), gas-station attendants exposed to air benzene and traffic drivers exposed to traffic-derived elemental carbon). By using the bisulfite-pyrosequencing method a higher methylation level within Phe-mtRNA and 12S rRNA coding regions compared to low airborne pollutant exposed subjects was found [61]. This finding is very intriguing, but it needs to be replicated in prospective cohort studies. It is noteworthy that 12S rRNA is a 959 long mitochondrial ribosomal RNA.

Correlation between 12S rRNA methylation and aging has also been reported by Giordano et al. [62]. Analysis of the presence of methylated cytosine residues in two mitochondria genes, 12S and 16S rRNA, performed in different aged subjects revealed that the 12S rRNA gene is subjected to different methylation levels with age. Methylation percentage significantly decreased with age in males.

4.2.3. ND2, ND4, ND4L, ND5 and ND6 (complex I subunits) in aging

Despite the body of knowledge on interaction between aging and mitochondria, particularly in the brain [63], mtDNA has been investigated mainly with respect to mtDNA mutations and mtDNA damage. Only recently, Dzitoyeva et al. [64] reported that the 5hmC but not 5mC mtDNA levels are lower in the frontal cortex of 4- compared to 24-month-old mouse brain. Transcript levels of selected mtDNA-encoded genes, including complex I components (ND2, ND4, ND4L, ND5 and ND6), increase during aging in the frontal cortex, but not in the cerebellum. However, whether 5hmC changes directly affect expression of the selected mitochondrial genes has not been clearly demonstrated. They also showed that aging affects the expression of mtDNMT1, and ten–eleven-translocation, TET1 and TET3, respectively. In the frontal cortex, mouse brain aging is associated to a decreased mtDNMT1 mRNA levels without affecting TET1–TET3 mRNA levels. In the cerebellum, TET2 and TET3 mRNA content is increased but mtDNMT1 mRNA levels were unaffected. All together these findings strongly suggest that mitochondrial epigenetic mechanisms in the mammalian brain are susceptible to aging.

5. Functional consequences of mtDNA methylation

Despite direct evidence is still lacking, it is likely that mtDNA methylation influences mitochondrial gene expression, biogenesis and function. Considering that mitochondria are essential in almost all cell types for ATP production, any epigenetic modification affecting expression of respiratory chain complex subunits is destined to impact on the energy production machinery. We have reported that expression of NADH dehydrogenase subunit 6 (ND6), a crucial subunit for complex I assembly, is suppressed through hypermethylation of its gene by upregulation of DNMT1 expression [12]. With this respect it is worth mentioning that methylation is present in the D-loop which contains three promoters required for transcription initiation [38]. Recently, methylated cytosines have been found in the promoter region of the heavy strand within conserved sequence blocks (CSB-III), located at 5'-end of the D-loop [65]. This region is considered to be implicated in the processing of the RNA primer during the replication of the H-strand [66]. The functional relevance of this epigenetic event and the mechanisms by which it occurs are not known. However, other functional consequences of mitochondrial epigenetic markers are still under investigation. For example, the exact role of 5hmC by itself and with respect to 5mC, how compaction of mtDNA (mitochromosome) is affected by methylation, and whether full length polycistronic primary transcripts processing to generate mitochondrial tRNA, rRNA and mRNA are affected by epigenetics are not clear. With respect to the latter, it should be mentioned that mtDNA transcription requires one of two orthologous rRNA methyltransferase-related transcription factors, mt-TFB1 and mt-TFB2, to initiate promoter-specific transcription [67,68]. They not only possess a transcriptional activity, but also act as methylating enzymes. In particular, mt-TFB1 is primarily the 12S rRNA methyltransferase, important for ribosome biogenesis and mitochondrial translation [69].

