



Interplay between environmental exposure and mitochondrial DNA methylation in disease susceptibility and cancer: a comprehensive review

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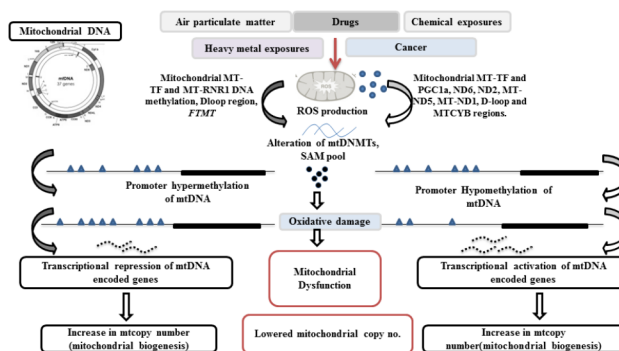
Abstract

Nuclear epigenetics has been a major area of research through decades, involved in the pathogenesis of several human diseases. Current studies brought to light the role of mitochondrial epigenetics in disease initiation and progression. Mitochondrial DNA methylation mark, considered as one of the major epigenetic modifications, has been lately studied in various health outcomes. Herein, this review aims to explore the effects of several environmental factors (including air particulate matter, chemical compounds and heavy metal exposures) and to find their role in influencing diseases through epigenetic alteration, especially focusing on mitochondrial DNA methylation. However, little is known about mitochondrial gene regulation with disease association, which opens up a novel area of research. In addition to this, potential epi-therapeutic approaches have also been discussed. Further, in-depth mito-epigenetic studies will be helpful for clinical research.

Keywords Mitochondria · Epigenetics · mtDNA methylation · Environmental exposure · Health disorders · Cancer

Graphical Abstract

Schematic representation of mitochondrial DNA methylation regulation in response to environmental toxicants and diseases.



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Abbreviations

MtDNA	Mitochondrial DNA.
D-Loop	Displacement Loop
TFAM	Transcription and mtDNA Maintenance Factor
TERF	Mitochondrial Transcription Termination Factor
POLRMT	Mitochondrial RNA polymerase
TFB1M	Mitochondrial Transcription Factor B1
TFB2M	Mitochondrial Transcription Factor B2
L-strand	Light Strand
H-strand	Heavy strand
ETC	Electron Transport Chain
OXPHOS	Oxidative phosphorylation
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
SAM	S-adenosylmethionine
DNMT	DNA methyltransferases
ROS	Reactive Oxygen Species

Introduction

Epigenetics can be explained as the study related to alteration in gene expression with unchanged DNA sequences. Presently, the field of epigenetics is rapidly expanding. Understanding its role in various diseases and therapeutic interventions is an emerging field of research worldwide [22, 62]. Alteration in expression of target genes depends on the epigenetic status of the chromatin structure, involving DNA promoter methylation or hydroxymethylation, histone modifications, promoter–enhancer interactions, and non-coding RNA–mediated regulation. De-regulation of epigenetic machinery leads to inappropriate onset of genes causing either activation or inhibition of downstream protein complexes [22, 62].

Present researches on epigenetics can be divided into two key areas: epigenetic-regulation of nuclear DNA and mitochondrial DNA [89]. Epigenetic-regulation of nuclear DNA involves DNA methylation, post-translational histone modifications and non-coding RNAs; whereas epigenetic-regulation of mitochondrial DNA seems to be alike in the aspect of DNA methylation and noncoding RNAs but lacks histone moieties. Previously it has been established that mitochondrial epigenetic-mechanisms in coordination with nuclear epigenetic regulation influence transcription activation or repression of crucial genes and protein synthesis [20].

Mitochondria possess their own genome, along with its transcription-translation machinery; communicates intricately with the nuclear genome for maintaining normal cellular homeostasis [20]. Human mtDNA consists of ~16,569 base pairs (bp) double stranded circular DNA that encompasses 37 genes. The Heavy strand (or H-strand) consisting of 28 genes is guanine rich; whereas the Light strand (or

L-strand) consisting of 9 genes is cytosine rich. Out of these, 13 genes encode for polypeptides that comprises the electron transport chain (ETC). These include 7 mitochondrial genes encoding for complex I subunit (*MT-ND1*, *MT-ND2*, *MT-ND3*, *MT-ND4*, *MT-ND4L*, *MT-ND5*, *MT-ND6*), one for complex III subunit (*MT-CYB*), three for complex IV subunits (*MT-CO1*, *MT-CO2*, *MT-CO3*) and two for complex V subunits (*MT-ATP6*, *MT-ATP8*), along with two ribosomal RNAs (*MT-RNR1* and *MT-RNR2*) and 22 transfer RNAs [20, 26, 90]. Nuclear DNA possess single promoter for the transcription of a target gene, whereas total mtDNA has only three promoter regions (LSP, HSP1 and HSP2) responsible for transcribing all the genes from both L-strand and H-strand. Mitochondrial displacement loop (D-loop) is a unique region which contains all three promoters along with the replication start site (or RSS) controlling mitochondrial gene expression. Replication and transcription of mtDNA is regulated by multiple nuclear encoded proteins, like TFAM, POLRMT, TFB1M, TFB2M and mTERF etc. [90].

Based on previously established facts, mitochondrial epigenetics comprises of four areas: mtDNA methylation/hydroxymethylation, mitochondrial nucleoid modifications, mitochondrial RNA modifications and mtDNA-derived or nuclear DNA-derived non-coding RNA modulations during mtDNA-encoded gene transcription and protein synthesis [26, 90]. In this review, we highlighted the role of mtDNA methylation in various diseases including cancer and in exposures to environmental pollutants in order to provide an in-depth mechanistic insight.

