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REVIEW

Role of mtDNA-related mitoepigenetic phenomena in cancer

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ABSTRACT

Background Abnormal mitochondrial function has long been associated with the development and the progression of cancer. Multiple defects in the mitochondrial genome have been reported for various cancers, however the often disregarded mitochondrial epigenetic landscape provides an additional source of deregulation that may contribute to carcinogenesis.

Design This article reviews the current understanding of mitochondrial epigenetics and how it may relate to cancer progression and development. Relevant studies were found through electronic databases (Web of Science and PubMed).

Results and conclusions The remarkably unexplored field of mitochondrial epigenetics has the potential to shed light on several cancer-related mitochondrial abnormalities. More studies using innovative, genome-wide sequencing technologies are highly warranted to assess whether and how altered mtDNA methylation patterns affect cancer initiation and progression.

Keywords Cancer, DNA methylation, mitochondria, mitoepigenetics, mtDNA.

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Introduction

Cancer is a complex, heterogeneous disease characterized by multiple molecular and cellular transformation events that ultimately lead to the formation of tumours. A growing body of evidence suggests that defects in the collective mitochondrial genome (i.e. both nuclear and mitochondrial encoded) may be one such collection of transformation events that contribute to cancer initiation and progression. Multiple insults to the mitochondrial genome associated with cancer have been described in the literature [1,2]. For instance, characteristic mutations and deletions in control and coding regions of mitochondrial DNA (mtDNA) specific to particular cancers are also commonly reported [3], as is depletion of mtDNA [4]. In this regard, Petros *et al.* [5] showed that cytoplasmic hybrids (cybrids) made by fusing mitochondrial DNA depleted prostate cancer cells with mitochondria containing a well-characterized point mutation in the mtDNA-encoded ATP6 gene, known to impair ATP production and increase ROS formation, were more tumorigenic in mice than wild-type prostate cybrids. In contrast to the mitochondrial genome, little to no research efforts have been directed at the role of alterations to the mitochondrial epigenome in cancer. This review explores the potential role of the emerging field of mitoepigenetics in carcinogenesis, with particular emphasis on mtDNA epigenomics.

Mitoepigenetics

The term mitoepigenetics encompasses all bidirectional phenomena between the mitochondrial and the nuclear genomes [6]. This includes all epigenetic events that affect the expression of nuclear-encoded mitochondrial genes and, in the opposite direction, the amount of cellular mtDNA copies and the specific mtDNA haplotype – both known to significantly alter the nuclear epigenetic landscape [7]. Mitoepigenetic events may also include the interplay between mitochondrial-derived substrates and the nuclear epigenetic landscape [8,9] and the often overlooked methylation and hydroxymethylation of mtDNA, which are discussed in detail below.

Mitochondrial DNA: genomics and epigenomics

Human mtDNA is a double-stranded, closed-circular molecule of 16 569 bp that encodes for 2 rRNAs and 22 tRNAs of the mitochondrial translation machinery, as well as 13 polypeptides of the oxidative phosphorylation (OxPhos) system (Fig. 1) [10]. Similarly to nuclear DNA (nDNA), mtDNA is found in a packed protein–DNA structure called nucleoid, in which

