Mitochondrial DNA copy number alterations: Key players in the complexity of glioblastoma (Review)

ABDUL AZIZ MOHAMED YUSOFF, SITI ZULAIKHA NASHWA MOHD KHAIR and SITI MUSLIHAH ABD RADZAK

Department of Neurosciences, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan 16150, Malaysia

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Abstract. Renowned as a highly invasive and lethal tumor derived from neural stem cells in the central nervous system, glioblastoma (GBM) exhibits substantial histopathological variation and genomic complexity, which drive its rapid progression and therapeutic resistance. Alterations in mitochondrial DNA (mtDNA) copy number (CN) serve a crucial role in GBM development and progression, affecting various aspects of tumor biology, including energy production, oxidative stress regulation and cellular adaptability. Fluctuations in mtDNA levels, whether elevated or diminished, can impair mitochondrial function, potentially disrupting oxidative phosphorylation and amplifying reactive oxygen species generation, thereby fueling tumor growth and influencing treatment responses. Understanding the mechanisms of mtDNA-CN variations, and their interplay with genetic and environmental elements in the tumor microenvironment, is essential for advancing diagnostic and therapeutic strategies. Targeting mtDNA alterations could strengthen treatment efficacy, mitigate resistance and ultimately enhance the prognosis of patients with this aggressive brain tumor. The present review summarizes the existing literature on mtDNA alterations, specifically emphasizing variations in mtDNA-CN and their association with GBM by surveying articles published between 1996 and 2024, sourced from databases such as Scopus, PubMed and Google Scholar. In addition, the review provides a brief overview of mitochondrial genome architecture, knowledge regarding the regulation of mtDNA integrity and CN, and how mitochondria significantly impact GBM tumorigenesis. This review further presents information on therapeutic approaches for restoring mtDNA-CN that contribute to optimized mitochondrial function and improved health outcomes.

Correspondence to: Dr Abdul Aziz Mohamed Yusoff, Department of Neurosciences, School of Medical Sciences, Universiti Sains Malaysia, Health Campus, Raja Perempuan Zainab II Road, Kubang Kerian, Kelantan 16150, Malaysia
E-mail: azizmdy@yahoo.com

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1. Introduction

Cellular metabolic alterations represent a well-known aspect of cancer, which have been linked to controlling the invasion and spread of cancer cells (1,2). Glioblastoma (GBM), which is characterized by high invasiveness and metabolic flexibility, demonstrates this connection. Known as the most aggressive type of primary brain tumor in adults, GBM rapidly progresses, infiltrates nearby healthy brain tissue and is associated with a poor prognosis (3).

The recurrence of GBM, driven by its invasive behavior, typically occurs in close proximity to the initial tumor location, ultimately resulting in the death of almost all patients, which is globally reflected in the fact that >200,000 patients die from this condition each year (3). The invasiveness of GBM poses a significant challenge to its treatment, as the tumor cells infiltrating healthy brain tissue evade surgical removal and show limited responsiveness to existing therapies (4,5). Enhancing the understanding of how invasion and metabolism function, along with their connections, in the progression, maintenance and dissemination of tumors, may result in the identification of innovative therapies that can ultimately prolong patient survival.

Previous research has highlighted that mitochondria, fundamental organelles present in the majority of eukaryotic cells, are integral to cellular metabolism, energy generation and communication pathways, with implications extending

to disease progression, including cancer (6,7). Various alterations in mitochondrial function have been detected in GBM, encompassing structural and functional modifications that influence mitogenic, hemodynamic, bioenergetic and apoptotic signaling, suggesting that the oxidative phosphorylation (OXPHOS) system and energy coupling in glioma cells may operate with diminished efficiency (8).

Alterations in mitochondrial DNA (mtDNA) copy number (CN) have an important role in tumorigenesis, including in GBM, as they influence the quantity of mitochondrial genome copies within cells, potentially impacting cellular metabolism, energy production and overall mitochondrial function (9,10). Enhanced mitochondrial biogenesis and metabolic flexibility are associated with an increased mtDNA-CN, supporting the proliferation and survival of tumor cells (11). Conversely, a reduced mtDNA-CN leads to mitochondrial dysfunction, altered bioenergetics and increased resistance to apoptosis (7,12). These mtDNA-CN alterations are associated with clinical outcomes, including tumor aggressiveness, therapy response and patient prognosis (13,14). Understanding these changes may provide insights into mitochondrial dynamics in GBM and could suggest potential therapeutic strategies that target mitochondrial function. Therefore, comprehending the significance of mtDNA-CN alterations in the development of GBM carcinogenesis is crucial. To address this, the present article reviewed these occurrences, with a particular focus on variations in mtDNA-CN and their association with GBM, based on articles published between 1996 and 2024 from databases such as Scopus, PubMed and Google Scholar.

2. GBM

Derived from glial cells in the brain and spinal cord, gliomas are the most prevalent type of primary tumors in the central nervous system, varying in aggressiveness and posing significant global public health risks due to their high mortality rate, impact on patient quality of life and association with reduced life expectancy (15). Gliomas, which are classified based on their histological origin into types such as ependymoma, oligodendroglioma, astrocytoma, GBM or mixed glioma, have been reported to account for 26.3% of all tumors, which refers to a global figure, and have been graded by the World Health Organization (WHO) from grade 1, the least aggressive, to grade 4, the most aggressive, reflecting their degree of cellular alteration and malignancy (16).

Astrocytomas, originating from astrocytic cells, are the most common type of glioma in adults. The WHO classifies astrocytomas into three grades: i) Diffuse astrocytoma (WHO grade 2) is characterized by diffuse infiltration, high cellularity and atypia, but lacks significant endothelial proliferation, mitoses or necrosis; ii) anaplastic astrocytoma (WHO grade 3) exhibits increased cellularity, more pronounced nuclear atypia, hyperchromasia and mitotic activity, but shows no notable endothelial proliferation or necrosis; iii) GBM (WHO grade 4) is highly aggressive with dense cellularity, pleomorphism, mitotic activity and microvascular proliferation or necrosis, often invading adjacent brain areas but rarely spreading beyond the brain (17,18).

GBM is not only one of the most severe malignancies but also the most prevalent malignant primary tumor of the brain and central nervous system, constituting 14.5% of all central nervous system tumors and 48.6% of malignant central nervous system tumors, with a notably short median overall survival time of 15 months (19). Global research conducted by Ostrom *et al* (20) revealed that GBM has an incidence rate of <10 cases per 100,000 people worldwide, with a rising trend over the last decade. According to projections by the American Cancer Society, 25,400 individuals in the United States are expected to be diagnosed with malignant brain and spinal cord tumors in 2024, leading to 18,760 deaths from this condition (21).

Distinguished by several defining characteristics, this aggressive tumor features unique histological traits, rapid cellular proliferation and a notable capacity to invade surrounding healthy brain tissue, while also demonstrating pronounced angiogenesis that enhances blood supply and supports tumor growth (22). The high likelihood of recurrence, combined with an inherent resistance to apoptosis, complicates treatment efforts and verifies the status of GBM as one of the most treatment-resistant types of cancer, ultimately leading to a particularly unfavorable prognosis for affected patients, with typical survival rates of <2 years (5,23).

At present, the primary treatment modalities for GBM include surgery, chemotherapy and radiotherapy; however, this tumor type is considered particularly challenging to treat, as it exhibits resistance to conventional chemotherapy, is frequently identified at advanced stages and predominantly displays invasive growth, which results in partial tumor removal through surgery (24). The inadequate effectiveness of existing glioma treatments is associated with several critical factors, including modifications in intricate signaling pathways that are essential for cellular communication, the significance of the blood-brain barrier, which restricts the delivery of therapeutic agents, and the persistence of stem-like cells within GBM that contribute to tumor resilience and recurrence (25).

Historically, GBM had been classified into primary and secondary types based on its clinical presentation (26). Primary GBM has been established to account for ~90% of GBM cases, emerging *de novo* in patients without a preceding history of brain tumors and being more prevalent among older adults, characterized by specific molecular alterations, such as amplification of the epidermal growth factor receptor (EGFR) gene and loss of the tumor suppressor gene phosphatase and tensin homolog (PTEN) (26). By contrast, secondary GBM constitutes ~10% of cases, tends to occur in younger patients and develops from lower-grade tumors, exhibiting a more favorable prognosis and often harboring mutations in the isocitrate dehydrogenase (IDH)1 and tumor protein 53 (TP53) genes (26). In the 2016 update of the WHO classification system for CNS tumors, experts formally classified GBM based on the presence or absence of IDH gene mutations (either IDH1 or IDH2), acknowledging that IDH-mutant GBM, closely aligned with secondary GBM, and IDH-wild-type GBM, defined as primary or *de novo*, differ fundamentally in tumorigenesis, biological progression and therapeutic outcomes (27). The classification of GBM has been further updated in the fifth edition of the WHO classification of CNS tumors, released in 2021 to include more molecular features, such as EGFR

gene amplification, telomerase reverse transcriptase (TERT) promoter mutations, and the simultaneous gain of chromosome 7 along with the loss of chromosome 10 (+7/-10), all of which meet the criteria for diagnosing IDH-wild-type GBM (28). Additionally, the 2016 classification of secondary GBM was revised to astrocytoma, IDH-mutant, central nervous system WHO grade 4, marked by mutations in IDH1 or IDH2, along with ATRX and TP53 gene alterations, and CDKN2A/B homozygous deletions (28).