Mitochondrial epigenetics

Despite decades of research work, mitochondrial epigenetics remained a controversial area of study. Major modifications in mitochondrial epigenome include DNA methylation or hydroxymethylation, demethylation and alteration in mitochondrial non-coding RNAs [16, 43, 68, 88]. However, as in nuclear genome, mitochondrial genome lacks histone packaging; therefore, post translational histone modifications are not a part of mitochondrial epigenetic regulation as in nuclear epigenetics. It is already established that, there exists a cross-talk between mitochondria and nucleus which plays an important role in maintaining overall cellular homeostasis. Key regulatory genes for biogenesis, fission-fusion, mitophagy and also mtDNA methyltransferase enzymes (responsible for mito-epigenetic modification) are encoded by nuclear genome. In response to specific cellular micro-environmental condition, synchronized anterograde (nuclear to mitochondrial) and retrograde (mitochondrial to nuclear) signalling pathways are found to be activated. Epigenetic changes in mitochondrial DNA may induce

alterations in the nuclear epigenome leading to an array of downstream activities including differential gene expression, genetic instability and tumorigenesis [34, 61, 71]. Furthermore, in response to extracellular signals like environmental exposure towards toxic substances, mitochondria may play an essential role in modification of nuclear epigenetic regulation, which in turn affect mitochondrial functionality [68]. Thus, the reciprocity between nuclear and mitochondrial epigenome thought to be a crucial factor in understanding the aspects of mitochondrial functionality under normal and pathological conditions [51].

Mitochondrial DNA methylation and associated methyltransferases

Earlier, DNA methylation occurring in the mitochondrial epigenome has been a topic of debate since 1970s. In 1973, Nass MM had first reported the evidences of mtDNA methylation in *in-vivo* and *in-vitro* model systems [73, 74]. Gradually, technical advancements along with new methodologies have evolved for quantifying methylation on CpG- or non-CpG-specific sites that substantiated the existence of mtDNA methylation mark [90]. The conclusive evidence suggesting the presence of methylated CpG islands came from the study of Infantino and co-workers [44] who used a mass spectrometry-based system which overcame the technical limitations of former studies. On the contrary, few studies showed the absence of mtDNA methylation mark [27, 42, 59, 69]. Several factors such as circular nature of mitochondrial genome, different experimental model systems, disease occurrences, exposures to various external and internal stimuli, stressors etc. play a vital role in determining the presence or absence of mitochondrial cytosine methylation [27, 42, 59, 69].

DNA methylation occurs on cytosine residues that are followed by guanine residues near the promoter regions or transcriptional start sites (CpG islands or CpG clusters or CpG sequences) catalyzed by DNA methyltransferases (DNMTs) in presence of methyl donor, S-adenosylmethionine or SAM [86, 87]. Presence of mtDNA methyltransferase activity was first recorded by Nass MM [73]. Nuclear DNA methyltransferase 1 (DNMT1), the most abundant methyltransferase, is translocated to the mitochondria via signalling by a mitochondrial targeting sequence (MTS) located immediately upstream of the translational start site (TSS). MTS has been found to be conserved across mammals, encodes a peptide required for translocation of nuclear DNMT1 to mitochondrial matrix. Mitochondrial DNMT1 (mtDNMT1) binds to mtDNA, proving the presence of mtDNMT1 in the mitochondrial matrix. However, MTSs are not present in the primary sequences of de novo

methyltransferases DNMT3A and DNMT3B that are specifically found in mitochondria [87].

Mitochondrial DNA methyltransferase 1 or mtDNMT1 plays a significant role in the epigenetic mechanism of mtDNA methylation and in the regulation of transcription of mitochondrial genes. MtDNMT1 regulates the mitochondrial gene expression through mtDNMT1 binding to the mitochondrial D-loop control region and promoting transcription of target genes [27, 42, 59, 69, 87]. Interestingly, recent studies have highlighted the role of DNMT1 mutations and its effect on mitochondrial epigenome [36, 67]. Dominant mutations in Replication foci targeting sequence (RFTS), present in the N-terminal domain of DNMT1 has been previously reported, deletion of which has been shown to alter DNMT1-dependent DNA methylation in cancerous cells [96]. Currently, a study by Maresca et al., 2020 showed that *DNMT1* mutations lead to mitochondrial hyper-function and increased oxidative stress, resulting in neurodegeneration [67]. Also, *DNMT1* mutation was reported to cause epigenetic de-repression of the γ -globin gene in β -thalassemia elevating the fetal hemoglobin levels [36]. In addition to this, mutations in DNMT3A and DNMT3B have also been reported to cause significant alterations in mtDNA methylation thereby affecting mitochondrial functionality [24, 31, 48, 52, 78, 80].

Apart from mtDNA methylation, hydroxymethylation has also been detected in mitochondrial genome. MtDNA hydroxymethylation occurs through direct addition of 5-hydroxymethyl group to cytosine residues catalyzed by mtDNMT1. Also, mitochondrial DNMT3A and DNMT3B contribute to mtDNA hydroxymethylation. Current reports also suggested the existence of N^6 -Deoxyadenosine methylation in mammalian mtDNA [40]. METTL4, a mammalian methyltransferase mediated mtDNA N^6 -Deoxyadenosine (6mA) methylation promotes reduced mtDNA transcription along with a decreased mitochondrial biogenesis and mtDNA copy number [40].

Technologies involved in the detection of mtDNA methylation

MtDNA methylation is associated with various physiological aspects such as cardiovascular diseases, neurodegenerative diseases, cancer and many more. Various technical approaches are involved for the assessment of methylation of mtDNA. The most widely used technique for the study of mtDNA methylation is bisulphite sequencing. Prior to this, bisulphite conversion of mtDNA has been done that converts the unmethylated cytosine into uracil, whereas the methylated cytosines remain unchanged. The bisulphite converted DNA undergoes PCR amplification prior to bisulphite sequencing procedure. The methylation level is

