EPIGENETICS OF MITOCHONDRIA – THE OVERVIEW OF mtDNA METHYLATION

EPIGENETYKA MITOCHONDRIÓW – PRZEGLĄD DONIESIEŃ DOTYCZĄCYCH METYLACJI mtDNA

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Summary: One of the basic manifestations of epigenetic is tissue-specific gene regulation, which determines the proper functioning of the cell metabolism. This regulation is primarily connected with the presence of chromatin and DNA modifications, without alterations in the underlying DNA sequence, which have influences on the correct gene expression. The uniqueness of epigenetics modifications results from the possibility of their regulation throughout human life and susceptibility to environmental factors. One of the most studied epigenetic modification is DNA methylation. This process involves the covalent attachment of methyl group to the nitrogen bases of nucleotides, especially to the 5-position of cytosine. Several studies have already proven that the mitochondrial genome (mtDNA), the only genetic material which exists outside the cellular nucleus, like nuclear genome (ntDNA) is also subject to epigenetic modifications.

The mammalian mitochondria are cellular organelles, which nondeceptive size is misleading in comparison to the role which they play in the cell. Mitochondria constitute the fundament energetic support and take part in the process of the cellular respiratory. These organelles have a significant influence on the proper maintenance of the cell metabolism playing a role in a variety of key biological processes like apoptosis, the regulation of calcium homeostasis and the production of reactive oxygen species (ROS). The proper functioning of the mitochondria translates into a properly functioning the whole cell, thus any potential dysregulating of genes expression encoded by mtDNA may have surprisingly serious effects.

Numerous recent data indicate the impact of mtDNA methylation on the functionality, number, and size of mitochondria. In this review, we have compiled a wide range of scientific reports summarizing current knowledge in mtDNA methylation and the influence of epigenetic regulation in the functioning of mitochondria in health and disease.

Keywords: mtDNA methylation, mitochondrial DNA, epigenetic regulation

Streszczenie: Jednym z podstawowych przejawów regulacji epigenetycznej jest specyficzna tkankowo regulacja genów, która decyduje o prawidłowym funkcjonowaniu metabolizmu komórkowego. Regulacja ta jest związana przede wszystkim z obecnością modyfikacji w obrębie chromatyny i DNA, bez zmian w sekwencji DNA, które mają wpływ na prawidłową ekspresję genów. Jedną z najbardziej zbadanych zmian epigenetycznych jest metylacja DNA. Proces ten obejmuje kowalencyjne przyłączenie grupy metylowej do zasad azotowych nukleotydów, zwłaszcza do pozycji 5 w cząsteczce cytozyny. Doniesienia naukowe sugerują że genom mitochondrialny (mtDNA), jedyny materiał genetyczny, który istnieje poza jądrem komórkowym, podobnie jak genom jądrowy (ntDNA), również podlega modyfikacjom epigenetycznym.
Mitochondria stanowią fundament energetyczny w komórkach, a także biorą udział w procesie oddychania komórkowego. Struktury te mają znaczący wpływ na prawidłowe utrzymanie metabolizmu komórkowego, odgrywając rolę w wielu kluczowych procesach biologicznych, takich jak apoptoza, regulacja homeostazy wapnia i wytwarzanie reaktywnych form tlenu (ROS). Prawidłowe funkcjonowanie mitochondriów przekłada się na prawidłowe funkcjonowanie całej komórki, a zatem wszelkie potencjalne zaburzenia ekspresji genów kodowanych przez mtDNA mogą mieć bardzo poważne skutki.
Liczne doniesienia naukowe wskazują na wpływ metylacji mtDNA na funkcjonalność, liczbę i rozmiar mitochondriów. Praca ta podsumowuje i porządkuje aktualną wiedzę na temat metylacji mtDNA i jej wpływu na funkcjonowanie mitochondriów, zarówno u zdrowych organizmów, jak i w określonych jednostkach chorobowych.