Although the exact role of mt-TFB1 and mt-TFB2 has not yet been fully elucidated, it is likely that mt-TFB1 and mt-TFB2 have downstream biogenesis and function. A point mutation that eliminates the rRNA methyltransferase activity of mt-TFB1 prevents the increase in mitochondrial mass associated with its overexpression in HeLa cells, pointing to a novel role for ribosome 12S methylation and assembly in controlling overall mitochondrial biogenesis key target for mitochondrial biogenesis signaling pathways that do not act alone, but rather synergistically promote different aspects of mitochondrial gene expression [70]. Overexpression of mt-TFB1 causes hypermethylation of the 12S rRNA which is very relevant to its identification as a nuclear genetic modifier of the A1555G mtDNA mutation that causes maternally inherited non-syndromic and antibiotic-induced deafness [71].

Finally, it should also be mentioned that tRNAs and rRNAs are post-transcriptionally methylated in mammalian mitochondria [72,73]. rRNA modifications, including methylation sites, are highly conserved, suggesting that they serve important functions [74] and their impairment can lead to disease [71].

Modifications of tRNA, including methylation, are critical for proper folding, recognition and base-pairing. However, a comprehensive survey of all mitochondrial tRNA modification, especially in human, has not yet been performed.

6. Methods for measuring DNA methylation

Methods to quantify methylation levels of DNA have been established based on different techniques, with the goal of overcoming the many challenges linked to its detection. Indeed the features of DNA methylation are very complex. Not only the distribution of 5mC in a DNA sample is dependent on the methylation pattern of different cells but also methylation itself is not evenly distributed along the target sequence. Additionally, DNA amplification does not provide information on methylation since the hybridization-based methods do not distinguish 5mC from unmethylated cytosine and do not contemplate the presence of DNA methyltransferases activities. Consequently, techniques to detect DNA methylation need to be applied before amplification or hybridization [75]. Targeting mtDNA with respect to methylation introduces a new level of challenges which have hampered for many years mtDNA methylation detection. However, recent evidences reported by us [11] and others [12,52,55,59,61,65] have demonstrated that methylation of mtDNA can be measured. The successful methodological approaches rely either on

deeply increasing sensitivity of 5mC detection or affinity enriching methylated regions. Additionally, for very small regions of mtDNA bisulfite method coupled to pyrosequencing has been proved useful to test methylation patterns [61].

The specific methods applied to mtDNA will be discussed with respect to the recent innovative approaches to detect methylation of nDNA, ranging from global to region or gene-specific approaches.

6.1. Global quantitation of DNA methylation

Nonspecific (or global) measurements of DNA methylation provide an overall picture of cellular DNA methylation levels and are crucial for understanding the relationship between genome wide alterations in DNA methylation, gene specific methylation patterns, and genome stability [76]. When applied to mtDNA sensitivity is crucial. Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) offers a fundamental tool for the determination of genomic and mitochondrial DNA methylation [77]. This technique produces an excellent LC separation of DNA and RNA and easily distinguishes 5mdC from 5mC providing unambiguous quantification of 5mdC with high reproducibility. Indeed as little as 4 ng of DNA is requested for measurement of samples with 5mdC levels as low as 0.05% [77]. More importantly, this method directly quantitates the modified base into hydrolized DNA samples, without any sequence-limitation. By applying this method we were able to detect global methylation level of mtDNA, demonstrating that human mtDNA undergoes this modification [11].

6.2. Gene specific methods

Gene-specific methods interrogate the methylation patterns of specific genetic loci. Their results provide genome DNA methylation profiling and allow integration of DNA methylation patterns with gene expression, chromatin modifications, and assembly of transcription factors at gene promoters. These methods rely on different experimental approaches, mainly based on affinity enrichment, restriction enzymes and bisulfite reaction. The limited sensitivity associated to these methods have hampered their application on mtDNA until recently, when these approaches, alone or in combination, have been proved useful to detect methylated mtDNA.