assessed using the percentage of methylated cytosine over the total number of methylated and non-methylated cytosine residues. MtDNA methylation analysis has been commonly done through bisulphite PCR pyrosequencing [46]. Global DNA m5C content can be assessed using ELISA assay by capture and detection antibodies [21, 37]. Such technique allows the detection of methylated mtDNA fluorimetrically by measuring the intensity of fluorescence. MtDNA methylation can also be evaluated using targeted next-generation bisulphite sequencing (TNGBS) [87, 98]. MassARRAY platform also can be used to detect the methylation of mtDNA which is more efficient as compared to methylation specific PCR and bisulphite sequencing. hMeDIP Assay can be performed using hMeDIP kit which provide three spikes or internal immunoprecipitation controls such as, an unmethylated DNA, a methylated DNA, and a hydroxymethylated DNA [6]. Post bisulphite conversion, the DNA fragments can be hybridized to the Infinium Human Methylation 450 BeadChip array [95]. In addition to this, there are targeted bisulphite deep sequencing and shotgun bisulphite deep sequencing methods to assess the methylation pattern of mtDNA [75]. Another option for evaluation mtDNA-methylation pattern is to use immunoprecipitation reaction using specific antibodies for 5mC followed by microarray hybridization technique [90]. Sequence specific restriction enzymes may be helpful in detecting the methylation sites residing in mtDNA as these enzymes are inhibited by 5mC, they can indicate the methylation pattern more effectively [90]. In combination with high-throughput sequencing, methylation specific restriction enzyme digestion has also been reported for the detection of specific methylation sites [38]. An alternative approach to hybridization and sequencing is to use mass spectrometry; however, it requires gene specific amplification. Also, nanopore sequencing is one of the most effective methods which allow direct sequencing of 5mC without the need for the bisulphite conversion of mtDNA [89, 90]. New approaches such as ELISA along with restriction digestion procedure results in better yield of mitochondrial methylation pattern [90]. Recent advancements include Bisulphite-PCR–single stranded DNA conformation polymorphism (SSCP) and methylation sensitive-high resolution melting (MSHRM) that uses a CFX96 Real-Time PCR detection system [70, 92]. Recently identified easiest methods for mtDNA methylation analysis is Illumina Infinium Bead Chip array and Methylation Epic Bead Chip [79]. Interestingly, it has been found that the closed circular topology of mtDNA inhibits bisulphite conversion resulting in false-positive detection of mtDNA methylation [75]. At times, incomplete bisulphite conversion produces false-positive measurement which becomes a major drawback [75]. The adequacy of mtDNA methylation can be affected by the purity of the bisulphite converted

pyrosequencing templates, presence of incompletely converted DNA and the topology of the mtDNA. To avoid these discrepancies and false-positive results it is suggested to use selective primers and the evaluation of conversion efficiency must be calculated accurately for identification and analysis of mtDNA methylation [75].

Regulation of mtDNA methylation in response to environmental pollutants

Humans are being continuously exposed to environmental toxins in daily life which is a growing concern affecting cellular and genomic integrity. Nuclear and mitochondrial DNA damage is the most widely studied impact of environmental or occupational exposures to xenobiotics [12, 23, 32, 65, 66]. Mitochondria play a crucial role in the cellular response to environmental pollutants or stressors, including both intrinsic and extrinsic pathways. Earlier, few reports have revealed how mitochondrial epigenome gets frequently altered primarily through DNA methylation in response to various environmental toxins [2, 53, 72, 81, 83]. Differential tissue specific mitochondrial DNA methylation has been previously reported in placenta, brain, liver and blood cells as a result of environmental exposures involving heavy metal exposures, air particulate matter, various chemical compounds [2, 53, 72, 81, 83]. In the following sections, a detailed account of association of altered mtDNA methylation and its downstream cellular effects have been discussed.

MtDNA methylation in response to air particulate matter exposure

Epigenetic mechanism tune gene expression in response to various environmental and occupational pollutants such as different air particulate matters [3, 10, 13, 19, 84]. Such exposures are associated with several human diseases including neurodegeneration, diabetes, obesity, cardiovascular diseases and different types of cancers. Environmental exposures to air particulate matter (PM) or smoke has been previously reported to induce ROS generation that is shown to cause oxidative stress and promote mitochondrial damage. Evidences support alteration in methylation pattern of mitochondrial DNA affects mitochondrial biogenesis [15, 17, 47, 94].

Interestingly, some studies (Table 1) on PM exposure have previously reported a higher level of mtDNA methylation along with increase in mtDNA copy number [15, 47, 94]. Earlier, a study was performed on steel workers exposed to metal-rich particulate matter demonstrated higher mtDNA methylation (in *MT-TF* and *MT-RNR1* genes)

Table 1 Mitochondrial DNA methylation in response to airborne particulate matter exposure

Exposure	Model	Dose/Exposure	Bio-logical Sample	Method	Observation	Refer-ence
Ultrafine par-ticulate matter (<200 nm in diameter)	In vitro:SH-SY5Y cells In vivo: Liver tissues from C57B1/6 males N= 82 (Mother infant pairs) from Los Angeles, California	In vitro:: 2 h with 20µM MDPs (HNG, SHLP2, MOTS–c) followed by 24 h treatment with the aqueous suspension of 10 ug/ml TRAP–UFP In vivo: 5 h per day, 3 days per week, for 10 weeks at a constant concentration of 340 µg/m ³ of TRAP–UFP. Human studies: Envi-ronmental exposure	Cell line Cell line Cord Blood	Pyrosequencing	1. ↓ mtDNA copy number 2. HyperDNA methylation of two distinct regions of the D-Loop in the mitochondrial genome.	[10]
PM2.5	N=381 (mother-newborn pair) from Belgium	Environmental exposure	Pla-cental Tissue	Bisulphite pyrosequencing	(1) Placental mtDNA meth-ylation is positively associated with PM2.5 exposure during gestation. (2) ↓ mtDNA content reflecting mitophagy and mitochondrial death.	[46]
PM2.5	N=48 (Male workers) from Massachusetts	Exposure in workplace	Periph-eral Blood	Bisulphite Sequencing	↑ mtDNA methylation (<i>MT-TF</i> and <i>MT-RNR1</i> genes).	[15]
Airborne pollutants 1. Metal-rich PM 2. Air benzene 3. Elemental carbon	N=40 male participants (20 high and 20 low exposure) from each of 3 studies STUDY 1: metal-rich particulate matter (measured as PM1) in Brescia, Italy STUDY 2: gas-station attendants exposed to air benzene in Milan, Italy STUDY 3: truck drivers exposed to traffic-derived Elemental Carbon (EC) in Beijing, China	Environmental exposure	Peripheral Blood	Bisulphite pyrosequencing	1. ↑ Methylation of <i>MT-RNR1</i> and <i>MT-TF</i> 2. ↓ mitochondrial translation 3. ↑ mtDNA copy number Found no effects on mtDNA methylation from air benzene and traffic-derived EC exposure as well.	[16]

detected through bisulphite pyrosequencing and higher mtDNA copy number [16]. Consistent with the earlier results, exposures to PM (2.5) induced higher oxidative stress and mtDNA methylation that affects mitochondrial biogenesis with increased risk for cardiovascular diseases [15]. However, mother-newborn pairs exposed to PM (2.5) during the gestational period revealed hypermethylation of placental mtDNA tested through bisulphite pyrosequencing which resulted in lower mtDNA content [46]. An *in-vivo* and *in-vitro* study performed by Breton et al., 2019 reported that exposure to PM (2.5) caused the hypermethylation of mitochondrial D loop regions that affect mitochondrial respiratory function and mtDNA in SH-SY5Y cells and in liver tissues from C57B1/6 males [10].