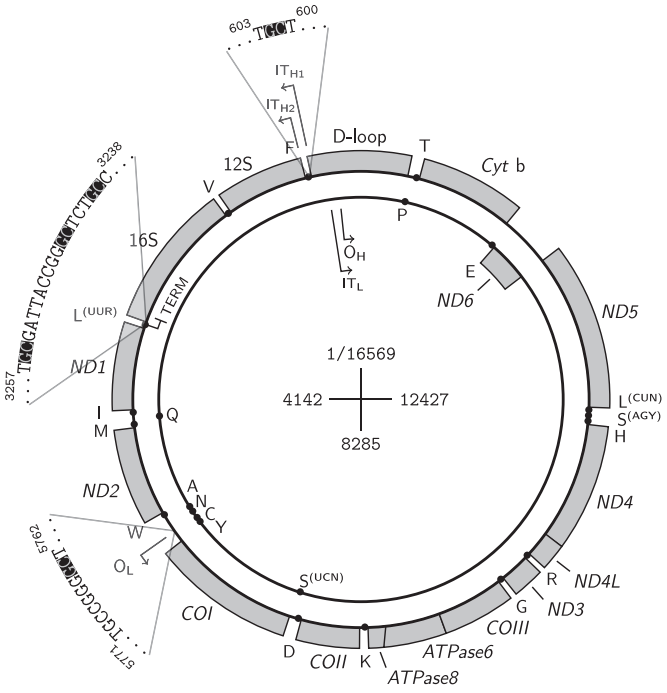

Figure 1 Map of the human mitochondrial genome, according to the revised Cambridge Reference Sequence (NC_012920, [56]). mRNA- and rRNA-coding genes (grey boxes) are interspersed with tRNA genes (black dots, single-letter code) on both the H- and L-strands (outer and inner circles, respectively). Duplicate tRNA genes are distinguished by their codon recognition sequences (parentheses). ND, Cyt *b*, CO and ATPase refer to genes that encode to OxPhos complexes I, III, IV and V, respectively. Locations of the various translation initiation sites (IT_L, IT_{H1}, IT_{H2}), replication origins (O_H, O_L) and the termination site (TERM), indicated by bent arrows, are in agreement with various publications (see [10,57] and references therein). CpGs that display protection from methylation by exogenous bacterial DNMTs are highlighted in black boxes (data from [30]).

mitochondrial transcription factor A (TFAM) is the major proteic component [11]. While it is conceivable that mtDNA nucleoids may undergo epigenetic post-translational modifications in their protein scaffold in a similar fashion to histones in nDNA nucleosomes, there is currently no evidence for the occurrence of this mechanism. Thus, mtDNA epigenetic phenomena currently comprise only DNA methylation and hydroxymethylation [6].

mtDNA methylation and hydroxymethylation. The first comprehensive map of methylated cytosines (m^5C) in the human mitochondrial genome was recently published [12]. Similarly to nDNA, human mtDNA displays a low frequency of CpG dinucleotides (435 in 16 659 nucleotides) [13]. Although mtDNA methylation was initially reported to occur exclusively in CpG dinucleotides [14], recent data shows that m^5C is found predominantly in non-CpG sites [15]. The origin and functional roles of non-CpG methylation are currently unknown. Some controversy regarding mtDNA methylation still remains, however, as a recent study applying regionally specific and genome-wide analyses found that both CpG methylation and hydroxymethylation were absent from human mtDNA in two different cell lines, suggesting that CpG methylation plays no role in mtDNA function [16].

Presumably, CpG mtDNA methylation is carried out by mitochondrial DNA methyltransferase (mtDNMT1), an isoform of the maintenance methylase DNMT1 that contains a mitochondrial targeting sequence [17]. The *de novo* methyltransferase DNMT3a may also be involved, as it has been found to colocalize with mitochondria in mouse neuronal cells when overexpressed [18]. The required methyl donor *S*-adenosyl-L-methionine (SAM) is synthesized in the cytosol and imported to the mitochondrial matrix via the mitochondrial SAM carrier, likely via exchange for its metabolized variant *S*-adenosyl-L-homocysteine (SAH) [19]. SAM synthesis is regulated in part by the mitochondrial one-carbon (folate) metabolism [20]. In postmitotic differentiated cells, the mitochondrial bifunctional enzyme (MBE, a protein that participates in mitochondrial folate metabolism) is not expressed, allowing the flow of one-carbon units towards SAM synthesis. Conversely, cells with a more proliferative phenotype (e.g. embryonic or cancer cells) express MBE, and thus, one-carbon units are shuttled predominantly towards nucleotide rather than SAM synthesis [21,22]. Reduced SAM availability may explain, at least in part, the global nDNA hypomethylation patterns seen in cancer cells [23] and mtDNA hypomethylation in lymphoblasts derived from Down syndrome patients [24].