6.2.1. Affinity enrichment

Affinity-based methods are based on different techniques able to enrich the methylated portion of the nuclear and mitochondrial genomes. Chromatin immunoprecipitation (ChIP), which can be coupled to microarray hybridization (ChIP–chip) or next-generation sequencing (ChIP–seq) [78–82] is the most common affinity-based approach for nDNA but is obviously not feasible when applied to mtDNA. However, mtDNA-packaging proteins such as Abf2 have also been successfully characterized using ChIP-like procedures [83]. Another option is the enrichment of methylated regions by immunoprecipitation of denatured DNA with an antibody specific for 5mC [84] or methyl-binding proteins, such as MECP2 [85] or MBD2, which have been applied in combination with microarray hybridization (MeDIP, [86]) and mDIP [87], leading to a fast and efficient evaluation of the methylation status with no information on individual CpG dinucleotide. This approach has been successfully experimented on mtDNA [12], confirming our finding on methylation of human mtDNA [11]. Randomly sheared mtDNA was precipitated with antibodies to 5mC and 5hmC and probed by qPCR to determine the presence and relative abundance of methylated bases. Immunoprecipitated samples were enriched up to 20 fold for 5mC and up to 85–580 fold for 5hmC relative to IgG controls.

6.2.2. Bisulfite conversion

Sodium bisulfite is a chemical reagent that reacts with DNA by preferentially modifying unmethylated cytosine (to dU) compared to methylated cytosine [88,89]. Through this reaction DNA methylation is translated into a difference in DNA sequence [42,75,90,91], providing an indirect indication of the DNA methylation status. However bisulfite treatment not only requires DNA denaturation before treatment but also causes substantial DNA degradation, and requires sample purification. Bisulfite does not distinguish between 5mC and 5hmC. Since the product of bisulfite conversion of 5hmC tends to stall DNA polymerases during PCR, hydroxymethylated regions of DNA are destined to be underrepresented in quantitative methylated analyses [92]. Additionally, methylated cytosine conversion occurs only at CpG islands, indicating that underestimation of methylation located at C-sites different than CpG islands might occur.

The bisulfite methods are the classical approach to evaluate the methylation status with single base resolution in nDNA. Recently it has been associated with innovative platforms to read the methylation signal. The Illumina technology bisulfite-converted DNA can be subjected to multiplexed methylation specific primer extension and hybridization to bead arrays [93–98]. Recently the Infinium platform from Illumina has also been adapted to DNA methylation analysis [99]. The procedure requires whole-genome amplification after bisulfite conversion, fragmentation and hybridization of the sample to methylation-specific probes linked to specific bead types, each bead corresponding to a specific DNA CpG site and methylation state [100]. Moreover, the whole-genome shotgun bisulfite sequencing (WGsBs) has been achieved by using the Illumina Genome Analyzer platform for small eukaryotic genomes, such as *Arabidopsis thaliana* (*A. thaliana*) [101] and for mammalian DNA [102] providing single-base-pair resolution.

Many scientists have questioned the sensitivity of the bisulfite method when applied to mtDNA, concluding that this chemical approach might not be suitable to detect methylated mtDNA [103]. On this point, recently, the debate on the existence of mtDNA methylation was again opened. Hong et al. [104] questioned the existence of mtDNA methylation, concluding that, at present, methylation of mtDNA is of no biological significance. This rash conclusion is based on the lack of significant level of methylated cytosines obtained by sodium bisulfite sequencing and not supported by functional studies. In opposition, by simultaneously applying the bisulfite and other methods to mtDNA samples from human and murine blood and cultured cells, Bellizzi et al. [65] confirmed that mtDNA is indeed methylated (5mC and 5hmC), particularly at very crucial regions such as the D-loop. Interestingly, the authors found that the majority of methylated cytosine are located outside the CpG sites. This possibility has been already suggested by us [11] and pointed out by others [104], since the 5mC/C found in human cells with the direct LC–MS/MS method is inconsistent with the methylation being located exclusively at CpG sites.