A hallmark trait of GBM is its marked molecular and cellular heterogeneity, which includes a variety of genetic alterations, epigenetic modifications and cellular phenotypes (29). This heterogeneity is widely recognized as a significant factor contributing to the high malignancy and likelihood of recurrence of GBM, creating considerable challenges in the development of effective treatment strategies. Traditionally, studies on tumor initiation and progression have primarily focused on nuclear genetic changes, with GBM serving as a key example where these alterations have been thoroughly documented (8,9,29).

The genetic alterations in GBM are typically defined by three fundamental biological mechanisms associated with triggering tumor growth, circumventing cellular aging and facilitating sustained proliferation, and flaws in each mechanism appear vital for glioma tumorigenesis through essential signaling pathways. Three core signaling pathways, the activation of the receptor tyrosine kinase/Ras/PI3K pathway, the inhibition of the p53 pathway, and the disruption of the retinoblastoma protein pathway, are commonly dysregulated in GBM (30). The primary genetic alterations recognized in GBM thus far encompass TP53 mutations, IDH1 mutations, alterations in the promoter region of the TERT gene, ATRX mutations, deletions of the PTEN tumor suppressor gene, O6-methylguanine DNA methyltransferase promoter methylation, and both the amplification and overexpression of EGFR, all of which have demonstrated promise in forecasting survival outcomes and treatment responses in patients with GBM (31).

A previous study revealed that the TP53 mutation spectrum in IDH-mutant astrocytoma, as opposed to IDH-wild-type astrocytoma, is primarily driven by a single enriched hotspot mutation, R273C, which is associated with poor outcomes, especially in male patients, in spite of histologic and transcriptomic findings indicating lower proliferation (32). The unique IDH1 R132H mutation has been identified as a robust prognostic and predictive biomarker linked to improved clinical outcomes for patients with glioma, as those diagnosed with secondary GBM harboring this mutation exhibit a more favorable prognosis compared with patients with primary GBM possessing the wild-type IDH1 gene (33). Additional significant genetic alterations noted in GBM comprise CDKN2A/B, H3F3A, NF1, PDGFRA and PIK3CA (34).

Despite significant efforts to clarify the etiology of GBM and advancements in molecular assessment, considerable uncertainty persists regarding the specific mechanisms underlying its tumorigenesis, while the identification of effective therapies remains challenging, yielding limited improvements in survival rates. The intricate genomic changes observed in GBM cells have led researchers to redirect their attention toward another genome; beyond the nuclear genome, the mitochondrial genome also warrants investigation. Exploring alterations in the mitochondrial genome has created new possibilities for targeted therapies in GBM.

3. Mitochondrial genome: Heredity, organization and preservation

Mitochondria are essential organelles within mammalian cells, responsible for generating energy via adenosine triphosphate (ATP) through the Krebs cycle and OXPHOS (35). They also regulate key physiological processes, such as apoptosis, β-oxidation of fatty acids, iron-sulfur cluster biogenesis, and maintenance of calcium and redox homeostasis (36,37). Mitochondria-derived reactive oxygen species (ROS), byproducts of OXPHOS, are implicated in several conditions, including neurodegenerative disorders, diabetes, cancer and the aging process (38).

Mitochondria house their own genetic material, mtDNA, a circular double-stranded DNA (dsDNA) structure of 16,569 base pairs that lacks introns (39). Unlike nuclear DNA, which exists in two copies per cell, mtDNA is present in numerous copies per cell, varying based on tissue-specific energy requirements. MtDNA is encapsulated in nucleoids, which are organized nucleoprotein complexes, rather than existing as a naked molecule such as bacterial chromosomes (40). Super-resolution microscopy has shown that most nucleoids contain only a single mtDNA molecule, indicating that mitochondria operate autonomously (41,42).

Various proteins interacting with mtDNA nucleoids have been identified using advanced techniques, such as immunoprecipitation and biotinylation (43,44). One key protein, mitochondrial transcription factor A (TFAM), serves an essential role in both packaging and transcribing mtDNA (45). TFAM completely coats mtDNA and induces bends at specific locations, facilitating the regulation of mitochondrial transcription and replication (46-48).

Throughout evolution, most mitochondrial genes were either lost or transferred to nuclear DNA; however, mtDNA has retained 37 genes. These include genes encoding two ribosomal RNAs, 22 transfer RNAs and 13 proteins (ND1-ND6, ND4L, cytochrome *b*, COI-COIII, ATPase-6 and ATPase-8), which are integral components of the electron transport chain (ETC) in the OXPHOS complexes (49). The majority of mitochondrial proteins are encoded by nuclear DNA, translated in the cytoplasm, and imported into the mitochondria, serving crucial roles in mitochondrial biogenesis and function (50).

Human mtDNA consists of light (L) and heavy (H) strands with two non-coding regions (NCRs). The displacement loop (D-loop), spanning 16,024 to 576, contains elements essential for replication and transcription (10). Another NCRs, including the L-strand origin of replication (OriL), consists of 30 nucleotides. Within the NCRs, the L-strand promoter (LSP) and H-strand promoter (HSP) are located 150 bp apart (51). HSP drives transcription of 12 mRNAs, 2 rRNAs and 22 tRNAs, whereas LSP regulates ND6 and 8 tRNAs in the opposite direction (52). These details are illustrated in Fig. 1.

The existence of a second HSP (HSP2) downstream of HSP was suggested based on early guanylyltransferase capping studies (53); however, its functionality has been questioned due to discrepancies in its transcription start site between *in vivo* and *in vitro* studies, as well as the lack of TFAM requirement