Levels of mtDNMT1 may also influence mtDNA methylation status. Interestingly, transcription of the mitochondrial variant of DNMT1 has been shown to be influenced by various factors related to cancer such as loss of p53 and increased signalling by the oxidative stress-responding transcription factors nuclear respiratory factor 1 (NRF1) and peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) [17]. Similarly to nDNA methylation [25], mtDNA methylation can also be affected by several factors, suggesting that abnormal mtDNA methylation can be used as a biomarker [26].

Hydroxymethylation of mtDNA has also been described [17]. In nuclear DNA, m^5C is oxidized into 5-hydroxymethylcytosine (hm^5C) by the ten-eleven translocation (TET) family of methylcytosine dioxygenases [6]. The biological significance of this modification awaits further characterization, although it is possible that in mitochondria it promotes demethylation by preventing mtDNMT1-mediated remethylation after a replication cycle.

mtDNA transcription. The individual strands of mtDNA are distinguished based on their different buoyant densities in denaturing CsCl gradients, in which they may be separated into the guanine-rich 'heavy' (H) and cytosine-rich 'light' (L) strands. Transcription and replication of mtDNA in both strands are largely controlled by the regulatory noncoding displacement loop (D-loop) region, which contains the H-strand promoter 1 (HSP1), the L-strand promoter (LSP) and the H-strand origin of replication (O_H) [10]. HSP2 is located close to the D-loop, roughly ~60 bp upstream of HSP1. In many types of cancer, the bulk of the reported mtDNA mutations occur predominantly in the D-loop region [3]. This indicates a trend favouring the segregation of heteroplasmic mutant D-loop mtDNA populations towards homoplasmy, as they may confer a survival advantage to cancer cells. Likewise, mtDNA variants displaying aberrant D-loop methylation could also follow this selection pattern.

Transcription of mtDNA is initiated at specific sites within the promoters (IT_L, IT_{H1} and IT_{H2}) and is carried out by the mitochondrial DNA-directed RNA polymerase (POLRMT), which requires the assembly of a complex with TFAM and mitochondrial transcription factor B2 (TFB2M) [27] at the promoter sites. The initial mitochondrial transcripts are polycistronic pre-mRNAs that must be further processed to produce the individual mRNA, tRNA and rRNA molecules [28]. HSP1 produces a relatively short pre-mRNA transcript that terminates at the 3′ end of the 16S rRNA gene in the termination site (TERM) region, generating two tRNAs and the two rRNAs [29]. CpGs in the TERM region are strongly protected against methylation, presumably due to occupancy by the mitochondrial transcription termination factor (mTERF) that arrests HSP1-derived transcript progression at the TERM site [30]. mTERF also appears to stimulate serial HSP1 transcription [31], possibly via simultaneous binding to both the TERM and IT_{H1} regions (Fig. 2) [32]. Because HSP1 encodes for rRNA and tRNA only, mTERF stimulated transcription may underlie the higher rate of rRNA synthesis relative to mRNA in mitochondria [33]. HSP2 produces a much longer transcript that encompasses the majority of the H-strand, which is ultimately processed into 10 mRNAs, 13 tRNAs and the 2 rRNAs. LSP produces a transcript that spans all coding regions of the L-strand, generating 1 mRNA and 8 tRNAs. Termination of L-strand transcription is also mediated by mTERF at the TERM site [31]. The organization of mtDNA promoters is different from that of their nuclear counterparts; nDNA promoters are enriched with clusters of CpG dinucleotides (the so-called 'CpG islands') [34], whereas mtDNA promoters are virtually devoid of CpG dinucleotides. While this suggests that mtDNA methylation plays no significant role in mtDNA transcription regulation, the observation that mtDNMT1 overexpression asymmetrically alters transcription rates of both mtDNA strands suggests