Słowa kluczowe: metylacja mtDNA, DNA mitochondrialne, regulacja epigenetyczna

INTRODUCTION

One of the basic manifestations of epigenetic is tissue-specific gene regulation, which determines the proper functioning of the cell metabolism. This regulation is primarily connected with the presence of chromatin and DNA modifications, without alterations in the underlying DNA sequence, which have influences on the correct expression of genes [2]. The uniqueness of epigenetics modifications results from the possibility of their regulation throughout human life and susceptibility to environmental factors, both internal and external. One of the most important and the most studied epigenetic modification is DNA methylation. This process involves the covalent attachment of methyl group to the nitrogen bases of nucleotides, especially to the 5-position of cytosine, more rarely to adenine. The DNA methylation patterns usually include cytosines that are part of symmetrical C-phosphate-G dinucleotides. Regions in the genome with increased content of these dinucleotides are called CpG islands. Moreover, when the CpG islands within the genome promoters undergo methylation, they can affect the expression by silencing it [35,37]. Several studies have already proven that the mitochondrial genome (mtDNA), like nuclear genome (ntDNA), is a subject of epigenetic modifications. The presence of methylation DNA in mitochondria may suggest that the expression of genes encoded by mtDNA is also subject to epigenetic regulation [11].

The mammalian mitochondria are cellular organelles, which nondeceptive size is misleading in comparison to the role which they play in the cell. Mitochondria constitute the fundament energetic support and take part in the process of the cellular respiratory. Being the main place of ATP generation, these organelles have a significant influence on the proper maintenance of the cell metabolism and play a role in a variety of key biological processes like apoptosis, the regulation of calcium homeostasis and the very important role of the production of reactive oxygen species (ROS) [10, 33, 36].

Mitochondrial DNA is the only genetic material which exists outside the cellular nucleus. A relatively small mitochondrial genome with a total length of 16 569 bases encodes 22 tRNA, 2 rRNA, and 13 respiratory chain polypeptides, which are subunits of enzyme complexes of the oxidative phosphorylation system [38]. The proper functioning of the mitochondria translates into properly functioning whole cell metabolism, thus any potential dysregulating of genes expression encoded by mtDNA may have surprisingly serious effects.

THE METHYLATION PATTERNS OF mtDNA

Within human mtDNA occurs 435 CpG sites and 4747 cytosines outside them. Theoretically, both can undergo methylation process [23]. The first research of epigenetic modifications within mtDNA dates to the 70s of the previous centuries, but despite their over 40-year history they still arouse a lot of controversies. The earlier studies showed either a low level of mtDNA methylation [17, 28, 32, 34] or its presence was excluded at all [7, 13, 21]. Mentioned studies carried out on a wide range of organisms, e.g. human mortal and immortal cells like mutant skin fibroblasts [34], HeLa cells, frogs [7], mouse and hamster cell line [28, 32], rats [13, 17], cows [17] and even calf [13]. At this stage of research, it had already been found that methylation within mtDNA, if present, was nonrandom and applies primarily to CpG sites at a frequency of 3-5% [32].

The research published 30 years later showed that the methylation was not only found in the mtDNA, but the pattern and distribution of methylated cytosines are relatively constant across the mtDNA. Particularly noteworthy is the fact that in this study was analyzed methyl-cytosine profiles from 39 different human cell and tissue types [11]. Recent scientific reports with a significant prevalence indicated the presence of methylation within mtDNA [1, 19, 23, 35, 37]. In addition, even if the presence of methylation has been confirmed, the methylation pattern of mtDNA has not been fully understanding yet. In the majority of published studies, which were carried out on human cells (blood cells, skin fibroblasts, SKOV3, C33A), the observed methylation was related to the cytosine within the CpG sites. It was also shown regional differences in methylation levels across the mitochondrial genome [23, 35, 40, 42].

A different look at the methylation pattern of mtDNA was given by the study conducted by *Bellizzi* et al., which not only confirmed the presence of methylated cytosines within D-loop region of mtDNA mouse and human blood or cultured cell, but also found that the majority of them were located outside of CpG dinucleotides [1]. The research carried out two years later by *Blanch* et al. also found non-CpG methylation pattern (CHG and CHH, where H = A, T, or C) in human brain mtDNA [4]. What is more, both studies state unanimously that despite the observed methylation also within CpG sites, this type of pattern represents a small population of mtDNA [1,4]. The non-CpG methylation pattern was observed also in the endothelial human cells. Moreover, in this study was observed comparable methylation level on the L and H strand of mtDNA [3].

Studies, which contradict the presence of methylation within mtDNA are still turned out. *Hong* et al. find no evidence of CpG methylation at a biologically significant level in the analyzed regions. Furthermore, these results were confirmed by bisulfite sequencing and also by next-generation sequencing [14].