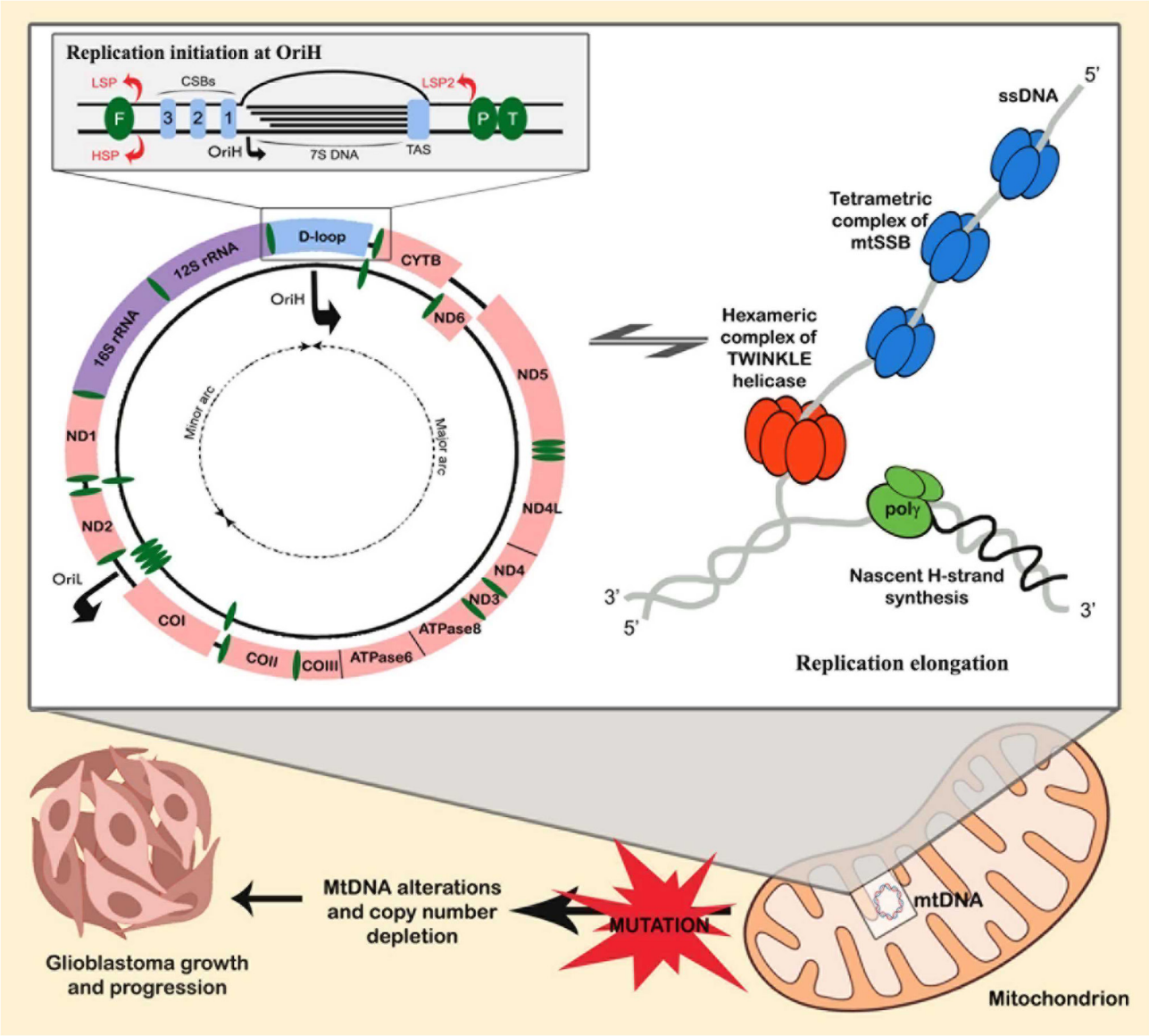

Figure 1. mtDNA replication mechanisms and copy number variations in GBM. The mtDNA-encoded genes are depicted as separate strands on the H-strand and L-strand, with the replication origins (OriH and OriL) marked accordingly. A close-up view of the D-loop shows replication initiation at OriH, highlighting all essential components: The HSP, LSP and LSP2; CSBs 1, 2 and 3; 7S DNA; and TAS. The 5' end aligns with the OriH region, while the 3' end is located at the TAS. It is postulated that mtDNA mutations from endogenous damage may impair the regulation of mitochondrial replication elongation, potentially leading to further mtDNA alterations and depletion of mtDNA-copy number. This disruption could impact overall mtDNA biogenesis, potentially promoting GBM growth and progression. GBM, glioblastoma; OriH, heavy-strand origin of replication; OriL, light-strand origin of replication; HSP, heavy-strand promoter; LSP, light-strand promoter; CSBs, conserved sequence blocks; TAS, termination-associated sequence; mtDNA, mitochondrial DNA; mtSSB, mitochondrial single-stranded binding protein; polγ, polymerase γ.

during initiation (54,55). Recent cappable-seq data have provided no evidence for transcription initiation at the HSP2 site (56,57). Additionally, a new LSP, LSP2, has been identified opposite LSP in the NCRs, suggesting a potential role in transcribing mtDNA genes (57). Further studies are required to confirm the involvement of LSP2 in mtDNA replication and transcription.

Transcription in mitochondria follows the polycistronic model, with long primary transcripts produced from the LSP and HSP. These transcripts are processed by mitochondrial RNase P and RNase Z, leading to the release of individual mRNAs, tRNAs and rRNAs necessary for protein synthesis (52,58). The mitochondrial RNA polymerase (POLRMT), along with TFAM and TFB2M, is critical for initiating transcription. TFAM binds to the promoter regions, facilitating POLRMT recruitment, while TFB2M aids in unwinding the promoter DNA to initiate transcription (59). The transcription elongation factor TEFM supports POLRMT during transcription, enhancing the production of long RNA transcripts.

Human mtDNA replication proceeds continuously and independently of the cell cycle, which is crucial for maintaining a high mtDNA-CN per cell (60). While the replication process has been extensively studied (55,61,62) it remains incompletely understood and relies on two distinct canonical replication origins, the H-strand origin of replication (OriH) and the OriL, which are oriented oppositely. OriH resides in the NCR, downstream of the LSP, whereas OriL is positioned

amidst a cluster of five tRNA genes, situated around two-thirds of the genome's distance (~10 kb downstream of OriH) (63).

The minimal essential proteins necessary for mtDNA synthesis include the heterotrimeric DNA polymerase γ (POLG), comprising POLGA and POLGB subunits, the hexameric helicase, TWINKLE, and the mitochondrial single-stranded DNA (ssDNA)-binding protein, mtSSB (64,65). During replication of the mtDNA leading strand, dsDNA unwinding occurs in a 5' to 3' direction by TWINKLE. mtSSB binds to protect the ssDNA, thereby enhancing TWINKLE-induced dsDNA unwinding and POLG-mediated DNA synthesis, as shown in Fig. 1. POLRMT is responsible for producing the RNA primers needed to initiate synthesis on both DNA strands (64). Replication starts at OriH, where POLRMT initiates transcription from the LSP, ending at conserved sequence blocks that form R-loops essential for initiation (59). RNase H1 processes the 3' ends of the R-loop, providing primer sites for POLG to begin mtDNA synthesis (66). At OriL, POLRMT generates an RNA primer from a poly(T) stretch, forming a stem-loop structure that enables lagging strand replication (67,68). This process ensures proper mtDNA replication, with replication from OriH displacing the OriL region to begin lagging-strand synthesis (65,66).

4. Regulation of mtDNA integrity and CN

In humans, the number of mtDNA copies per cell varies from 100 to 1,000, depending on the cell type, aligning with tissue-specific metabolic demands. Tissues with higher energy requirements typically exhibit a higher mtDNA-CN (69). For example, the heart contains 2,000-5,000 copies per nucleus, skeletal muscles have 1,000-3,000 copies, the liver ranges from 500 to 1,000 copies, and blood leukocytes typically have between 150 and 600 copies (69). By contrast, mammalian erythrocytes lack mtDNA, sperm have ~5 copies per cell and oocytes may contain >500,000 copies (70). Researchers have proposed that the mtDNA-CN per cell serves as an indicator of mitochondrial health.

Notably, the normal mtDNA-CN in a particular tissue is not constant and can vary considerably, with numerous studies (71-73) indicating that, in apparently healthy individuals, mtDNA-CN can fluctuate between 2 to 10 times the standard value, and mtDNA content ranging from 40 to 150% of the average is considered within normal clinical parameters (74).

By exposing cells to intercalating agents, such as ethidium bromide or the POLG inhibitor dideoxycytidine, mtDNA replication is inhibited, allowing researchers to achieve decreased mtDNA-CN in laboratory experiments; however, certain cells, such as cancer cells, may inherently exhibit resistance to these treatments or acquire resistance during therapy, resulting in mtDNA-CN recovery (75). Traditionally, scientists quantify mtDNA levels in samples by using quantitative PCR (qPCR) to count nuclear DNA and mtDNA gene copies, with droplet digital PCR recognized as an advanced method for determining mtDNA content, each method having resolution thresholds of 50-60% and 30%, respectively (70,76).

The role of specific proteins in regulating mtDNA-CN has been debated, with TFAM being particularly scrutinized. TFAM not only serves a critical role in packaging mtDNA into nucleoids but also acts as a significant controller of mtDNA-CN, supported by direct associations observed in diverse genetic models, both *in vitro* and *in vivo* (77-79). Therefore, researchers have hypothesized that the balanced levels of TFAM and mtDNA observed in certain studies (78-80) may result from their mutual stabilization within mitochondrial nucleoids, contributing to a model that explains the role of TFAM in mitochondrial biogenesis. Moreover, the regulation of mtDNA biogenesis is believed to be indirectly affected by Lon protease, as it targets TFAM for degradation (81). Findings from a previous study indicated that a moderate rise in TFAM levels can result in an increased mtDNA-CN, with no impact observed on mitochondrial gene expression in a BAC-TFAM transgenic mouse model (80). By contrast, another study observed that knockdown of TFAM in 293 cells could result in a significant decrease in mtDNA-CN and aggregation of mtDNA nucleoids (82). Despite the evidence supporting the strong positive association between TFAM expression and mtDNA-CN, there is considerable conflicting data. Previously, Matsuda *et al* (83) illustrated that an overabundance of TFAM reduced lifespan in *Drosophila*, although it had no impact on the mtDNA-CN or transcriptional activity. In another experiment using human cultured cells, researchers consistently indicated that transiently boosting TFAM expression may be adequate to enhance mtDNA transcription but does not result in an increase in mtDNA-CN (84). Furthermore, in a patient with myoclonic epilepsy with ragged red fibers, a study revealed that mtDNA-CNs were increased by 3-7 fold in the predominantly affected brain areas (hippocampus, cortex and putamen) as well as in skeletal muscle, whereas TFAM levels remained low (85). Kozhukhar and Alexeyev (86) found conflicting evidence regarding TFAM expression compared with mtDNA-CN, mitochondrial transcription and mitochondrial mass. They found that TFAM expression does not consistently correlate with mtDNA-CN or the expression of mtDNA-encoded proteins across various experimental systems. Therefore, it is recommended to cautiously validate the use of TFAM as a marker of mitochondrial biogenesis (86). Research has indicated that in certain mtDNA-deficient cells, TFAM expression is lower than in parental cells with mtDNA, and TFAM release from mtDNA complexes is facilitated by Lon-mediated degradation (87). These findings imply a necessary proportional balance between TFAM and mtDNA. Nevertheless, findings from another study indicated that in a tissue-specific knockout of POLRMT, TFAM expression remains consistent despite a significant reduction in mtDNA-CN (88). TFAM in this scenario remains detached from mtDNA and is unaffected by Lon-mediated degradation, indicating that the stoichiometric balance between TFAM and mtDNA may not be universally applicable.