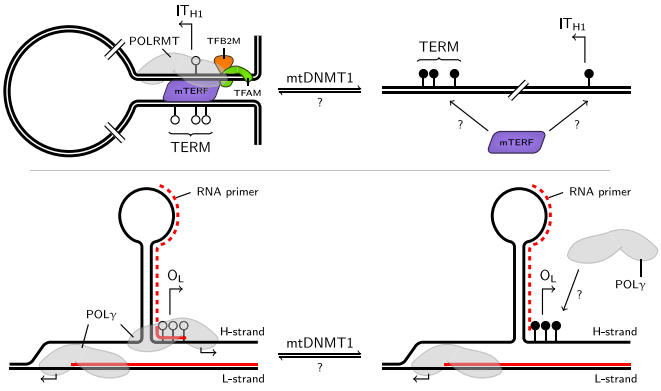

Figure 2 Schematic representation of mtDNA regulatory hubs containing CpG sites protected from methylation. Top: methylation of the TERM and IT_{H1} regions may alter the binding efficiency of mTERF, thereby altering normal rates of rRNA: mRNA synthesis and ultimately compromising mitochondrial translation dynamics. Bottom: methylation of O_L may prevent POLγ binding and impair mtDNA replication. Black and red strands represent parental and nascent strands, respectively. Open and solid lollipops represent unmethylated and methylated CpGs, respectively. Presently, no data are available linking mitochondrial transcription and replication dynamics to regional mtDNA methylation status; therefore, this model should be regarded as purely hypothetical.

otherwise [17]. Moreover, some of the few CpGs that do occur in mitochondrial promoters have been found to be moderately protected from methylation by TFAM occupancy [30]. This raises the possibility that short-term reductions in TFAM levels may lead to long-term epigenetic imprinting in the transiently exposed promoters, thereby disturbing mitochondrial transcriptomics.

mtDNA replication. Replication of mtDNA occurs continuously and is independent of the cell cycle. mtDNA is replicated by a protein complex composed of polymerase γ (POLγ), an accessory 55 kDa subunit (p55), the replication factors mitochondrial single-stranded binding protein (mtSSB) and Twinkle, the mtDNA helicase [35]. Methylation of the POLγ gene (*POLG*) promoter was shown to regulate mtDNA copy number. Replication of mtDNA begins in the O_H, upon extension of a cleaved LSP-derived RNA–DNA primer. Although the L-strand origin of replication (O_L) is located well outside the D-loop, L-strand replication is only initiated when the growing daughter H-strand displaces the parental H-strand [28]. Thus, replication of both strands is dependent on the integrity of the D-loop. As such, abnormal methylation of the D-loop and O_L regions could explain, at least in part, the depletion of

mtDNA commonly observed in cancer. The displaced H-strand adopts a stem-loop structure with a CG-rich region at the base of the stem (Fig. 2) that is critical for L-strand replication, as mutations in this regions were shown to impair the transition from primer RNA to DNA synthesis [36]. The functional implication of O_L methylation state remains to be addressed.

Mitochondrial regulation of nDNA epigenetics in cancer

Interestingly, retrograde crosstalk between mitochondria and nucleus appears to have a role on tumorigenesis [37]. Mitochondrial dysfunction can promote changes in the expression of nuclear genes involved in cellular signalling, metabolism, growth, differentiation and apoptosis [38]. There is increasing evidence that alterations in mitochondrial function severely influence nDNA methylation and histone methylation and acetylation, possibly via disturbing the normal cellular metabolome [39].

Epigenetic mechanisms involving protein acetylation are highly dependent on mitochondrial function, which affects acetyl coenzyme A availability. Tumour proliferation is promoted by upregulation of the mitochondrial citrate transport protein, which shuttles acetyl moieties from mitochondria to the cytoplasm [40,41]. Altered mitochondrial acetyl group export rates disrupt histone acetylation dynamics and may partly account for the enhanced tumorigenic phenotype.