Discrepancies in results in the earliest periods of mtDNA methylation studies may relate to technological limitations. However, this argument loses the power in relation to recent discrepant date, because the continuous development of technology enables to use very sensitive and well-developed methods. These various results though may be related to the structure of the analyzed mtDNA, especially in the case of bisulfite pyrosequencing. The research indicated that the circular structure of mtDNA had significantly lower bisulfite conversion efficiency compared with linear mtDNA. Moreover, *Liu* et. al validated the results obtained by pyrosequencing by using an alternative method and showed that the methylation results obtained from linear mtDNA were significantly lower compared to the circular one. This fact may be indicative of overestimated values of methylation in the case of circular mtDNA analysis [19]. Moreover, bisulfite conversion-based methods used by researchers cannot measure the methylation of another nucleotide, like adenine, while the possibility of occurring of this type of methylation in mtDNA cannot be excluded.

THE ROLE OF ENZYMES IN CREATING OF CpG METHYLATION PATTERNS

The mechanisms of mtDNA methylation have still been unknown, but previous reports suggest that DNA methyltransferases (DNMTs) could take responsibility for this action [1, 8, 35, 37]. *Shock* et al. assigned role in the formation of the CpG methylation pattern within mtDNA to isoform 1 of DNMT1. The research, which was carried out on mouse (MEFs) and human cells (HCT116), not only confirmed the presence of DNMT1 in mitochondria but also ruled out the presence of the

other forms i.e. DNMT3a and DNMT3b in this organelle. Because all known today catalytically active DNA methyltransferases (DNMT1, DNMT3a, DNMT3b) are encoded by nuclear DNA, so if one of them is responsible for mtDNA methylations, it must be transported to this organelle. Focusing on isoform 1 of DNMT1, related to the fact that this isoform contains an additional upstream open reading frame (uORF) which encoded for a mitochondrial targeting sequence (MTS). This sequence, located at the N-terminal, is presumably responsible for mitochondrial transport of this nuclear-encoded enzyme. Another reason why the role in the formation of mtDNA methylation has been attributed to methyltransferases is the fact that DNMT1 is bound to mtDNA strand, in a place rich in CpG dinucleotides. Moreover, the intensity of the interaction between mtDNA and DNMT1 is proportional to CpG density [26, 29, 37].

Saini et. al also took a closer look at DNMT1 and their available isoforms. Their study suggests that isoform 3 of DNMT1 instead of isoform 1 localizes to mitochondria and initiates the process of methylation. It turned out that DNMT1-isoform 1, with strong mitochondrial targeting sequence, is located exclusively in the nucleus, not in the mitochondrion. The structure of isoform 1 was studied in detail and discovered area multiple patches of nuclear localization signal sequences (NLS) in its, which probably interfered with the effect of MTS on the location of the enzyme in the mitochondrion, ultimately targeting them to the nucleus. The process of mtDNA methylation and the ability to localize within the mitochondrion was assigned to isoform 3 of DNMT1, the shorter form of DNMT1 devoid of NLS. It was also confirmed that this isoform was bound to the mtDNA [35]. The subsequent studies not only confirmed the presence of DNMT1 in the mitochondria but also, although in much lower levels, the presence of DNMT3b. Whereas DNMT3a has been demonstrated inside the mitochondria of skeletal muscles and NSC-34 cells [1, 5, 41].

In mitochondrial protein, the fraction has also been detected the presence of TET1 (Ten-eleven translocation methylcytosine dioxygenase 1) and TET2 (Ten-eleven translocation methylcytosine dioxygenase 2), the enzymes which would have also a potential role in creating epigenetic modifications within mtDNA[1, 8]. The group of the Ten Eleven Translocation (TET) enzymes is able to modify 5-mC by oxidation them to 5-hmC and as a result, potentially erase ntDNA methylation [6,16,18,39]. The presence of these enzymes within mitochondria may suggest that 5-hmC, which were detected within mtDNA, are formed in the same way. *Dzitoyeva* et. al observed that the increased in *Tet2* (*tet methylcytosine dioxygenase 2*) and *Tet3* (*tet methylcytosine dioxygenase 3*) expression in the cerebellum and in *TET3* in the Purkinje cell layer of these mice, was accompanied by a rise in 5-hmC content in both nDNA and mtDNA. However, in the same study was found a negative correlation between age and 5hmC content in mtDNA, but it was not accompanied by the expected decrease in the expression of TET enzymes in old mice [8,37].

Bellizzi et al. also showed the presence of methylation in the D-loop region of mtDNA in mouse cells deficient for genes responsible for the synthesis of methyltransferases, like *Dnmt1* (*DNA methyltransferase 1*), *Dnmt3a* (*DNA methyltransferase 3 alpha*), *Dnmt3b* (*DNA methyltransferase 3 beta*). This finding may be indicative of co-existing methylation mechanisms independent of these enzymes or suggest the complexity of one mechanism [1].