Findings regarding other proteins have also yielded inconclusive results. Studies have documented that mutations in mitofusin 2 are linked to varying levels of mtDNA content, both higher (89) and lower levels (90), in the skeletal muscle of affected patients.

In a recent meta-analysis, researchers identified two nuclear gene regions near HBS1L/MYB and within the GSDMA gene that were associated with mtDNA-CN across all participants, as well as two loci in sex-specific analyses (72). The study also detected one rare mitochondrial variant [MT:9548_A:

corresponds to the cytochrome c oxidase subunit III (MT-CO3)] linked to mtDNA-CN. These findings imply that genetic variants in the mitochondrial genome likely have limited influence on regulating mtDNA-CN, given the rarity of the associated mitochondrial variant (72).

Researchers have extensively documented alterations in mtDNA content in the tissues of elderly individuals (91,92), yet the direction of these changes remains contentious and cannot be directly linked to any specific protein. The Leiden Longevity Study, with 2,734 participants, discovered that mtDNA content declines with age, and low mtDNA content was shown to be associated with familial longevity, indicating that long-lived families prioritize preserving mitochondrial function over boosting mitochondrial biogenesis (91). Moreover, research has indicated that decreased mtDNA-CN in older individuals is linked to a gradual decline in both cognitive and physical functions, along with an elevated risk of mortality (92). In a recent study, lower levels of mtDNA-CN were revealed to significantly reduce cumulative survival rates, revealing a particularly higher mortality risk in middle-aged individuals with low mtDNA-CN levels, underscoring the association between leukocyte mtDNA-CN and future mortality risk (93).

5. Role of mitochondria in governing GBM tumorigenesis

Comprehensive research has focused on the importance of mitochondria in GBM, particularly concerning their genomic contributions to the advancement of cancer, with increasing evidence indicating that these organelles experience genetic modifications, functional disturbances and metabolic reprogramming within the context of this aggressive malignancy, highlighting their essential role in tumor development and progression (94). A previous study on the mitochondrial cancer genome demonstrated that hypermutation, structural variations, CN alterations and the somatic relocation of mtDNA into the nuclear genome elevate the risk of cancer progression, growth and metastasis (95).

Horizontal mitochondrial transfer from astrocytes has been recognized as a mechanism that promotes tumorigenesis in GBM, which relies on intercellular connections formed between GBM cells and astrocytes, mediated by growth-associated protein 43, which serves a role in neuronal axon regeneration and astrocyte reactivity, eventually leading to increased mitochondrial respiration and enhanced tumorigenic potential (96).

The relevance of mtDNA alterations in cancer progression and sustainability has been extensively studied, revealing that most mtDNA mutations in brain tumors, particularly in GBM, are missense transitions irregularly allocated between HSP and LPS strands, potentially playing a pivotal role in modulating GBM tumorigenesis (Fig. 1) (8,9). Most somatic point mutations in mtDNA occur within the D-loop region, disrupting replication and transcription processes, which cause fluctuations in mtDNA content and elevated ROS levels, and may subsequently lead to mitochondrial impairment; notably, researchers have identified mutations linked to GBM throughout nearly the entire mitochondrial genome (8,97). The D-loop region acts as the primary hotspot for somatic mtDNA mutations, particularly within the polycytosine tract (D310), a reiterated sequence of cytosines spanning nucleotides 303 to 315, potentially aiding in the clinical monitoring of GBM (98,99). The T16189C polymorphism represents another hotspot in the D-loop region that, despite its common occurrence, fails to display an association with the pathogenesis of GBM.

Apart from the D-loop, mutations have also been observed in the coding regions of mtDNA, particularly in those that participate in the ETC and OXPHOS. It has been documented that genes encoding the subunits of complex I of the ETC, notably NADH dehydrogenase 4 (ND4) and NADH dehydrogenase 6 (ND6), are highly prone to mutations in GBM, indicating that mtDNA variants in complex I may function as triggers for GBM and confer benefits that facilitate tumorigenesis (100,101). The A10398G alteration in the NADH dehydrogenase 3 gene has also been identified in GBM, although its precise involvement remains uncertain, with hypotheses suggesting that this defect, in combination with other genetic and environmental factors, may enhance electron leakage and oxidative stress, leading to increased ROS levels that promote carcinogenesis (102).

Genes encoding complexes III and IV have commonly been identified as being susceptible to mutations in GBM, with several anticipated to be functional and affecting mitochondrial respiratory chain performance (103). A previous study revealed that the germ-line mtDNA mutation T14798C, identified in GBM, may influence the activity and drug sensitivity of complex III in the mitochondrial ETC by causing an amino acid substitution (F18L) in the cytochrome b subunit, thereby potentially altering ROS production, cellular behavior, and patient outcomes (104).

Large-scale 4,977-bp mtDNA deletions, designated as the 'common deletion', have also been reported in patients with GBM, but a significant association with the etiology of GBM has not been established (105).

The significant involvement of mitochondria-related genes (MRGs) in the onset and development of GBM has been underscored by multiple studies (8,106,107); however, the precise roles of proteins encoded by these genes in GBM pathology remain inadequately defined. Prognostic MRGs have been discovered, and a novel prognostic model for GBM was previously verified using 12 differentially expressed MRGs in a study by Su *et al* (108), which demonstrated that the risk score was associated with inflammatory responses, extracellular matrix interactions, and pro-cancer and immune-related pathways, as well as being strongly linked to gene mutations and immune cell infiltration. Additionally, single-stranded DNA-binding protein 1 was found to be significantly upregulated in GBM, and its knockdown caused mitochondrial dysfunction and elevated ROS levels, subsequently enhancing the responsiveness of GBM cells to temozolomide (TMZ) by promoting ferroptosis. In another study, Peng *et al* (109) identified nine prognostic MRGs and created an MRG-based prognostic model validated as an independent risk predictor for patients with GBM, potentially serving as a dependable diagnostic tool. Moreover, this previous study revealed that p66Shc, the longest isoform of SHC1, was upregulated in GBM tissue, and its silencing impeded the proliferation and migration of GBM cells by altering mitochondrial ROS synthesis and morphology (109).

The oxidant-antioxidant balance within a cell is regulated by mitochondria, and oxidative damage, which is associated with tumorigenesis, is typically caused by mitochondrial impairment. It is essential to highlight that a marked tendency to generate ROS is exhibited by numerous tumors with mutations in ETC components, emphasizing the vital function of this machinery in influencing the cancer cell phenotype (110). For example, increased superoxide production may arise from mutations in complex I components, such as the ND4 subunit, which can support ROS-dependent oncogenic pathways and cause damage to mtDNA, thereby promoting tumorigenesis and metastasis in GBM (111).

The mitochondrial enzyme glutamate dehydrogenase 2 (GLUD2), which is predominantly expressed in the brain, functions as an essential component in catalyzing the reversible interconversion of glutamate to α-ketoglutarate and ammonia, significantly contributing to the tricarboxylic acid cycle, energy production and ammonia homeostasis, while also being considered to modulate GBM progression (112). Alterations in GLUD2 expression levels are known to influence changes in mitochondrial functions and metabolic phenotypes in human GBM cells. A study by Franceschi *et al* (112) demonstrated that overexpression of GLUD2 was significantly associated with improved overall survival and lower glioma grades. Furthermore, *in vitro* functional studies conducted with human GBM cell lines revealed that GLUD2 overexpression was linked to elevated oxygen consumption and enhanced ROS production. The rise in ROS generation resulting from GLUD2 overexpression may account for subsequent cell cycle blockage in G_0/G_1, caused by the reduced expression of cyclin D1/E (113,114).

The increased glucose uptake and ATP production through glycolysis, which are subsequently accompanied by lactic acid fermentation even in the presence of oxygen, are identified as significant characteristics of cancer cells, with this metabolic transition from mitochondrial OXPHOS to glycolysis commonly referred to as the Warburg effect. This phenomenon demonstrates an enhanced rate of glycolysis coupled with suppressed mitochondrial metabolism, a pattern that is prominently evident in a range of malignancies, particularly GBM (115). Recent research combining bioinformatics techniques and laboratory assays has established a link between the Warburg effect and the prognosis and immune microenvironment of GBM, indicating that targeting genes associated with this metabolic shift could offer new therapeutic options (116).