Impaired function of succinate dehydrogenase (SDH), a component of both the mitochondrial respiratory chain and the Krebs cycle, promotes nDNA hypermethylation in multiple tumour lineages, including gastrointestinal stromal tumours, gliomas, paragangliomas and pheochromocytomas [42]. Similar effects were shown to occur in absence of another Krebs cycle enzyme, isocitrate dehydrogenase (IDH), suggesting that mutations or malfunction of these two Krebs cycle enzymes in distinct tumour types disturb epigenomic patterns [42]. In addition, the oncometabolite d-2-hydroxyglutarate (2HG), produced by mutant IDH enzymes, promotes neoplasia development by competitive inhibition of histone demethylation and m^5C hydroxylation, leading to alterations in histone and DNA methylation in gliomas and leukaemias [43]. Human breast tumours and cancer cell lines with elevated 2HG also exhibit a hypermethylation phenotype, which in some cases is associated with MYC activation [44].

Absence of mtDNA in the prostate cancer cell line LNρ0-8 was shown to induce methyltransferase 1 expression and hypermethylation of the nDNA promoters, including the CpG islands of endothelin B receptor (EDNRB), O^6-methylguanine-DNA methyltransferase (MGMT) and E-cadherin (CDH-1) [45]. In addition, in 90% of prostate cancers, the GLI pathogenesis-related 1 (GSTP1) promoter is found methylated [46]. Distinct works show either increased or decreased mtDNA content in malignant tumours [47–49], which might depend on tumour status and origin. One such study focused on D-loop demethylation, which was pointed out as an early event in colorectal cancer that might affect mtDNA content, because the D-loop regulates mtDNA replication [47].

As several key subunits of the mitochondrial respiratory chain are coded by mtDNA, mutations or methylation of the mitochondrial genome may promote aberrant expression of those subunits, altering mitochondrial metabolism. Being the source of fundamental metabolites for enzymes that modify the epigenetic landscape of nuclear DNA, disruption of mitochondrial homeostasis may activate proto-oncogenes and/or inactivate tumour suppressing genes, leading to tumour development. In fact, oxidative phosphorylation, Krebs cycle, β-oxidation of fatty acids and amino acid and lipid metabolism are altered metabolic pathways observed in several human cancers, including colon, breast, lung, prostate, pancreas, liver, kidney and brain [50]. Thus, mtDNA methylation may occur spontaneously in normal conditions, and when a minimal threshold of mtDNA heteroplasmy is reached, changes in mitochondrial metabolism homeostasis may occur, inducing nuclear epigenetic landscape transformation, which may in turn lead to tumour formation and development.

There are some studies showing mtDNA methylation in distinct diseases [17,51]. As this is a rather new subject, reports regarding mtDNA methylation in cancer are currently scarce. One of the few studies available suggests that hypermethylation of mtDNA only occurs at very low frequency in both gastric and colorectal cancer [52]. However, only 37 CpG sites were considered. A second study analysing both cervicovaginal cells of patients with cervix cancer and a cervix cancer cell line (SiHa) obtained similar results. Again, only three CpG sites were taken into account [53].

Nevertheless, it is well known that both metabolic reprogramming and epigenetic alterations are deeply involved in carcinogenesis [54,55], justifying and encouraging the search for alterations in mtDNA methylation in cancer cells using more sophisticated methodologies, such as mtDNA-wide bisulphite sequencing or LC-MS.

Final remarks

The considerations presented in this review support the idea that disrupted mitoepigenetics may contribute to tumorigenesis. Experiments aimed at elucidating the functional implications of mtDNA methylation and hydroxymethylation could help clarify the role of these epigenetic marks, deepening our understanding of mitochondrial biology in cancer and providing

a basis for experimental treatments aimed at epigenetic manipulation.

At this stage, it is important to ascertain whether the mtDNA methylome is deregulated in various cancers and at different progression stages. This would immediately indicate new avenues of research. For instance, can aberrant mtDNA methylation be used as a reliable biomarker for cancer? Do heteroplasmic methylomes develop into homoplasmic methylomes? Is there a functional link between mtDNA methylation and mitochondrial dysfunction in cancer? Finally, does mtDNA methylation in fact play a role in cancer initiation and progression? The extreme simplicity of the mitochondrial genome relative to the nuclear genome should make it an excellent experimental model to address these issues.

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Conflict of interest

The authors declare no conflict of interests.

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