MtDNA methylation in response to chemical exposures

Exposure to different chemical compounds has impactful effects on mtDNA methylation and thus in turn can influence various disease progressions (Table 2). Earlier, an *in*

vivo study by Byun et al., 2015 [13] experimentally demonstrated the exposures to PBDEs (Polybrominated diphenyl ethers) and mtDNA methylation in rats PBDE induced ROS generation and hypomethylation of mitochondrial *Mt-CO2* (*Cytochrome c oxidase gene*) led to mitochondrial dysfunction in the frontal brain of rats [13]. Maternal smoking during pregnancy has been reported to affect placental trophoblast tissues, placental activity and foetal development by altering the methylation pattern of mtDNA identified by bisulphite pyrosequencing [3]. Doxorubicin, a well-known chemotherapeutic drug, was found to be associated with decreased SAM levels and consequently the hypomethylation of *PGC1a* and *TFAM* genes [29]. Next generation bisulphite sequencing (TNGBS) and ELISA of 5mc DNA content has helped the identification of methylation alteration of mtDNA by the psycho stimulant drug cocaine [25]. Cocaine has been reported to alter mtDNA methylation pattern, lowering the protein levels of DNMT3A and DNMT3B affecting mitochondrial biogenesis [25]. Also, tobacco smoking and air pollution during pregnancy has been reported to cause epigenetic modifications of mtDNA. Hypermethylation of

mtDNA in two regions of displacement loop control region (*D-loop* and *LDLR2*) has been reported to lower the mtDNA content in placental tissues [94]. Exposures to PAH (Polycyclic aromatic hydrocarbons), also caused significant alterations in the expression of epigenetic modifiers and thus is responsible for the altered level of methylation of mtDNA. Methyl Flash Global DNA methylation (5-mC) ELISA is used for the determination of this epigenetic modification of mtDNA [7].

However, the exact mechanisms responsible for changes in mitochondrial methylation due to different exposures need to be investigated and further studies are needed to substantiate the role of DNA methylation as potential mechanism for different adverse health effects and toxicities.

MtDNA methylation in response to heavy metal exposure

Environmental or occupational heavy metal exposures are associated with diverse mitochondrial dysfunctions. Extensive research has been performed in the field of heavy metal exposure and nuclear DNA epigenetics. However, information regarding how heavy metal contaminants affect mitochondrial epigenome through DNA methylation remains very limited (Table 3). Previous research work demonstrated the role of mtDNA hypomethylation through Mass ARRAY Platform in response to chromium in chrome-plate workers. The methylation levels of *MT-TF* and *MT-RNR1* genes were negatively associated with blood Cr ion concentrations; however, no change in mitochondrial copy number was observed [58]. A recent study by a group reported a significant association between arsenic contamination and hypomethylation (of D-loop region and *ND6*) in exposed individuals, as detected through Methylation specific PCR. Hypomethylation of D loop and *ND6* led to the transcriptional activation of mitochondrial genes along with higher mitochondrial copy number in arsenic exposed individuals [2, 81]. Consistent with the above results, significant hypomethylation of both *TFAM* and *PGC1 α* in arsenic exposed individuals along with the higher level of gene expression and increased mtDNA copy number was also observed [83]. Transcriptional activation of these crucial genes are important mediating factors for mitochondrial biogenesis and are associated with tumour cell proliferation and development of arsenical skin cancer [81, 83]. Another study have reported Cd²⁺ and Cr⁶⁺ induced methylation in the mitochondrial DNA in an invertebrate, *Exopalaemon carinicauda*. Heavy metal stress in *E. Carinicauda* has resulted in been reduced gene expression through mtDNA methylation [63].

Mitochondrial epigenetics and its relation with different health disorders

Nuclear epigenetics and its relation with various diseases have been studied thoroughly; however, the association between mitochondrial epigenetics and different diseases has not been given much importance. Here in, we have tried to shed light over this area to look into various means of association between altered mtDNA methylation and different human diseases (Table 4). Mitochondrial epigenetic alterations have been linked to various disease types including Type 2 Diabetes mellitus, neurological disorders (Alzheimer's disease, Parkinson disease), Down Syndrome, NASH (Nonalcoholic Steatohepatitis), cardiovascular diseases (CVD), cancer etc. [5, 8, 45, 55, 77]. Increased oxidative stress has been shown to damage to mitochondrial genome and epigenome leading to mitochondrial dysfunction as observed in neurodegenerative disorders [8, 47, 97].

Mitochondrial DNA methylation and its association in disease outcomes have evolved as a new area of research in the last few years. Higher mtDNA methylation was observed in *ND6* and D loop region of mitochondria during the early stage of diabetes through Methylation specific-PCR. Altered mtDNA methylation was found to be responsible for the development of insulin resistance in individuals with diabetes [99, 100]. The abnormalities in methyl group donors such as S-adenosylmethionine (SAM) and mtDNA methylation status probably led to mitochondrial dysfunction as widely documented through mass spectrometry in patients with Down syndrome [44]. Higher mtDNA methylation at *ND6* region due to high level of *DNMT1* mRNA in the liver of NASH (Nonalcoholic steatohepatitis) patients has been reported by Pirola et al., 2013 [77] along with increased oxidative stress. Hypermethylation of *ND6* gene in the mitochondrial genome caused its transcriptional inactivation that promoted mitochondrial dysfunction. Higher mitochondrial methylation in platelets has been reported to promote the dysfunction of mitochondria and in turn has been shown to be responsible for platelet dysfunction and cardiovascular risks [5]. These mitochondrial epigenetic de-regulations are associated with the prognosis of the various diseases. A greater understanding of how DNA methylation in these regions of mitochondria affects mtDNA genes, evolution that needs to be investigated and pharmacologic manipulations is needed which could provide better insights to the patho-physiology of the diseases.