Mitochondria have been identified as key regulators in maintaining the quiescent state of GBM stem cells (GSCs), which are major contributors to the resistance of GBM to therapy, positioning them as a potential target for overcoming resistance. OXPHOS serves as a critical metabolic pathway for GSCs, enabling them to sustain their elevated rates of proliferation, resilience to therapies and retention of stem-like properties (117). Conversely, when mitochondrial function is compromised, it can significantly contribute to tumorigenesis through several interconnected mechanisms, including disrupted cell cycle regulation, impaired calcium homeostasis, promotion of the shift of GSCs into a quiescent phase and suppression of apoptosis. Under conditions of stress, such as irradiation and hypoxia, GSCs activate mitochondrial stress pathways as a cytoprotective mechanism to endure the hostile environment, while proliferating GBM cells display heightened cytoplasmic glycolysis, in contrast to quiescent GSCs and fully differentiated GBM cells, which increasingly depend on OXPHOS (118). A recent study has revealed that GSCs can uptake exogenous mitochondria from mesenchymal stem cells, a process that not only provides significant resistance to TMZ chemotherapy but also triggers a crucial metabolic transition from metabolizing glucose to glutamine, ultimately resulting in enhanced orotate synthesis (119).

6. Implications of mtDNA-CN alterations in GBM tumorigenesis

Changes in mtDNA-CN are considered key factors in the development of GBM, a highly aggressive and frequently treatment-resistant brain tumor. These alterations can disrupt cellular metabolism, energy generation and oxidative stress, thereby impacting tumor growth and survival. Exploring the consequences of mtDNA-CN variations could provide valuable insights into the progression and clinical severity of GBM, potentially paving the way for more precise and targeted treatment strategies.

Research has demonstrated that dysfunctional mtDNA POLG can induce changes in mtDNA-CN, disrupting OXPHOS and reducing ATP production (120). This decline in mitochondrial respiration and ATP synthesis, coupled with an increase in glycolysis, commonly occurs in both mitochondrial diseases and cancer (120,121). Dysregulated mtDNA-CN in GBM may impair OXPHOS efficiency, driving a metabolic shift toward aerobic glycolysis that supports rapid tumor growth, survival and malignancy (122,123).

Tumorigenesis is promoted by mtDNA alterations through the modification of ROS generation, which subsequently influences the expression of apoptosis-related proteins, such as cytochrome *c* and BCL-2 family members (124,125). In GBM, reduced mtDNA-CN may impair the initiation of apoptosis, enabling cells to escape programmed cell death and maintain malignancy. In addition, changes in mtDNA-CN influence the expression of mitochondrial-encoded genes and the signaling pathways between the nucleus and mitochondria (126). This disruption may affect genes regulating cell proliferation, migration and invasion, fostering tumorigenesis and enhancing GBM malignancy. It has been reported in previous studies that altered mtDNA-CN can result in defective ETC activity, leading to elevated ROS production (127-129). Consequently, excessive ROS levels may cause DNA damage, genomic instability, and mutations in both nuclear DNA and mtDNA, potentially driving GBM progression. A reduction in POLG and yopoisomerase levels, combined with increased TFAM, has been shown to alter mtDNA-CN and gene expression (130). Declines in mtDNA-CN are believed to trigger epigenetic modifications of nuclear DNA through retrograde signaling, leading to a reduction in OXPHOS capacity and the subsequent shift of stem cells toward glycolytic metabolism and aggressive GBM behavior (130).

7. Variation in mtDNA-CN in GBM

Mitochondrial mechanisms experience alterations in numerous prevalent pathologies, including cardiovascular diseases,

neurodegeneration, metabolic syndrome and cancer. These ailments exhibit specific changes in mtDNA, including variations in CN, rearrangements, deletions and point mutations, which researchers have scrutinized as potential risk factors or early diagnostic markers (8-10,35). Nevertheless, the precise impact of these alterations on disease progression remains unclear.

Oxidative stress more readily damages mtDNA than nuclear DNA, potentially due to its close proximity to OXPHOS, where excessive ROS are regularly produced (131), a phenomenon frequently observed in cancer cells. Efficient base excision repair mechanisms predominantly restore damaged mtDNA molecules, while mtDNA suffering from double-strand breaks undergoes rapid degradation rather than repair, resulting in a substantial reduction in mtDNA-CN compared with nuclear DNA (132). Under various physiological and environmental conditions, mtDNA-CN serves as a relative indicator, reflecting variations in mitochondrial health, and can fluctuate according to energy demands (133). Despite this, mtDNA mutations can accumulate due to faulty replication and repair, resulting in mitochondrial impairment and transmission of signals to the nucleus (134).

mtDNA-CN, acting as a surrogate marker for mitochondrial function, demonstrates extensive connections with various diseases, and documented alterations in its levels have been linked to a higher cancer risk (10,127). Nevertheless, the fluctuating nature of the association of mtDNA-CN with cancer occurrence, whether positive or negative, is heavily influenced by diverse factors such as the source of the sample and the type of cancer. Although mtDNA exists in multiple copies per cell due to mitochondrial dynamics, such as fusion and fission, cells regulate its content within a stable range to sustain energy levels and ensure proper cell function (10,135). Reports have indicated that variations in mtDNA content occur early in carcinogenesis, indicating crucial mutations for neoplastic transformation, which then cause disruptions in OXPHOS and ATP generation (10,136).

mtDNA-CN alterations have been reported across multiple types of cancer, including breast cancer, colorectal cancer, gastric cancer, lung cancer, esophageal cancer and brain tumors (10). However, the present review specifically focuses on providing a comprehensive summary of the existing data and previous findings related to mtDNA level changes reported in brain tumors, with particular emphasis on GBM.

Research has provided limited evidence on how mtDNA content influences GBM tumorigenesis, and its association with clinicopathological factors and patient outcomes. A comprehensive literature search was performed using PubMed, Google Scholar, Scopus and Web of Science platforms to compile information regarding the involvement of mtDNA-CN alterations in GBM (Table I).

In 1996, Liang (137) initially documented that cDNA homologous to mtDNA at positions 1,679-1,948 and 2,017-2,057 had been applied to evaluate 15 low-grade glial tumors, demonstrating an elevated mtDNA-CN in these tumors compared with in normal brain tissue controls. In an additional study, Liang and Hays disclosed that up to a 25-fold increase in mtDNA-CN had been observed in 39 out of 45 (87%) assessed glial tumor specimens, both low and high grade, and asserted that this prevalence exceeded the erb-b gene amplification found in only 18% of the tumors, implying that changes in mtDNA were more widespread than alterations in nuclear-encoded genes in malignant glioma (138).

Correia *et al* (139) revealed that diffusely infiltrating astrocytoma exhibited a significant decrease in mtDNA-CN compared with that in non-neoplastic brain tissues, with the most pronounced depletion occurring in GBM as malignancy progressed. This decrease, when associated with the overexpression of TFAM and POLG, was linked to prolonged survival in patients with GBM, suggesting that these factors may serve as possible indicators of more favorable outcomes. Consistent findings by Soltész *et al* (140), where brain tissue DNA and plasma-derived exosomal DNA from 44 patients with GBM and 40 control individuals were examined, demonstrated that lower mtDNA-CN was found in GBM cases compared with in the control subjects.

In 2013, oncocytic changes in GBM were identified through histology, immunohistochemistry and ultrastructural observation, and high levels of mtDNA-CN were detected, as reported by Marucci *et al* (141). A series of GBM cases was analyzed to establish any association with morphology and survival, resulting in the identification of 10 cases where most cells exhibited characteristic oncocytic traits at histological, immunohistochemical and ultrastructural levels, and nine of these cases showed significantly higher mtDNA content compared with in control tissue (141).

