Exploring the Utility of Mitochondrial DNA Copy Number as a Quantitative Biomarker in Health and Disease

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Abstract

Mitochondrial DNA copy number (mtDNA-CN) refers to the total number of mitochondrial DNA molecules within a cell, which varies depending on cell type and environmental factors. This variation provides critical insights into cellular health and function. A reduction in mtDNA-CN can signal changes in the cellular environment, potentially triggered by various factors, including stress or disease. The regulation of mtDNA-CN is governed by mitochondrial replication processes and influenced by factors such as energy demand, nutrient availability, and aging.

Several methods are used to measure mtDNA-CN, including quantitative PCR (qPCR), digital PCR (dPCR), and next-generation sequencing (NGS). These techniques enable accurate quantification, although each has limitations. MtDNA-CN is increasingly recognized as a potential biomarker for various health conditions, including cancer, neurodegenerative diseases, and aging-related disorders. Research indicates that mtDNA-CN could reflect mitochondrial biogenesis, oxidative stress, or cellular aging, making it a valuable tool in disease diagnosis and monitoring.

Despite its potential, challenges remain in standardizing mtDNA-CN measurements and understanding its role in different tissues and conditions. Further research is needed to fully realize its clinical utility and to explore its implications in health and disease.

Keywords: Biomarkers, Cellular health, Mitochondrial DNA copy number, Quantification methods

Introduction

Oxidative phosphorylation (OXPHOS) takes place in the mitochondria, resulting in the production of adenosine triphosphate (ATP) following respiration. This process can cause accumulation of free radicals, resulting in the initiation of membrane lipid peroxidation and the production of human serum malondialdehyde (MDA) from these lipid hydroperoxides, where a standard colorimetric method can be used to quantify serum MDA levels. It has been

reported that systemic MDA levels are increased in diseases such as cardiovascular disease, impaired glucose metabolism, and Hashimoto's Thyroiditis, in addition to declining energy metabolism (lower OXPHOS function). The ability to measure MDA is thus a bio-indicator of either declining health or adaptation to the disease; hence, it is referred to here as a chronic'function' variable to OXPHOS levels [1]. However, the above chronic system response to declining OXPHOS does not give information on OXPHOS activity levels directly at the source, i.e. in human muscle tissue. Mitochondria have their own genetic code, i.e. mitochondrial DNA (mtDNA), which is the sole genetic source of OXPHOS enzyme production in human tissue. This is useful as mtDNA levels are not only highly variable but also have a high heritability of between 42-78% [2]. It is here proposed that mtDNA copy number (cn) in muscle can be used as a 'state' variable, reflecting OXPHOS activity levels at the source/organs of energy metabolism, particularly if analyzed with other complementary biological and physical measures.

Mitochondrial DNA Copy Number: Basics and Regulation

Mitochondrial DNA (mtDNA) copy number is comprised of the total number of mtDNA molecules within a cell and can provide valuable insights into several cellular characteristics. There is no specific "normal" or "mile" range for the copy number as it varies widely and depends mostly on the cell type and environmental factors. A decrease in copy number describes smaller differences in mtDNA, but it is vital to appreciate that these can also be triggered by other factors, such as a change in cellular environment that activates a reduction in mass. Moreover, under certain conditions like cancer in which mtDNA variations are increased, their analysis is very complex [3].

Several cellular processes are responsible for the regulation and homeostasis of mtDNA copy number. Firstly, mtDNA replication initiates at the mitochondrial displacement loop (D-loop), which is rich in replication origins. On average, it transcribes and replicates single-stranded RNA and DNA strands that compete for initiating replication. Secondly, mtDNA replication is affected by a multitude of physical and cellular characteristics. High energy in cells in a hypoxic state will be expended in the generation of ATP, glycine, and fumarate, leading to depletion of the inhibiting nucleosides and the mitochondrial replication restart. Additionally, the electron transport chain of a cell's mitochondria is affected by exogenous and endogenous changing properties like nutrients, environment, and age have affected or can affect mitochondrial copy number [4, 5].