Table 2 Mitochondrial DNA methylation response to environmental exposure of several chemical compounds

Exposure	Model	Dose/Exposure	Biological Sample	Method	Observation	Reference
Doxorubicin (DOX)	8 week old male Wistar rat (N = 6/group)	7 weekly injections with DOX (2 mg/kg)	Cardiac tissue	Global DNA methylation by ELISA	(1) ↓ SAM production (2) ↓ methylation of mtDNA (3) ↓ levels of transcripts (PGC-1 α and TFAM)	[29]
Maternal smoking during pregnancy	N = 96 mother- newborn pair (Rhode island birth cohort) N = 62 infants (Kentucky birth cohort)	Environmental exposure	Placenta from 96 mother-newborn pair and foreskin samples from 62 infants	Bisulphitepyrosequencing	1. In both placenta and foreskin, mtDNA methylation in the light chain D-Loop region 1 was positively associated with MSDP in placenta 2. ↑ methylation at specific D-loop mtDNA CpGs in placenta and 2 D-loop segments in a second tissue, foreskin.	[3]
Endocrine disrupting chemical (Polybrominated diphenyl ethers PBDEs)	Seven weeks old Wistar female and male rats	BDE-47 (0.002 and 0.2 mg/kg body weight) at gestation days 9 and 16, and postnatal days 1, 8, and 15.	Frontal lobes of their offsprings at postnatal day	Pyrosequencing	The prefrontal cortex of rats treated with BDE-47 showed decreased 5mC levels of the mitochondrial-encoded gene Mt-co2	[14]
PAH (Polycyclic Aromatic Hydrocarbon)	Peripheral Lymphocytes	PAHs (ANT and Bap) for 0 h, 30 min, 1, 3, 6, 12, 24, 48 and 72 h	Peripheral lymphocytes	1. Bisulphite sequencing 2. MethylFlash Global DNA Methylation (5-mC) ELISA	The exposure of BaP also caused significant alterations in the expression of epigenetic modifiers and lowers the methylation of mtDNA.	[87]

Table 2 (continued)

Exposure	Model	Dose/Exposure	Biological Sample	Method	Observation	Reference
HIV 1 Tat and psychostimulant drug cocaine	In vitro : Human brain cerebellum tissue collected from patients in Florida. In vivo: adult male Tat-inducible transgenic mice	1. In mice HIV 1 Tat expression was induced by administered Dox via intraperitoneal injection of a single daily dose of 100 mg/kg dissolved in 0.9% saline in a volume of 0.3 ml/30 g body weight for 14 days. 2. Astrocytes (1 × 10 ⁶) were separately treated with HIV-1 Tat (50 ng/ml) and cocaine (0.5 μM) for 24 h.	Human primary astrocytes and brain samples from the mice	1. Next generation Bisulphite sequencing (TNGBS) 2. ELISA of 5mC DNA content 3. Pyrosequencing methylation analysis	1. Altered levels of DNA methyltransferases and Ten-eleven translocation (TET) enzymes 1, 2, and 3, and mitochondrial DNMTs (mtDNMTs) both in vitro and in vivo. 2. Alterations in mtDNA methylation levels at CpG and non-CpG sites in human primary astrocytes i.e. ↓ of methylation in MT-RNR1, MT-ND5, MT-ND1, D-loop and MTCYB regions.	[25]
Tobacco smoke and air pollution during the pregnancy	N = 60 mothers-newborn pairs three groups of participants in East Limburg Hospital, Genk, Belgium (n = 20/group) 1st group with mothers who smoked during pregnancy, 2nd group with high air pollutant exposure and 3rd or control group with low air pollutant exposure.	1st group (average 13.2 cig/day) 2nd group (PM2.5: 16.0 ± 1.4 μg/m ³ , black carbon: 1.8 ± 0.3 μg/m ³) Control group (non-smokers, PM2.5: 10.6 ± 1.7 μg/m ³ , black carbon: 0.9 ± 0.1 μg/m ³)	Placenta	1. Bisulphite sequencing 2. qPCR	1. Absolute D-loop [94] methylation levels were higher for mothers that smoked extensively and for mothers that were highly exposed to air pollutants 2. D-loop methylation levels were correlated with placental mtDNA content and associated with birth weight	

Mitochondrial DNA methylation and cancer

Nuclear epigenetics and its association with diseases especially cancer has been studied widely and given much importance till date; however, the field of mitochondrial epigenetics and its relation with cancer has not been studied and reported thoroughly. Herein, we have made an attempt to elaborate the relationship between mtDNA methylation and cancer progressions (Table 5) elucidated in recent studies [28, 33, 45, 91, 93]. Previously, studies have reported that colorectal cancer tissues with hypomethylated mitochondrial D-loop might be associated with higher expression of mtDNA along with higher mtDNA copy number [28, 33]. MtDNA methylation study was done using Methylation

specific PCR and mitochondrial copy number was estimated using quantitative PCR (qPCR). A study by Feng et al., 2012 [28] showed increased expression of *ND2* (a subunit of NADH, encoded by mtDNA) is associated with hypomethylation of mtDNA D-loop region, a crucial factor for mtDNA replication and transcription. Hypomethylation of D loop region is associated with the binding of TFAM on mtDNA and thus the expression of *ND2* is upregulated leading to oxidative phosphorylation which is required for the enhanced growth of cancer tissues [28]. Consistent with the earlier results, similar finding was observed in colorectal cancer cases and *Caco-2* cell line (colorectal cancer cell line) in a study by Gao et al., 2015 [33]. In this study, the demethylation of the D-loop region has facilitated the

Table 3 Mitochondrial DNA methylation in response to environmental exposures of heavy metal pollutants