6.2.3. Restriction enzyme method

Sequence-specific restriction enzymes are associated to a DNA methyltransferase that protects the endogenous DNA from the restriction defense system by methylating bases in the recognition site. Since many of these enzymes are inhibited by 5mC, the restriction pattern of a strand represents an effective indication of DNA methylation. The advantage of these methods is their potential applicability to native DNA before purification, starting from serum or plasma samples. The most widely used methylation-sensitive restriction enzymes for DNA methylation studies are *Hpa*II and *Sma*I. This endonuclease-dependent approach coupled to two dimensional gel electrophoresis (restriction landmark genome scanning (RlGs) identified methylated sites in a cancer- or tissue-specific manner [105–108]. Methylation-sensitive arbitrarily primed PCR (Ms-AP-PCR) [109] and amplification of inter-methylated sites (AIMs) [110] are other examples of this approach. Array-based analysis has been also successfully coupled to digestion methods. Methylated CpG island amplification (MCA) exploits the different methylation sensitivities and cutting behaviors of *Sma*I and *Xma*I and can be coupled with array hybridization (MCAM) [111–113]. MethylScope [114], comprehensive high-throughput arrays for relative methylation (CHARM) [115], and microarray-based methylation assessment of single samples

(MMAss) [116] are other examples of array based detection. Methyl–seq [117] and methylation-sensitive cut counting (MsCC) [118]) are examples of next generation sequencing coupled to enzyme restriction.

This approach is relevant for nDNA application but is obviously limited when applied to shorter genomes such as mtDNA. However, the restriction pattern method, associated to affinity enrichment, has been proven useful for site specific detection of 5hmC in mtDNA (12) by exploiting the couple Gla1-T4 5hmC-β-glucosyltransferase in which glycosylated methylated bases hamper Gla1 cleavage [119].

6.2.4. Other approaches

An alternative approach to sequencing or hybridization is detection by mass spectrometry [120,121], which can be automated but so far it requires gene specific amplification. Additionally, nanopore sequencing offers the potential for direct sequencing of 5mC without bisulfite treatment [122,123], and this might represent a revolution in high-throughput DNA methylation analysis.

7. Conclusions and perspectives

The large body of evidence on the existence of mtDNA methylation, together with the identification of the mitochondrially-localized DNMTs and TET, strongly indicates that the controversy on mtDNA is probably at its end. The discrepancies in the results suggest that a combination of different, global, direct and sequence-specific methods is the successful strategy to evaluate the extent of mtDNA methylation. Furthermore studies on mtDNA methylation should be unbiased from previous knowledge on nDNA methylation, as mtDNA methylation might follow specific/unique mechanisms of occurrence in response to the specific functional relevance of mitochondrial epigenetic modifications.

Occurrence of mtDNA methylation has changed our perception of methylation with respect to mitochondrial physiology. Thus, a whole systematic epigenetic characterization of cell is now better defined, although some specific aspects regulating mtDNA methylation are still unclear.

First of all the organization of mtDNA is different from the structural organization of nDNA. Histone modifications, which are important for nDNA, are not applicable to mtDNA, stressing the different mechanisms of 5mC and 5hmC formation for mtDNA.

Second, current data show that expression of DNMT1 is crucial in regulating mammalian mtDNA methylation and that epigenetic regulation of mtDNA might respond to a signaling pathway mechanism [12]. Future investigations will be aimed at the understanding of how mitochondrial versus total DNMT1 expression is regulated and distributed among tissues in normal and pathological conditions.

Third, validation of some reported data is essential for the development for mtDNA methylation as a biomarker tool. This will allow to establish disease-specific mtDNA methylation profiles in many diseases, in particular neurodegenerative and cancer, leading to the discovery of certain types of epigenetically modified mtDNA as specific biomarkers. It will also provide insights into the cross-talk between the nucleus and mitochondria, which might be significant in different conditions (Fig. 4). For example, DNMT1 activity is perturbed in cancer [124].