Mitochondrial DNA copy number (mtDNA-CN) is increasingly making headway as a potential marker for several conditions affecting human health and behaviour. This review aimed to illuminate the knowledge surrounding mtDNA-CN in normal and diseased states in humans.

Methods for Measuring Mitochondrial DNA Copy Number

The quantitative assessment of mtDNA copy number holds significant importance in understanding various physiological and pathological processes. The measurement of mtDNA copy number is essential for investigating cellular responses, disease progression, and therapeutic interventions. There are a variety of techniques and strategies to quantify mitochondrial DNA copy number as a quantitative biomarker, and it is important to understand the ways to perform such measurements in order to interpret the evidence. The limitation of a given method needs to be considered in the execution and results interpretation, to assess correctly mitochondrial DNA copy number's role in biological and pathological processes.

quantitative PCR (qPCR): qPCR has now become the most widely used technique for determination of mitochondrial DNA copy number because it is rapid, reliable, easy to perform, and generally less time-consuming compared with other methods. More importantly, qPCR allows to quantify the mitochondrial DNA copy number identified as the ratio of two DNA molecule distributions (mitochondrial DNA and nuclear DNA) using calibration curves. qPCR standard curve should be generated using a serial dilution of human mitochondria and nuclear DNA for estimating the absolute amount of gene of interest [6]. However, qPCR's reliance on standard curves can introduce variability, and it may not distinguish between different mtDNA populations.

Digital PCR (dPCR) has emerged as a robust technique for quantifying mitochondrial DNA (mtDNA) due to its unique attributes. Unlike traditional PCR, dPCR partitions the sample into thousands of individual reactions, allowing for absolute quantification of DNA molecules.

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The utilization of EvaGreen in dPCR experiments has been shown to reliably quantitate mtDNA copy number by directly using cell lysates, eliminating the need for a nuclear reference gene and reducing compounded errors. This method has demonstrated the ability to detect subtle changes in mtDNA levels, making it particularly relevant for analyzing differences in mtDNA content between cell states and pathological conditions.

The dPCR method's performance surpasses many prevalent quantitative methods, making it an affordable and powerful tool for quantifying genetic material and serving as a non-invasive biomarker for various diseases.

Next-generation sequencing (NGS) has revolutionized the analysis of mitochondrial DNA (mtDNA) copy number by enabling highly sensitive detection of low-level mtDNA variants. To ensure accurate variant calling and avoid false positives induced by nuclear mitochondrial DNA sequences (numts), it is crucial to enrich the DNA sample for mtDNA. One commonly used method for mtDNA enrichment is the differential centrifugation (DC) technique, which allows for abundant mitochondrial isolates. However, the harsh nature of high-speed centrifugation in DC can lead to disruptions and potential nuclear DNA contamination. An alternative approach involves the use of magnetic bead isolation, which yields whole mitochondria with intact membrane machinery and reduces contamination compared to DC. Additionally, amplification of the mitochondrial genome using long-range PCR with one to two primer pairs is frequently employed for mtDNA sequence enrichment.

Furthermore, next-generation sequencing (NGS) offers the advantage of absolute quantification, which provides a simple method to calibrate optical measures of mtDNA copy number in single cells, as demonstrated by the mCN assay. This precise quantification capability enhances the accuracy of mtDNA copy number analysis and expands the potential applications of NGS in this context.

Mitochondrial DNA Copy Number in Health and Disease

Mitochondrial DNA (mtDNA) copy number (mtDNAcn) has emerged as a robust quantitative biomarker, and normative reference range values are becoming established. Expression

values and various parameters of inflammation either do not or only account for a fraction of the variance in mtDNAcn. While an appreciable fraction of the between-subject variance in some somatic tissues, such as whole blood mtDNAcn, is the result of random variation across cell types in a population, somatic tissue mtDNAcn estimations are generally reproducible for an individual over time in nonpathological states. Additionally, some mtDNAcn alterations have been consistently found to be characteristics of a cell or tissue [7]. Thus, tissue mtDNAcn can be measured in some cases to estimate the relative latitudinal (and/or chronological) age of a tissue.