Exposure	Model	Dose/Exposure	Biological Sample	Method	Observation	Reference
Arsenic (As)	N = 221 Arsenic exposed individuals (106 individuals with skin lesion and 115 subjects without skin lesion) from West Bengal	Environmental exposure	Blood, urine	Methylation specific PCR	1. Hypomethylation of D-loop region and <i>ND6</i> in arsenic-exposed individuals 2. ↑ expression of <i>ND6</i> gene 3. ↑ mtDNA copy number	[81, 82]
Arsenic (As)	N = 280 Arsenic exposed individuals (156 with skin lesion and 124 without skin lesion), N = 110 unexposed individuals from West Bengal	Environmental exposure	Blood, skin tumor tissue, urine	Methylation specific PCR	1. Promoter hypomethylation of two key biogenesis regulatory genes, <i>PGC1a</i> and <i>Tfam</i> 2. Increased expression of biogenesis regulatory genes <i>PGC1a</i> , <i>Tfam</i> , <i>NRF1</i> , <i>NRF2</i> 3. Altered mitochondrial fusion-fission regulatory genes <i>Mfn1</i> , <i>Mfn2</i> , <i>Opa1</i> , <i>Fis1</i> and <i>Drp1</i>	[83]
Arsenic (As)	N = 264 females, Puna N = 169 females, Chaco	Environmental exposure	Peripheral blood	Quantitative PCR	1. Increased mtDNA copy number 2. Telomere lengthening	[2]
Chromium (Cr)	N = 29 pairs (chrome plating workers) from Shenzhen City, China	Occupational exposure	Peripheral blood	Mas-ARRAY Platform	Decreased <i>MT-TF</i> and <i>MT-RNR1</i> gene methylation in chrome plate workers.	[58]

binding of the TFAM thus increasing the mtDNA expression i.e., the upregulation of *ND2*. Increased *ND2* expression is responsible for higher oxidative phosphorylation thus enhancing the growth of the colorectal cancer cells [33]. Further validation was done by a DNA hypomethylating agent, 5-aza-2'-deoxycytidine (5-AZA). It was performed to examine the effect of DNA methylation on the mtDNA copy number and the biological behaviours of cells by Sequenom MassARRAY platform using various colorectal cell lines [93]. 5-AZA treatment induced hypomethylation of mitochondrial D loop region at specific sites on CpG islands were found to be associated with higher mtDNA copy number that in turn is probably a triggering factor for increased cell proliferation in colorectal cancer cell lines [93]. Similarly, in breast cancer, it has been revealed that DNA methylation in D loop region is maternally inherited and eight aberrant methylation sites are associated with cancer progression detected through whole genome DNA methylation array and bisulphite sequencing [39]. According to a study by Lee et al., 2015 [54], cancer stem-like cells were found to be hypermethylated at exon 2 of the human mtDNA-specific polymerase (DNA polymerase gamma A (POLGA)) that accounted for low mtDNA copy number. Also, a study by Sun et al., 2018 [91], revealed a negative correlation between mtDNA methylation and mtDNA copy number during the progression of tumorigenesis in osteosarcoma and glioblastoma. Mitochondria, being a prognostic marker in carcinogenesis, further studies on the relationship between mtDNA epigenetic regulation and cancer should be clearly elucidated to better understand the clinical significance, function and mode of actions of mitochondrial epigenome in response to disease progression.

Mitoeigenetics, especially mtDNA methylation directly regulate gene expression, which was previously studied in different cancers [28, 33, 39, 54, 91, 93]. MtDNA possess only 13 protein coding genes and all of them encode the subunits of respiratory complexes associated with ETC. As a result, any small change in these gene expressions affects the cellular respiration. For malignant transformation and cancer progression, alterations in glucose metabolism pathway are one of the major phenomena that have been reported earlier [14, 30]. Up-regulation in the glycolytic pathway under hypoxic conditions is an important measure for tumour cell survival. Interestingly, even in the presence of sufficient oxygen, tumour cells preferentially use glycolysis rather than oxidative phosphorylation for energy production known as Warburg effect, leading to a shift in metabolism from aerobic respiration toward glycolysis [11, 30, 35]. This occurs via the induction of HIF-1 (hypoxia-inducible factor-1) which was shown to suppress mitochondrial respiratory activity, as a consequence, to suppress Krebs cycle and mitochondrial respiration [50, 76]. Also, mitoeigenetics may play essential role in mitochondria mediated biological processes. Alteration of mitoeigenetics alters the mtDNA encoded proteins which play essential roles in ETC/OXPHOS including glucose, lipid and amino acid metabolism [50, 76]. MtDNA of cancer stem-like cells has shown hypermethylation and low mtDNA copy number which provokes them to use glycolysis for cell proliferation [55]. It is reported that methylation of mtDNA may differentially suppress mtDNA encoded genes that are necessary for oxidative phosphorylation [85]. Oxidative stress such as ROS can trigger cellular proliferation and carcinogenesis by inducing mutation in mtDNA which further reduce the efficiency of