A Chinese case-control epidemiological study examined the link between leukocyte mtDNA content and glioma risk, finding that patients with glioma had markedly higher median mtDNA content than healthy controls, and that increased mtDNA content was strongly associated with a higher risk of glioma (142). This finding aligns with a case-control study from the USA, which showed that mtDNA-CN levels in whole blood were significantly higher in patients with glioma compared with in healthy controls, thereby reinforcing the role of mtDNA-CN in glioma carcinogenesis (143). Conversely, Zhang *et al* (144) demonstrated that an increased mtDNA-CN was significantly inversely associated with tumor grade, recurrence and cancer-related death, and critically, higher mtDNA content was closely linked to prolonged survival in patients with glioma. Additionally, Dardaud *et al* (136) corroborated these findings by observing that a higher mtDNA-CN was significantly associated with improved overall survival in young adult patients with GBM, which corresponded with the findings of Sourty *et al* (145), who reported that high mtDNA-CN was associated with longer survival in young adults but shorter survival in older patients with GBM. More recently, these findings were further supported by our previous study, which showed that longer overall survival periods and notably better outcomes were experienced by patients with higher mtDNA-CN, especially in high-grade brain tumor cases (146). Aligned with findings from glial tumors, Hua *et al* (147) analyzed 87 WHO grade III meningioma samples, revealing that high mtDNA content was associated with improved outcomes, while low mtDNA content was associated with enhanced progression-free survival in patients who received post-operative radiation therapy, indicating that mtDNA content in tumors could function as an indicator for anticipating the prognosis of patients with WHO grade III meningioma.

Table I. Summary of mtDNA copy number changes across brain tumor types, emphasizing alterations in GBM.

First author(s), year	Country	Brain tumor sample type	Technique	mtDNA gene	Nuclear DNA gene	mtDNA levels (relative ratio-fold changes)	Additional observation	(Refs.)
Liang, 1996	USA	15 low grade glioma tissues	Southern blot hybridization	MT-RNR2	ACTB	Increased	All tumors exhibited mitochondrial sequence localization, which was linked to elevated mtDNA content.	(137)
Liang and Hays, 1996	USA	45 glioma tissues	Southern blot hybridization	MT-CO1	ACTB	Increased	5/11 (46%) glioma tumors showed a recurrent deletion of a 1.2-kb *Eco*RI fragment.	(138)
Correia *et al*, 2011	Brazil	120 astrocytoma tissues (WHO grade II, III and IV)	qPCR	MT-ND1	HBB	Decreased	mtDNA reduction was predominantly observed in GBM samples. POLG expression was associated with low mtDNA content.	(139)
Soltész *et al*, 2022	Hungary	44 GBM tissues and plasma-derived exosomes	qPCR	MT-ND1 & MT-ND5	SERPINA1 & SLCO2B1	Increased	High mtDNA content was apparent in brain tissue and exosome samples from the control group.	(140)
Marucci *et al*, 2013	Italy	10 GBM tissues	qPCR	MT-ND2	FALSG	Increased	Longer median survival was observed in oncocytic GBM, at 16 months.	(141)
Zhang *et al*, 2014	China	414 blood lympho-cytes of glioma	qPCR	MT-ND1	HBB	Increased	Elevated mtDNA content was significantly associated with a higher risk of glioma.	(142)
Shen *et al*, 2016	USA	390 whole blood samples from glioma patients	qPCR	MT-ND1	HBB	Increased	High mtDNA levels were associated with a higher risk of glioma.	(143)
Zhang *et al*, 2015	China	124 glioma tissues	qPCR	MT-ND1	ACTB	Increased & decreased	High mtDNA content was significantly linked to seizures. Low mtDNA content was found in recurrent cases.	(144)

Table I. Continued.

First author(s), year	Country	Brain tumor sample type	Technique	mtDNA gene	Nuclear DNA gene	mtDNA levels (relative ratio-fold changes)	Additional observation	(Refs.)
Dardaud *et al*, 2019	France	67 GBM tissues	qPCR	N/A	N/A	Increased	High mtDNA content was associated with extended O in the young adult group.	(136)
Sourty *et al*, 2022	France	232 GBM tissues	qPCR	MT-CO1 & MT-ND4	B2M & GAPDH	Increased & decreased	High mtDNA level was associated with improved OS in younger patients. Low mtDNA levels were associated with better OS in older patients.	(145)
Ab Radzak *et al*, 2024	Malaysia	41 brain tumor tissues	qPCR	MT-ND1	ACTB	Increased	High mtDNA content was associated with longer OS, particularly in high-grade tumors.	(146)
Hua *et al*, 2020	China	87 meningioma III tissues	qPCR	MT-ND1	HBB	Increased	High mtDNA levels were associated with improved OS and PFS.	(147)
Sravya *et al*, 2020	ndia	20 GBM tissues	qPCR	MT-ND1	RNase P	Increased	Patients with low mtDNA Ilevels in blood revealed high mtDNA content in tumor tissue samples.	(148)
Sravya *et al*, 2020	India	162 GBM tissues	qPCR	MT-ND1	RNase P	Decreased	Low mtDNA levels were associated with IDH wild-type.	(149)
Chen *et al*, 2015	China	336 blood from patients with glioma	qPCR	MT-ND1	HBB	Increased	High mtDNA content was associated with poor OS and PFS	(150)
Dickinson *et al*, 2013	Australia	HSR-GBM-1, GBM-L1, GBM-L2, hNSCs	qPCR	MT-RNR2	HBB	Increased	High mtDNA content in hNSCs during differentiation was observed. -Prolonged mtDNA depletion led to defective mtDNA replication, decreased proliferation, and	(122)

Table I. Continued.

First author(s), year	Country	Brain tumor sample type	Technique	mtDNA gene	Nuclear DNA gene	mtDNA levels (relative ratio-fold changes)	Additional observation	(Refs.)
							stimulated the expression of OCT4 and SHH. -Cells recovered their mtDNA copy number after 7 days of depletion.	
Sun and St John, 2018	Australia	HSR-GBM-1 cell line	qPCR	MT-RNR2	HBB	Reverted	mtDNA content was restored to pre-depletion levels without significant differences.	(151)
Shen *et al*, 2020	Australia	60 high-grade glioma tissues	qPCR	D-loop & MT-CO2	ACTB	Decreased	Low mtDNA content led to tumorigenicity through increased cell migration and invasion, as well as resistance to therapy.	(123)
Braun *et al*, 2021	Germany	48 tissues from 22 patients primary and recurrent gliomas	qPCR	D-loop	B2M	Increased	High mtDNA content was observed in *IDH* mutant tumors.	(152)
Oliva *et al*, 2010	USA	U251 cell line	Semi-qPCR	MT-CO1	18S rRNA	Decreased	Temozolomide-resistant glioma cells exhibited reduced mtDNA content.	(153)
Luna *et al*, 2015	USA	48 tissues from pediatric brain tumor	qPCR	MT-ND1	18S rRNA	Increased	A high mtDNA copy number increased the probability of brain tumor expansion in female children by 51 times compared to controls.	(154)

18s rRNA, 18S ribosomal RNA; ACTB, β-actin; B2M, β-2-microglobulin; D-loop, displacement loop; GBM, glioblastoma; HBB, β-globin; hNSC, human neural stem cell; IDH, isocitrate dehydrogenase; MT-CO, mitochondrially encoded cytochrome *c* oxidase; MT-ND, mitochondrially encoded NADH; MT-RNR2, mitochondrially encoded 16S RNA; OS, overall survival; PFS, progression-free survival; POLG, DNA polymerase subunit γ; SERPINA1, serpin family A member 1; SLCO2B1, solute carrier organic anion transporter family member 2B1; WHO, World Health Organization; N/A, not applicable.

A study conducted in India by Sravya *et al* (148) indicated that improved overall survival in GBM was associated with a high mtDNA-CN in tumor tissue. In a distinct study conducted within the same year, Sravya *et al* (149) compared mtDNA-CN between newly diagnosed and recurrent GBM in paired samples, revealing that poor prognosis in patients with GBM was linked with low mtDNA content. Additionally, the research showed that increased neurosphere formation, indicative of higher stemness, and consequently, resistance to radiation and TMZ therapy in malignant glioma cell lines, was caused by mtDNA depletion.

In a study of 336 patients with glioma, it was reported that high mtDNA content was strongly linked to a poorer prognosis in younger patients, those with high-grade gliomas or those receiving adjuvant radiochemotherapy, and it was observed that these patients had markedly reduced natural killer cell frequencies and elevated concentrations of IL-2 and TNF-α, indicating that an immunosuppression-associated process may be engaged in mtDNA-mediated prognosis (150).

The research of Dickinson *et al* (122) on mtDNA content modulation in GBM cell lines highlighted that partial depletion of mtDNA may restore replication events and promote cell differentiation. However, extended depletion compromised mtDNA replication, decreased cell proliferation and triggered the expression of genes associated with early developmental processes. A phenotype that could not regenerate *in vitro* was produced by the gradual depletion of mtDNA content in human GBM cells (122). However, when researchers implanted these cells into immunocompromised mice, they observed that tumor development was delayed and mitochondrial function was recovered, depending on the extent of mtDNA copy reduction. In another study, Sun and St John (151) demonstrated that restoring mtDNA-CN during tumor development led to significant alterations in the nuclear genome, causing variations in DNA methylation and gene expression. These modifications enriched developmental processes and key metabolic pathways linked with GBM. Moreover, the interaction between the nuclear and mitochondrial genomes in reinstating tumorigenic potential was emphasized by the changes in nuclear-encoded mtDNA replication factors (151).