THE ROLE OF SAM IN THE REGULATION OF mtDNA METHYLATION

It is suggested that the availability of S-adenosyl methionine (SAM) in the cytosol of the cell also plays an important role in the formation of methylation both within ntDNA and mtDNA [15, 24, 31]. SAM is an organic chemical compound, a derivative of adenosine and methionine, and one of the most important substrates in the methylation process in the living body. It is the universal methyl donor for most methyltransferases, that methylation DNA, RNA, histones and another protein [12, 20]. It is transported to the mitochondrial matrix by the S-adenosylmethionine carrier (SAMC), a transport protein localization in the inner membranes of mitochondria, which is encoded by *SLC25A26* (*solute carrier family 25 member 26*) gene. This transport is based on exchange cytosolic SAM for mitochondrial S-adenosylhomocysteine [38]. The relationship between the level of methylation DNA and the level of SAM was observed in Down Syndrome (DS) patients, who suffer from methionine metabolism disorders. The lower level of DNA methylation was correlated with a lower level of SAM in the cytosol [31]. In addition, a similar correlation was observed by *Infantino* V. et al. in the case of mtDNA methylation. The lower level of cytoplasmic SAM was correlated with the lower level of mtDNA, also in DS patients [15]. Moreover, overexpression of *SLC25A26* promotes hypermethylation of mtDNA, by increasing the availability of SAM in mitochondria [24].

THE INFLUENCE OF EPIGENETICS ON mtDNA GENE EXPRESSION AND MITOCHONDRIAL BIOLOGY

GENE EXPRESSION

Regulation of expression of genes encoded by mtDNA is important for the proper functioning of the whole cell metabolism, especially cellular respiratory process. The mitochondrial genome contains only 37 genes, encoding 22 tRNA, 2 rRNA, and 13 respiratory chain polypeptides, which are subunits of enzyme complexes of

the oxidative phosphorylation system genes relevant for the respiratory chain [38]. Some scientific reports indicate the potential influence of mtDNA methylation on dysregulation of gene expression, encoded by mtDNA and ntDNA [35, 37].

A significant reduction in expression of *MT-ND6* (*mitochondrially encoded NADH dehydrogenase 6*) and *MT-ND3* (*mitochondrially encoded NADH dehydrogenase 3*) associated with the increase in mtDNA methylation was observed in human cells (H1299, H116) [30, 35, 37]. In addition, it is suggested that hypermethylation of *MT-ND6* correlated with reduced transcription of this gene and protein expression, and with increased *DNMT1*, may play an important role in the pathogenesis of the metabolic disorders like nonalcoholic fatty liver disease [30]. However, a decreased or no change in the expression with increased methylation was observed in the case of *MT-ATP6* (*mitochondrially encoded ATP synthase 6*) and *MT-CO1* (*mitochondrially encoded cytochrome c oxidase I*) [35, 37]. Interestingly, the expression of *MT-ND1* (*mitochondrially encoded NADH dehydrogenase 1*), the first protein-coding gene on the heavy strand of mtDNA, showed a positive correlation with the increased expression of *DNMT1* [37].

As it turns out, not only methylated cytosine but also 5-hydroksymethylationed cytosine (5hmC) influences gene expression. A similar relationship, but additionally correlated with the age, was noticed by *Dzitoyeva* et al. In this study was shown that increased expression of mtDNA genes like *mt-Nd2* (*NADH dehydrogenase 2, mitochondrial*), *mt-Nd4* (*NADH dehydrogenase 4, mitochondrial*), *mt-Nd4L* (*NADH dehydrogenase 4L, mitochondrial*), *mt-Nd5* (*NADH dehydrogenase 5, mitochondrial*), and *mt-Nd6* (*NADH dehydrogenase 6, mitochondrial*) in the brain from mice, but only in cortex cells, was associated with the decreased content of 5hmC [8].