Table 4 Mitochondrial DNA methylation in response to various diseases

Diseases	Model	Biological Sample	Method	Observation	Reference
Cardiovascular disease (CVD)	N = 17 (human plasma samples from healthy individuals) and N = 10 (Sample from individuals with CVD), Baltimore, USA	Blood plasma	Pyrosequencing	Higher platelet mtDNA methylation than healthy controls in <i>MT-CO1</i> , <i>MT-CO2</i> , <i>MT-CO3</i> , and <i>MT-TL1</i> genes in CVD patients.	[5]
Insulin resistance and obesity	N = 40 participants (with diagnosis of no diabetes or CVD) from Virginia	Blood	Methylation specific-PCR	1. ↑ DNA methylation in obese and insulin-resistant individuals. 2. ↓ mitochondrial DNA copy number (mtDNA) in the leukocytes from obese humans (BMI > 30) and strongly associated with insulin resistance.	[99]
T2DM (Type 2 Diabetes mellitus)	Human, N = 87 participants (12 = lean, 72 = obese/overweight), Virginia	Blood	Methylation specific- PCR	1. ↑ level of mt DNA methylation (in ND6 and D loop) is observed during the early stage of diabetes. 2. ↑ level of methylation is associated with the development of insulin resistance and higher risk of diabetes.	[100]
Alzheimer Disease (AD)	Male APP/PS1 transgenic mice and wild-type(C57BL/6J) mice were used at 9 months of age	Hippocampus	Pyrosequencing	1. Altered mitochondrial morphology in APP/PS1 transgenic mice 2. De-methylation mtDNA methylation D-loop in APP/PS1 transgenic mice 3. The mitochondrial DNA copy number decreases in APP/PS1 transgenic mice 4. The mitochondrial gene expression is reduced in APP/PS1 transgenic mice	[97]
Neurodegenerative disease (Alzheimer disease, Parkinson disease)	N = 30 (Human patients from Barcelona) and male APP/PS1 transgenic mice and wild type mice (C57BL/6J)	Human Brain samples (tissue samples) and mouse neocortex	hMeDIP Assay	1. DNA methylation ↑ in D-loop in entorhinal cortex in brain samples and ↓ in MT-ND1 gene in cases with early stage AD related pathology. 2. Dynamic DNA Methylation Pattern in the D-Loop in the Cerebral Cortex of APP/PS1 Mice along with AD Pathology Progression 3. DNA Methylation is reduced in D-Loop and maintained in MT-ND6 Gene in the SubstantiaNigra in PD.	[8]
NAFLD (Nonalcoholic fatty liver disease)	N = 45 patients and N = 18 near normal liver histology subjects from Argentina	Liver Tissue	Methylation specific-PCR	1. MT-ND6 methylation was higher in the liver of NASH(Nonalcoholic steatohepatitis) than SS(simple steatosis) patients 2. MT-ND6 methylated DNA/unmethylated DNA ratio was significantly associated with NAFLD activity score 3. Liver MT-ND6 mRNA expression was significantly decreased in NASH 4. Decreased expression levels of NT-ND6 mRNA expression levels in NASH patients	[77]
Down Syndrome	N = 6 DS children N = 6 Control children (1–13 years old)	Epstein-Barr virus-immortalized lymphoblastoid cells of the children	Mass spectrometry	1. Decreased SAM availability in DS mitochondria 2. Overexpression of the mitochondrial SAM carrier in DS cells 3. Hypomethylation of mtDNA	[44]

ETC and OXPHOS [18, 56, 60]. Taken together, changes in mitoeigenetics affect downstream gene expression, which in turn may cause altered cellular respiration associated with malignant transformation under certain circumstances.

Therapeutic interventions targeting mtDNA methylation

Epigenetic regulation has a reversible mode of action and can be regulated through direct epigenetic modifications. Several on-going drug therapies under research might provide a novel way to regulate mitochondrial epigenetic regulation particularly focussing on mitochondrial DNA methylation mark. Though several therapeutic drugs are validated that Potential ‘epitherapeutic’ drugs that are currently available

Table 5 Mitochondrial DNA methylation and cancer:

Diseases	Model	Biological Sample	Method	Observation	Reference
Colorectal Cancer	N = 30 colon and N = 35 rectal cancer patients from Sichuan, China and Caco-2 (colorectal cancer cell line) cell culture	Tissue and Cell line	Methylation specific PCR	In colorectal cancer tissues, the demethylation rate of the Dloop region increases the mtDNA copy number and ND2 expression and they were significantly higher than those in the corresponding noncancerous tissues. Demethylation of the Dloop region may be involved in the regulation of the mtDNA copy number and ND2 expression.	[33]
Colorectal Cancer	N=24 colon and N=20 rectal cancer patients from Sichuan, China	Tissue	Methylation specific PCR	1. Percentage of methylation in D loop is very low 2. Demethylation of the D-loop and regulation of ND2 expression during the initiation and/or progression of colorectal cancer. 3. Quantitative changes in ND2 expression exhibited a significant increase	[28]
Colorectal Cancer	Colorectal cancer cell lines (Lovo, Colo205, HCT116, SW480, LS174T, and HT290)	Cell line	Sequenom-MassARRAY platform for methylation analysis	De-methylation of specific sites on CpG islands of D-loop promoter region in specific cell lines	[93]
Breast Cancer	N = 10 individuals (5 independent breast cancer patients and their family members) from Tianjin, China	Blood	Whole genome DNA methylation array and bisulfite sequencing	MtDNA methylation was maternally inherited, in D-loop regions. Eight aberrant mtDNA methylation sites were correlated with breast cancer.	[39]
Glioblastoma and Osteosarcoma	HSR-GBM1 cells 143B cell lines	Cell line and tumors	mtDNA bisulfite sequencing by long PCR	mtDNA copy number in tumor-initiating cells affects mtDNA methylation	[91]

include 5-Azacytosine, Decitabine, Guadecitabine, Belinostat, Panobinostat, Vorinostat, and Romidepsin have shown promise in clinical and preclinical trials [49]. Azacitidine and Decitabine have been approved by FDA (Food and Drug Administration) and European Medicines Agency (EMA), are known to function as potent DNA demethylating agents [1]. Certain dietary components play a significant role and have a promising field of research in modulating epigenetic phenomenon in lessening the disease burden. Specific dietary factors and supplements can activate or inhibit DNA methyltransferase activity directly or indirectly to influence mitochondrial DNA methylation [9, 41]. Previous studies have suggested the role of folate and Vitamin B₁₂ supplementation that can regulate mitochondrial DNA methylation [64]. Few DNA methyltransferase inhibitors (DNMT

inhibitors) include dietary polyphenols, (-)-epigallocatechin-3-gallate (EGCG, from green tea), genistein (from soybean) and isothiocyanates (from plant foods), may be used for cancer prevention and therapy [57]. Implementation of these dietary factors in clinical trials against mitochondrial epigenetic de-regulations might prove to be helpful for therapeutic requirements. An important study done by Lee et al., 2015 [54] have demonstrated that cancerous cells with hypermethylated human mitochondrial *POLGA* region at exon 2 has been demethylated by applying DNA demethylating agent, 5-azacytidine. Various therapeutic drugs that are in medical practice have been reported to trigger mitochondrial toxicity leading to a wide range of clinical symptoms; hence development of non-toxic mitochondrial epitherapeutic drugs must be a primary concern. Further