Notably, in 2020, Shen *et al* (123) made a novel discovery by identifying a significant reduction in mtDNA content in most cases of pediatric high-grade glioma (pHGG) compared with in normal brain tissue, which was proposed as the molecular mechanism mediating the Warburg effect. Kinase modulators have significantly reduced pHGG viability by shifting glucose metabolism to mitochondrial oxidation, and combining this approach with metformin, which affects mitochondrial function, has disturbed tumor cell energy balance, resulting in elevated DNA injury and enhanced apoptosis (123).

A study conducted by Braun *et al* (152) investigated whether mitochondrial biomass differed between IDH-mutant and IDH-wild-type diffuse glioma, revealing that IDH-mutant tumors had higher mtDNA-CN associated with unique metabolic and epigenetic profiles compared with IDH-wild-type tumors, thereby highlighting that changes in mtDNA-CN correspond with these distinct profiles and suggesting that mtDNA levels could serve as valuable biomarkers for glioma characterization and prognosis.

In 2010, Oliva *et al* (153) studied the effects of TMZ on mtDNA and mitochondrial function in TMZ-resistant glioma cells and xenografts, indicating that TMZ reduced mtDNA-CN, heightened heteroplasmy, and disrupted mitochondrial ETC and bioenergetics, with similar changes observed in patient biopsies after adjuvant TMZ treatment, emphasizing their clinical importance. In a separate study, Luna *et al* (154) explored the impact of mtDNA-CN, oxidative damage and mtDNA variants as risk factors for pediatric brain tumors, using Bayesian network and Markov Chain Monte Carlo modelling. This analysis showed that the combined presence of certain mtDNA variants, oxidative damage and high mtDNA-CN significantly increased the likelihood of developing brain tumors in female children, with a 51-fold higher risk compared to the normal incidence (154).

Variability in mtDNA-CN findings across GBM studies is shaped by several factors inherent to the nature of the disease, and is often constrained by limitations in methodology, sample selection biases and study design inconsistencies. The complex and heterogeneous nature of GBM, which includes diverse molecular, genetic and metabolic characteristics, notably contributes to discrepancies in mtDNA-CN observations. As mtDNA-CN can vary considerably across different cell types, the cellular composition of the tissue under investigation becomes a crucial factor to consider.

Various molecular techniques, including qPCR, digital PCR and other genomic methods, such as whole exome sequencing and whole genome sequencing, have been employed to determine relative mtDNA-CN values (155). Each of these methods offers distinct advantages and limitations, and their application can influence the accuracy and consistency of mtDNA-CN quantification across different studies. Additionally, DNA extraction methods can significantly impact the accuracy of measurements, with classic procedures using phenol-chloroform or current commercial DNA extraction kits based on spin-column technology both altering the mtDNA:nuclear DNA ratio and potentially modifying the experimental results (156).

Another important consideration is that tissue composition can vary due to aging or disease, such as the neuronal loss seen in neurodegenerative disorders or fibrotic changes in aging tissues (156). In cancer, tumor samples typically consist of various cell types, thus complicating the analysis, although methods such as laser-capture microdissection can successfully isolate specific cells to a certain extent (157). However, the widespread use of these techniques is constrained by time limitations and the availability of the necessary technology, particularly when dealing with large sample sizes.

Study design also serves a crucial role in ensuring accuracy, requiring careful consideration of factors such as age, sex and lifestyle when choosing cohorts. Research has highlighted significant sex differences, with female individuals typically having higher mtDNA-CN than male individuals (91). Lifestyle factors, such as consistent aerobic exercise, can also increase mitochondrial mass and mtDNA levels in skeletal muscle through adaptive metabolic responses (156).

8. Integrating mtDNA-CN with GBM heterogeneity and therapeutic resistance

GBM, a complex and treatment-resistant brain tumor, is influenced by mtDNA-CN, with alterations contributing to its heterogeneity and enhancing its resistance to therapy (158). It has been suggested that variations in mtDNA-CN across different GBM subpopulations may facilitate adaptive responses to environmental stressors, including chemotherapy, radiation and metabolic changes.

Increased mtDNA-CN is necessary in the process of carcinogenesis. For example, it is possible that higher mtDNA-CN drives mitochondrial biogenesis to increase the mitochondrial activity needed for the increase in macromolecular synthesis required for cell growth and replication (159). Higher mtDNA-CN may be linked to increased mitochondrial biogenesis and enhanced OXPHOS, driving cellular energy production in the face of nutrient deprivation or therapeutic intervention. Conversely, low mtDNA-CN may favor glycolysis and other compensatory pathways that reduce reliance on OXPHOS, conferring resistance in a distinct subset of tumor cells. Mou *et al* (160) reinforced the idea that mtDNA depletion may trigger aerobic glycolysis and a reversible apoptosis-resistant phenotype in SW480 cells, with the Akt/mTOR pathway potentially serving a role in the drug-induced resistance to apoptosis.

Tumor cells exhibit heightened glucose uptake rates, even in oxygen-rich conditions, driven by a metabolic shift to aerobic glycolysis, known as the Warburg effect (161). A hypothesis has suggested that variations in mtDNA-CN may impact this shift in GBM cells, where glycolysis is elevated despite the presence of oxygen. Reduced mtDNA-CN levels have been reported to be associated with increased oxidative stress due to higher ROS production (71), which may drive cells to rely on glycolysis, shifting the metabolic burden to the cytoplasm and providing an alternative energy source that promotes GBM tumorigenesis and resistance to treatment. This is supported by evidence indicating that mtDNA-CN is notably reduced in pHGG, resulting in a glycolytic phenotype that is closely associated with increased cell migration, invasion, resistance to therapy and enhanced tumorigenicity *in vivo* (123).

mtDNA-CN serves a crucial role in regulating the stem cell-like properties of GBM cells. A previous study revealed that the HSR-GBM1 cancer stem cell line cannot increase its mtDNA-CN during differentiation, which may lead to impaired differentiation and abnormal expression of the astrocyte marker GFAP (162). This failure to expand mtDNA-CN likely prevents the cell from enhancing OXPHOS capacity and generating enough ATP for full differentiation. As a result, the inability of multipotent cells to differentiate appears to be directly linked to the failure to increase mtDNA-CN (162).

GBM cells with higher mtDNA-CN may have better self-renewal ability and resistance to chemotherapy through mitochondrial-dependent mechanisms. This includes the activation of pro-survival signaling pathways, such as PI3K/Akt/mTOR, which boost cell proliferation, growth, and resistance to chemo- and immunotherapy (163). By contrast, GBM subpopulations with low mtDNA-CN may display flexibility in mitochondrial function, allowing the cells to shift between oxidative and glycolytic metabolism in response to environmental stress (164). This metabolic adaptability helps the cells survive in low-oxygen or nutrient-deprived conditions, aiding in resistance to standard treatments (164). Future research on incorporating mtDNA-CN with GBM heterogeneity and resistance has the potential to reveal novel therapeutic strategies targeting the metabolic weaknesses of GBM.

9. Therapeutic strategies for restoring mtDNA-CN and improving health outcomes

Compelling evidence has suggested that total mtDNA levels have a crucial role in human pathology and aging, with researchers recognizing changes in mtDNA-CN, whether increases or decreases, as significant in cancer development and progression, and linking these fluctuations to various types of cancer, including breast, lung and colorectal cancer, and glioma (10). This has prompted the creation of approaches that modify mtDNA levels, either directly or indirectly, to mitigate or prevent disease progression, while ongoing research into mitochondrial dysfunction in cancer has led to the growing adoption of therapeutic methods targeting mtDNA-CN abnormalities, which hold promise for enhancing the effectiveness of conventional cancer treatments and improving patient outcomes. Researchers have employed two approaches to boost OXPHOS capacity and revive mitochondrial performance, either by altering total mitochondrial mass or by selectively adjusting mtDNA to affect mtDNA-CN and/or heteroplasmy levels; these methods, primarily aimed at managing primary mitochondrial disorders, may also tackle common age-related diseases if human trials prove effective (165,166).

Mitochondrial biogenesis, which expands mitochondrial mass, is driven by environmental factors such as exercise, calorie restriction, temperature changes, oxidative stress, cell division, renewal and differentiation, and boosts mtDNA-CN and metabolic enzyme subunits, enhancing metabolic capacity; this necessitates coordinated expression of both nuclear and mtDNA-encoded genes (167,168). Modulating mitochondrial biogenesis to rectify mtDNA-CN imbalances has emerged as a leading approach, with the enhancement of mitochondrial quantity through this process serving as a practical solution to address bioenergetic impairments resulting from mutations in either mtDNA or the nuclear genome (169). Experimental findings have indicated that the augmented mitochondrial mass can partially offset the diminished respiratory chain activity by sustaining overall ATP production in the skeletal muscle of mice with myopathy, acting as a compensatory response to respiratory chain deficiency and aiding in the enhancement of energy balance in the affected tissue (170).