Fourth, epigenetic alterations of mtDNA need to be evaluated in terms of prediction of therapeutic efficacy similar to those of nDNA. nDNA epigenetically targeted-drugs have already become an important part of the clinical armamentarium, and in some refractory conditions they are the only drugs of any activity available to patients [125]. Although no such research has been reported on mtDNA, it is conceivable to hypothesize that drugs capable of selectively crossing mitochondrial membrane, could be targeted to modify mtDNMT1 and DNMT3a activities and consequently mtDNA methylation.

Finally, for a global mitochondrial epigenetic assessment other aspects of mitochondrial genome regulation need to be elucidated. For example, it is not clear whether RNA interference mediated by microRNAs affects mitochondria epigenetic mechanisms. Although some mitochondrial functions are regulated by miRNAs [126,127], no evidence concerning miRNA mitochondrial epigenetic has been reported.

Crucial to these advances will be the development of technologies for the analysis of epigenetic variations in mtDNA. Even though global mtDNA methylation assessment can be today performed, large scale accurate mapping of mtDNA 5mC and 5hmC patterns obtained from blood samples are not yet available and still represent a challenge for next-generation sequencing technologies.

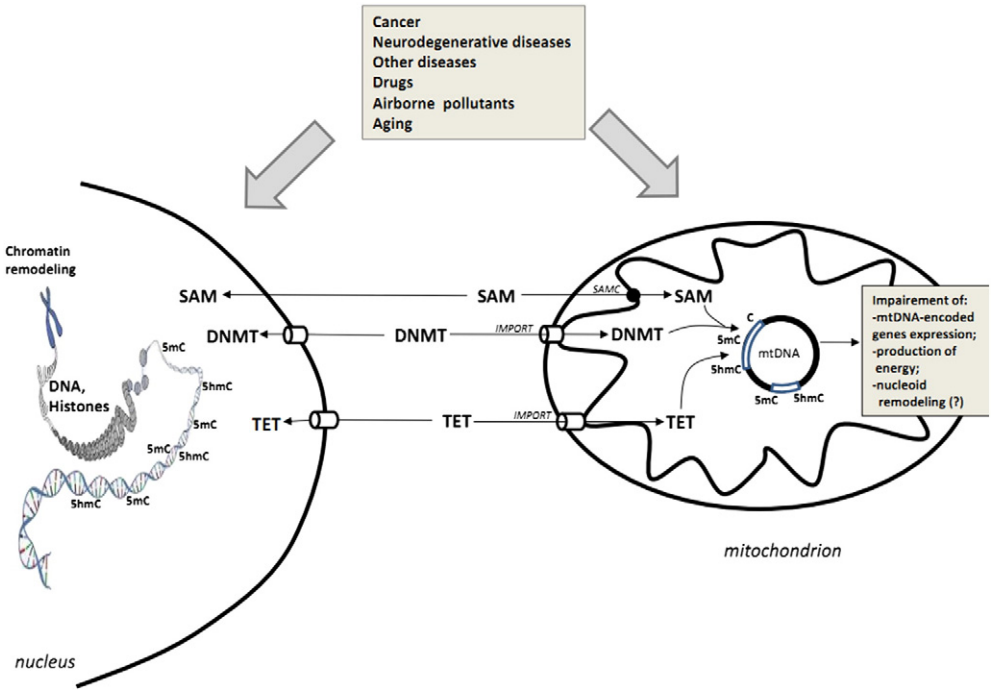

Fig. 4. Speculative model of nDNA and mtDNA methylation mechanisms in diseases and environmental exposure. White portions in mtDNA represent hypothetical methylated regions. White cylinders indicate the import machinery for DNMT and TET. Full black circle represents SAM carrier.

In conclusion, advanced studies are needed to understand whether and how changes in mtDNA methylation patterns, global and gene specific, are associated to diseases and risk factors, such as aging, oxidative stress and exposure to drugs and pollutants. Future development of new techniques for wide-methylation analysis from small samples will open a multitude of possibilities for biomarker development.

Conflict of interest

The authors declare that they have no conflict of interest.

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