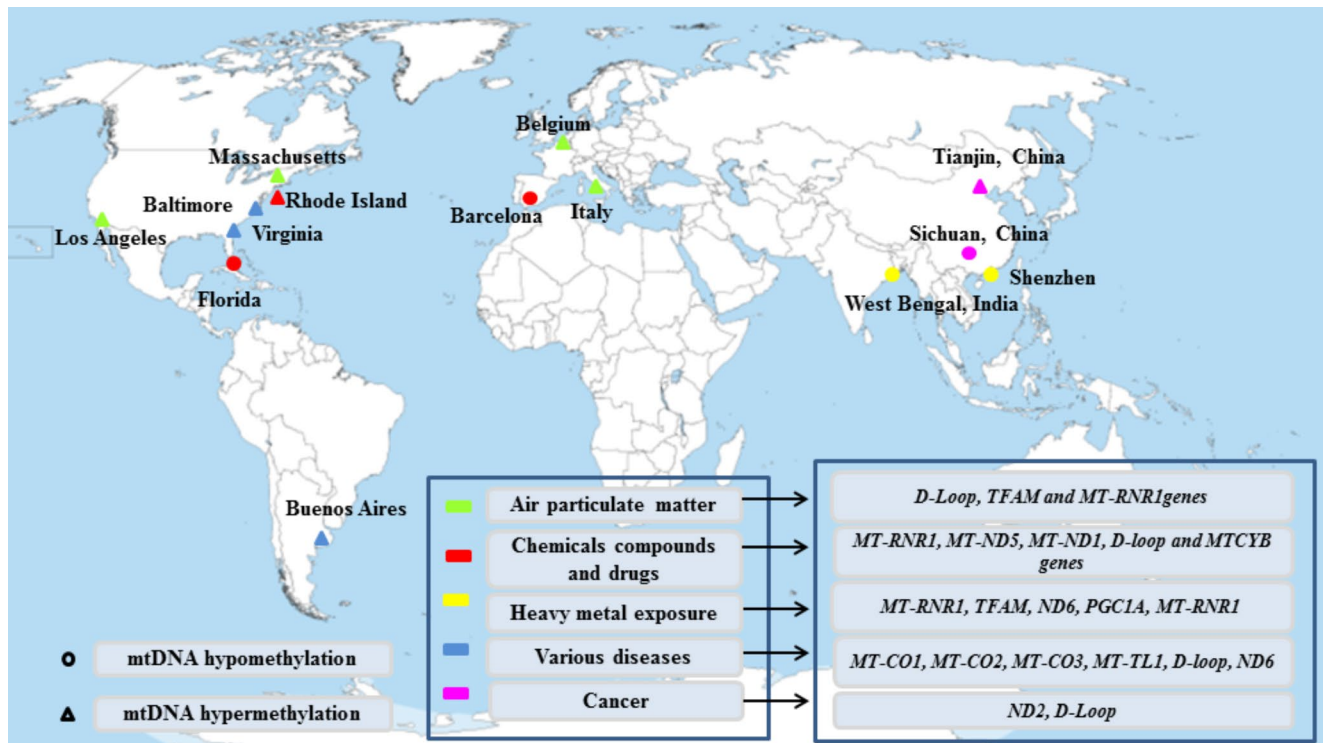


Fig. 1 A global (Country-wise) representation of methylation changes in mitochondrial DNA in human studies

in-depth high-throughput studies are warranted in this field along with several preclinical and clinical trials and accurate validation is required to obtain maximum health benefits.

Discussion

DNA methylation is a vital epigenetic mark affecting our physiological and biochemical process. Methylation-demethylation phenomenon influence gene expression of crucial signalling pathways required for normal functioning of cells. This review primarily aims to bring a holistic picture of the crosstalk between environmental factors, lifestyle disorders and adverse health outcomes including cancer with altered mtDNA methylation. MtDNA methylation is largely influenced by various environmental toxicants such as airborne particulate matter, exposures to heavy metals or chemical compounds or diseases [87]. These factors perturb the normal cellular homeostasis by genetic and epigenetic de-regulation of mitochondrial genome affecting the transcriptional-translational machinery and protein synthesis [4]. Mitochondrial dysfunction indirectly elevates ROS generation leading to an increased oxidative stress and in turn damage to the mitochondrial genome [17]. However, in-depth mechanistic insight about how these exposures alter the methylation pattern of mitochondrial DNA needs to be explored more in future studies. A global (country-wise)

representation of methylation changes in mitochondrial genome in human studies has been shown (Fig. 1).

Environmental or occupational exposure to different chemical compounds or drugs, heavy metal pollutants possesses potential risk factors for alterations in mitochondrial epigenome through differential mtDNA methylation pattern that affect the expression of significant mitochondrial genes [12, 53, 66]. Extensive studies are needed to comprehend the exact mechanisms responsible for methylation of mitochondrial genes to understand the role of ‘methylation mark’ as a potential risk factor. Previous studies have reported the association between mtDNA methylation and altered mtDNA copy number in response to various diseases, environmental exposures and cancer. Both hyper and hypo methylation of specific regions in mtDNA was found to be associated with higher mtDNA copy number [47, 70]. Those findings clearly indicate towards a complex mechanism involving not only the mitochondrial epigenetic modifications but also involvement of other confounding factors like nucleus-mitochondrial retrograde and anterograde signalling, accumulation of ROS, rate of mitochondrial biogenesis, fission-fusion, mitophagy status and individual genetic make-up, environmental exposure, cancer microenvironment (in case of malignant transformations) etc. in mtDNA copy number regulation. However, the exact intracellular molecular signalling pathway and identification of regulatory factors still remain obscure as majority of the studies

regarding this area were associative in nature. Evident from previous studies, that differential mtDNA methylation pattern can be used as a prognostic marker for cancer detection [28, 33, 93]. MtDNA methylation also play crucial roles in several other diseases like cardiovascular diseases, neurodegenerative diseases etc. which revealed that how disease progression is often associated with the altered mtDNA methylation pattern leading to an alteration in mitochondrial structural and functionality [5, 44, 77]. As epigenetic changes are known to be reversible, accurate implication of epitherapeutic drugs might reverse the alterations associated with neurodegenerative diseases, cancers etc.

Conclusions

MtDNA methylation plays an important role in disease development and progression. Further in-depth studies are needed to comprehend the alterations of mtDNA methylation pattern in response to various environmental stressors influencing various disease outcomes. Detailed research in the evolving field of mtDNA methylation may pave its way towards the development of potential epitherapeutic approaches in the immediate future.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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