Along with age, mtDNAcn has been linked to several human health outcomes and is a subject of active research in many diseases. In some cases, there is compelling evidence for a link between mtDNAcn and neurodegenerative diseases, cancer, and possibly other diseases associated with inflammation and metabolism. A potential utility of mtDNAcn is as an intermediate in knowledge discovery and validation of diseases suspected to have an inflammatory or metabolic component. Associations with cancer have generated considerable interest. Of particular interest concerning these articles is data indicating that mtDNAcn can be quite dynamic in whole tissues, or at least in blood. This is consistent with experimental data generated in cell culture and transgenic animals demonstrating that mtDNAcn changes in response to experimental treatments. In tissues, more cells may be obsolescent than in particular cell types, suggesting that both mtDNAcn and the utility of association analyses based on mtDNAcn may vary by tissue [8].

Mitochondrial DNA Copy Number in Aging

In the last 5 years, there has been a substantial number of publications that attempt to understand this parameter in a diverse range of tissues, in health and disease, and at different life stages. The work can be broadly categorized into that which is concerned with events of aging, its relatedness to any changes in commensal or adaptive mitochondrial biogenesis, and, if it is also altered with membrane potential, whether changes might contribute or result from the aging or disease process. Alternatively, some papers have approached the same subject concerned with falling solely under the aging process, representing a biologically predetermined mitochondria-to-cell number, the cause of which has not always been attributed to a functional or adaptive basis [9].

The published progression/correlation of mtDNA copy number with age has been in relation to either one or a combination of such factors as a surrogate marker of mitochondrial number, a reflection of mitochondrial biogenesis, results of oxidative stress, possible autophagy or mitophagy, or an adaptation to a decline in membrane potential. The observation of interindividual variation in mtDNA copy number has led to their determination in a variety of easily obtainable or accessible tissues, to identify their relationship with age and the variance of such numbers in the same samples or tissues from patients with diseases known to be either associated with pregnancy or preceding the development of aging/disease [10]. Further studies have also used mtDNA copy number to investigate tissue- or gender-associated differences in blood samples. However, the increasing amount of work now published means that it is also timely to question their utility - why of relevance? And what do they tell us about our biology?

Mitochondrial DNA Copy Number in Neurodegenerative Diseases

Research into the involvement of mtDNA copy number in neurodegenerative diseases (ND) is recent, but the mitochondrial theory of aging has implicated mtDNA mutation and content

status in age-related ND. This accumulation of damage to mtDNA can lead to a reduction in mtDNA copy number and increase the susceptibility of cells and tissues to undergo cell death. This association has only recently been considered an avenue of research to explore whether mtDNA copy number alone or as part of a ratio could be used as a marker of the pathophysiological mechanism of NDs [11]. Numerous studies demonstrate reductions in mtDNA copy number in common ND pathologies, behaviours, or symptomology.

Among Alzheimer's disease, the majority of studies report decreases in mtDNA copy number, even using different tissue samples. There are, however, exceptions to this finding. Within the blood, reports are mixed with elevations also being reported. In other NDs, reductions in mtDNA copy number are observed within HD and PD pathologies. Within the blood, the reduction is more consistent and found in PD and ALS. The multitude of reports across the interconnected NDs propel further inquiry to understand the potential of mtDNA copy number as a useful marker of metabolic activity during ND [3]. The explorative nature of these studies also implies that mtDNA copy number should be used as a quantitative measure aid for other well-established markers, even when no differences in ND are reported.

Mitochondrial DNA Copy Number in Cancer

An increasing number of studies have reported that mitochondrial DNA copy number is more likely to increase in cancer tissues and that it is frequently increased in many types of cancer. It has been shown that higher levels of mitochondrial DNA copy number are present in solid tumors compared to corresponding non-tumor tissues. Compared to healthy controls, cancer patients have a higher mtDNA copy number in peripheral blood leukocytes, subsequently conferring to be an important predictor for cancer diagnosis. However, increased levels of mtDNA copy number have also been associated with a poorer outcome [12]. These variations in the mode of regulation of mitochondrial DNA content across different cancer types strongly suggest the relevance of mitochondrial DNA. Additionally, recent evidence shows that mtDNA copy number levels are affected by inherited polymorphisms.