Epigenetic modifications not only within the encoding sequences can dysregulation of gene expression. Noncoding regions, which are responsible for proper expression, are also important. In the nuclear DNA, the CpG islands occur very densely within the promoter region and the hypermethylation of them is associated with gene silencing [27]. The crucial region for expression of mtDNA genes is the displacement loop (D-loop). It is the only noncoding region within mtDNA and the place of initiation replication and transcription of genes [38]. Interestingly, research proves that the D-loop region is subject to the action of epigenetic modifications. *Bellizzi* et al. detected methylated cytosines within this region in samples extracted from blood and cultured cells of both humans and mice. Furthermore, many of them were located outside of CpG sites [1]. Recently, *Mishra and Kowluru* reported that increased methylation in D-loop negatively regulated mitochondrial gene expression like *MT-CYB* (*mitochondrially encoded cytochrome b*), *MT-CO2* (*mitochondrially encoded cytochrome c oxidase II*), and *MT-ND6* [25]. On the other hand, the decreased methylation of D-loop correlated with the increased expression of the *MT-ND2* (*mitochondrially encoded NADH*

dehydrogenase 2) in the tumor cells. This study suggests that the process of demethylation D-loop region is probably involved in the regulation of *MT-ND2* expression during the initiation and progression of colorectal cancer [9].

A recently published study also demented the assumption that epigenetic modifications, like methylation, affect gene expression. The research was carried out on a human cell line (HCT116, C33A) and showed that methylation did not influence gene expressions like *MT-ND1, MT-ND6, MT-CO1, MT-CYB* (*mitochondrially encoded cytochrome b*)*, MT-RNR1*(*mitochondrially encoded 12S ribosomal RNA*) and *MT-RNR2* (*mitochondrially encoded 16S RNA*) [40].

The diverse influence of methylation on the expression on individual genes within mtDNA may indicate the gene-specific effect of epigenetic modifications within this genome. On the other hand, the different construction of the mtDNA compared to ntDNA could be a reason for this phenomenon. The fact that *MT-ND6* is the only protein-coding gene on the light strand of mitochondrial genome and methylation represses gene expression of this gene, may suggest a high sensitivity of the light strand to influence of methylations on change in transcription. In contrast, increased expression of the *MT-ND1* gene and no change in the expression of *MT-ATP6* and *MT-CO1* with increased methylation of these mtDNA regions may suggest a different mechanism of the effect of methylation on the heavy strand of mtDNA [35, 37].

BIOLOGY

The main function of mitochondria is the generation of energy in the production of ATP molecules during cellular respiration. Moreover, all the protein encoded by mtDNA exhibit enzymatic activity and constitute a part of the respiratory chain. If the mitochondrial biology is subject to epigenetic regulation, one of the best indicators of potential disturbances in mitochondrial function, resulting from disorders in the expression of the mtDNA encoded genes, should be the amount of generated ATP. *Saini* et al. were observed a correlation between the increased expression of *DNMT1- isoform 3* and the decreased ATP production. Moreover, the same report showed overexpression of methyltransferases affected also mitochondrial physiology by reducing mitochondrial membrane potential and mitochondrial activity, at the same time increased mitochondrial mass. It is also observed that the above changes were accompanied by an upregulation in the expression of mitochondrial biogenesis genes encoded by ntDNA, like *PGC1A* (*PPARG coactivator 1 alpha*)*, NRF1 (nuclear respiratory factor 1*)*, NRF2* (*nuclear respiratory factor 2*)*, TFAM* (*transcription factor A, mitochondrial*) and *TFB2M* (*transcription factor B2, mitochondrial*). It could suggest that in this way mitochondrial environment adapts to the changes induced by epigenetic modifications and this mechanism includes changes both in mtDNA and ntDNA [35]. The mitochondrial

dysfunction may also be evidenced by the decreasing number of mitochondria in the cell. It turns out that changes in the amount of mtDNA may be induced by epigenetic modifications. *Zheng* et al. observed a negative correlation between the amount of mtDNA and methylation within the D-loop in the mitochondrial genome [42].

AGE

Another important relationship on the ground of the epigenetic modifications is a potential correlation between methylation of the mitochondrial genome and age. *Mawlood* et al. observed the negative correlation between mtDNA methylation of *MT-RNR1* and age of human. Low methylation values, especially in the elderly, were detected using very sensitive and precise technology, like high throughput sequencing. This correlation was especially accurate for younger and middle-aged individuals. Whereas, after the age of 60, the predictive power was poorer, and it could be a result of individual lifetime metabolic or accumulated during live environmental influences, which cause methylation [23]. In addition, *Dzitoyeva* et al. observed the association between aging and the decreased content of 5-hmC in mtDNA of the mouse brain, and the increased expression of mtDNA encoded genes, like *mt-Nd2, mt-Nd4, mt-Nd4L, mt-Nd5*, and *mt-Nd6* in the cortex, what confirmed the previous report about aging-associated decreased expression of this gene in this part of brain [8, 22].

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