Peroxisome proliferator-activated receptor (PPAR)γ coactivator 1α (PGC-1α) is a co-transcriptional regulator that enhances mitochondrial production by interacting with nuclear transcription factors, such as PPARs, nuclear respiratory factors (NRFs, such as NRF-1 and NRF-2) and estrogen-related receptors, to stimulate the expression of nucleus-encoded genes, including TFAM and OXPHOS subunits. The activity of PGC-1α is regulated by sirtuin 1 and its deacetylation controlled by AMP-activated protein kinase (AMPK) (171). Research has revealed that elevating PGC-1α expression can increase mtDNA levels and amplify

the activity of mitochondrial respiratory chain complexes in COX-deficient mouse models (172), while also improving aging-related traits in mtDNA mutator mice (173), supporting the notion that the effects of mitochondrial diseases may be mitigated by enhanced mitochondrial biogenesis.

Reinforcing this perspective, research by Giordano *et al* (174) on Leber's hereditary optic neuropathy revealed that unaffected mutation carriers exhibited significantly higher mtDNA-CN and mitochondrial mass compared with their affected relatives and controls, implying that increased mitochondrial biogenesis in these carriers could potentially counteract some of the pathogenic effects of mtDNA mutations. Recently, Wu *et al* (175) uncovered in a mouse model of peritoneal dialysis fluid-induced peritoneal fibrosis that stimulating the AMPK-PGC-1α pathway elevated the expression of phosphorylated-AMPK, PGC-1α, NRF-1, NRF-2 and TFAM, increased mtDNA content, enhanced mitochondrial morphology, prevented apoptosis of peritoneal mesothelial cells, and reduced peritoneal fibrosis by promoting mitochondrial biogenesis.

In 2020, human trials on mitochondrial biogenesis enhancers, including niacin (a vitamin B3 derivative and NAD^+ precursor) were conducted. Scientists from the University of Helsinki demonstrated that niacin effectively increased NAD^+ levels and promoted mitochondrial biogenesis, thereby boosting muscle strength in patients with mitochondrial myopathy and NAD^+ depletion (176).

A previous study demonstrated that ZLN005, an innovative transcriptional regulator of PGC-1α, enhanced PGC-1α and downstream gene expression, boosted mitochondrial biogenesis and metabolic maturation in human embryonic stem cell-derived cardiomyocytes, and led to a higher mtDNA-CN in treated cardiomyocytes compared with the controls (177).

Researchers have also shown that electroacupuncture (EA) pretreatment safeguards mitochondria and enhances mitochondrial biogenesis by elevating mtDNA levels, and increasing mitochondrial volume and number through CB1R-dependent PGC-1α activation and upregulation of NRF-1 and TFAM expression, thus revealing a new mechanism for EA pretreatment-induced ischemic tolerance (178).

Research has also confirmed that TFAM protein levels precisely regulate mtDNA quantity, enabling the genetic modulation of TFAM expression to alter mtDNA-CN (80). It has been observed that a slight increase in TFAM levels in mice has led to an elevated mtDNA-CN while leaving mitochondrial gene expression, mitochondrial mass and respiratory chain capacity unaffected, thereby indicating that an increase in mtDNA-CN can occur independently of a rise in mitochondrial quantity (78,80).

In 2010, Nishiyama *et al* (179) conducted the initial study demonstrating that overexpression of TFAM in a mouse model with extensive mtDNA deletions elevated the total mtDNA-CN, mitigated severe mitochondrial disease symptoms and extended lifespan. Likewise, enhancing mtDNA quantities through TFAM overexpression in a study of male infertility partially restored spermatogenesis and OXPHOS functionality in the testes of mtDNA mutator mice, subsequently recovering their fertility phenotype (180). Both findings were corroborated by the work of Filograna *et al* (165), who experimentally manipulated mtDNA levels by boosting TFAM expression in mice with the C5024T mutation in the $tRNA^{Ala}$ gene, which alleviated the pathological outcomes of pathogenic mtDNA, emphasizing a potential therapeutic approach for mitochondrial diseases. Notably, these studies revealed that while the ratio of pathogenic mtDNA mutations remained constant, preserving the mutational load, the levels of wild-type mtDNA increased, which partially improved mitochondrial performance even though mutant mtDNA continued to prevail (165,180). Beyond its relevance to mitochondrial disease research, the advantageous impacts of TFAM overexpression have been shown to mitigate the reduction in mtDNA-CN and to maintain it at normal levels in cardiac tissues, while also restoring respiratory chain enzymatic activity in a myocardial infarction mouse model (181). Hayashi *et al* (182) previously reported that transgenic overproduction of human TFAM can diminish age-related motor learning and memory deficits, along with reducing 8-oxoguanine accumulation in aged mice. Furthermore, in a separate study, researchers from the same group revealed that TFAM may successfully disrupt the mitochondria-driven vicious cycle in Alzheimer's disease model neurons and mouse brains, leading to considerable progress in Alzheimer's pathology, with declined amyloid β accumulation and enhanced cognitive function (183). In contrast to stimulating the entire mitochondrial biogenesis process, regulating mtDNA quantities has been identified as a more targeted approach for enhancing OXPHOS, although thorough evaluation of the extent of TFAM expression remains crucial. Conversely, an *in vivo* study reported that TFAM overexpression could increase the mtDNA content, which was shown to have detrimental effects, such as nucleoid enlargement, disrupted transcription, age-related mtDNA deletions and impairments in the respiratory chain (184). Altering TFAM levels to regulate mtDNA-CN could offer a promising approach for addressing mitochondrial issues in both primary mitochondrial disorders and other related conditions.

Research has uncovered that a lower mtDNA level is linked to reduced chemotherapy efficacy in both clinical and laboratory studies, with evidence indicating that mtDNA depletion serves a role in triggering chemoresistance in various malignant tumors (185,186). However, a decrease in mtDNA levels may also enhance tumor cell sensitivity to chemotherapy in certain types of cancer, such as nasopharyngeal cancer (187), highlighting the unclear relationship between mtDNA-CN and chemotherapeutic resistance in cancer.

10. Conclusion and future perspectives

mtDNA-CN alterations have been increasingly recognized as significant factors in the development and progression of GBM. These variations can markedly influence mitochondrial function, energy metabolism and the behavior of tumor cells. Research has indicated that fluctuations in mtDNA levels, whether elevated or reduced, may disrupt OXPHOS, promoting the generation of ROS, which not only drives tumor growth but also affects how GBM cells respond to environmental challenges and therapies. Understanding the implications of mtDNA-CN alterations is essential for advancing therapeutic strategies. Although both increased and decreased mtDNA

levels have been observed in GBM, their precise effects on tumor biology and treatment outcomes remain to be fully elucidated.

From a clinical perspective, targeting mtDNA-CN alterations offers a promising therapeutic strategy for improving GBM treatment outcomes. Strategies aimed at restoring or modulating mtDNA levels, such as stabilizing mtDNA-CN through mitochondrial biogenesis enhancers or TFAM overexpression, have the potential to boost mitochondrial function, optimize OXPHOS, and heighten tumor cell sensitivity to chemotherapy or radiation. These interventions could serve as valuable additions to current treatments by addressing the metabolic vulnerabilities associated with mtDNA alterations in GBM.

Future research initiatives should focus on elucidating the fundamental mechanisms that connect mtDNA-CN variations to GBM pathology. This may include examining how these alterations interact with nuclear DNA mutations, epigenetic changes and environmental factors, as well as their influence on mitochondrial dynamics, ROS production and cellular metabolism in shaping tumor progression and resistance to treatment. Exploring these mechanisms could uncover novel biomarkers for prognosis or therapeutic response, contributing to the advancement of more personalized treatment strategies.

In conclusion, improving the understanding of mtDNA-CN alterations in GBM has substantial potential to enhance diagnostic and therapeutic strategies. By elucidating the roles of mtDNA variations, it may be feasible to develop treatments that not only optimize mitochondrial function but also counteract resistance to conventional therapies. This area of research could ultimately facilitate more effective management of GBM, improving patient outcomes and providing potential options in the treatment of this aggressive brain tumor.

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Competing interests

The authors declare that they have no competing interests.

Use of AI tools declaration

During the preparation of this work, AI tools (Grammarly) were used to improve the readability and language of the manuscript, and subsequently, the authors revised and edited the content produced by the AI tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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