In conclusion, although mitochondrial DNA copy number measurements highlight both the direct influence and contribution of mitochondrial content in cancer, the value of mtDNA copy number likely goes beyond only cancer. Mitochondrial DNA copy number can be easily assessed using easy-to-use biological materials, including circulating cell-free DNA, resulting in its promising role as a biomarker of disease and therapy response, supporting the strong interest and potential role in oncology for the future. More investigations are required to standardize mtDNA copy number analysis and interpretation in the clinic.

Challenges and Limitations in Using Mitochondrial DNA Copy Number as a Biomarker

Measuring variation in mitochondrial DNA (mtDNA) copy number has potential utility as a representational or quantitative biomarker. If mtDNA copy number can serve as a quantitative indicator of mitochondrial abundance or incorporation of mtDNA into the nuclear DNA of actively copying cells, mitochondrial parameters should be related to it. There are two reasons to be cautious of this association, and to consider alternative means to define mtDNA depletion in tissue: (1) diseased and control tissue can have different amounts of cellular heterogeneity in the distributions of mitochondrial and nuclear genes per cell; and (2) tested clinical phenotypes and sequences, all mRNA collected post-mortem from human middle frontal cortex, differ

Furthermore, inherent and induced heterogeneity among human tissues in mitochondrial and nuclear gene copy number per cell will not give identical relative levels of mtDNA and nuclear DNA with mitochondrial deletions (and without experimental depletion), and thus make mtDNA copy number of limited interest as a quantitative biomarker. Although mtDNA copy number is described in the human research literature as a measure of cellular and biological function, it does not rise to the rank of an established qualitative biomarker to track mitochondrial proliferation during muscle aging, disease, or adaptive remodeling, let alone a quantitative biomarker to match numbers against the severity of disease manifestation. A mathematical model can help differentiate whether a difference in the published results reflects a solution to differences of tissue or tissue pathologies, as opposed to differences in the emphasis given to one out of four alternative ways of estimating the copy number ratio. To sum up, while a pure- or skeletal muscle-derived mtDNA copy probably will not be useful as an ameliorative outcome biomarker from the research perspective, an mtDNA copy number may supplement measures of the rate of injury in a clinical context.

Conclusion

Mitochondrial DNA copy number has primarily been utilized as an outcome measure in human studies that are exploring mitochondrial biogenesis, life course epidemiology, aging, cell death, mitochondrial and nuclear DNA damage, or the association with one of a selection of common diseases and conditions. The evidence suggests that many of these will be relevant and that there is potential in this field of research, but current methodology and design in association with this fairly new field require substantial further work to achieve routine clinical use. There is a growing body of genetic studies in this area, which is mainly based on common variation and has not identified any single-nucleotide polymorphism that, aside from TFAM, is associated with levels of mitochondrial DNA copy number of the nuclear genome. No longitudinal studies in terms of genetic factors have been done, but it is clear that the association with mitochondrial DNA copy number is complex and is likely to contain many loci for differing physiological processes.

Environmental factors that are also thought to influence the copy number include drugs that cause mitochondrial toxicity, and heavy metals, especially lead. Replication of the genetics associations and some gene-environment causal inference fields in humans will result in a genetics-environment field and improve our understanding of the mitochondrial genome and the copy number in health and disease. The conclusion on utility is that copy number has potential, but the research is currently purely hypothesis generating. If health can be improved by maintaining the copy number, then health interventions to achieve the desired level could be imagined. In some diseases, the research into copy numbers is at the departure stage to health. In other areas, the research is certainly already of interest in terms of disease, particularly to tumors. That change in copy number or the absolute level reports on the level of damage in terms of apoptosis, or that an altered absolute level of copy number compared to the rest of the population might be a risk factor in cancer. The difference in the absolute level of copy number does, by the consensus of the field, not cause cancer but appears to be of interest as a field sensor.

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