Mitochondrial DNA copy number as a prognostic marker is age-dependent in adult glioblastoma

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Abstract

Background. Glioblastoma (GBM) is the most common and aggressive form of glioma. GBM frequently displays chromosome (chr) 7 gain, chr 10 loss and/or EGFR amplification (chr7+/chr10-/EGFRamp). Overall survival (OS) is 15 months after treatment. In young adults, IDH1/2 mutations are associated with longer survival. In children, histone H3 mutations portend a dismal prognosis. Novel reliable prognostic markers are needed in GBM. We assessed the prognostic value of mitochondrial DNA (mtDNA) copy number in adult GBM.

Methods. mtDNA copy number was assessed using real-time quantitative PCR in 232 primary GBM. Methylation of POLG and TFAM genes, involved in mtDNA replication, was assessed by bisulfite-pyrosequencing in 44 and 51 cases, respectively.

Results. Median age at diagnosis was 56.6 years-old and median OS, 13.3 months. 153/232 GBM (66 %) displayed chr7+/chr10-/EGFRamp, 23 (9.9 %) IDH1/2 mutation, 3 (1.3 %) H3 mutation and 53 (22.8 %) no key genetic alterations. GBM were divided into two groups, “Low” (n = 116) and “High” (n = 116), according to the median mtDNA/nuclear DNA ratio (237.7). There was no significant difference in OS between the two groups. By dividing the whole cohort according to the median age at diagnosis, OS was longer in the “High” vs “Low” subgroup (27.3 vs 15 months, P = .0203) in young adult GBM (n = 117) and longer in the “Low” vs “High” subgroup (14.5 vs 10.2 months, P = .0116) in older adult GBM (n = 115). POLG was highly methylated, whereas TFAM remained unmethylated.

Conclusion. mtDNA copy number may be a novel prognostic biomarker in GBM, its impact depending on age.

Key Points

• High mtDNA copy number correlates with better prognosis in young adult glioblastoma.
• High mtDNA copy number correlates with worse prognosis in older adult glioblastoma.
• POLG and TFAM genes are highly methylated and unmethylated, respectively, in glioblastoma.
Glioblastoma (GBM) is the most frequent primary brain tumor. This is the most aggressive form of diffuse glioma (WHO grade IV out of IV).\(^1\) Diffuse gliomas are classified according to their IDH1/2 (isocitrate dehydrogenase 1/2) gene status. Most GBM develop in patients older than 55 years and do not display IDH mutation. Those IDH wild-type (WT) tumors are characterized by gain of chromosome (chr) 7 (chr7+), loss of chr 10 (chr10-) and/or EGFR gene amplification (EGFRamp).\(^3\) The median overall survival is 15 months after standard of care treatments including maximum safe resection and concomitant radiochemotherapy.\(^2\) Some GBM (<10%) derive from lower-grade (II or III) IDH-mutant diffuse gliomas in young adults (secondary GBM). Occasionally, IDH-mutant GBM develop without past history of a lower-grade tumor (primary GBM). IDH mutation is associated with a longer survival (>2-3 years) compared to that of IDH-WT diffuse gliomas. Inversely, most pediatric GBM harbor histone H3 (H3F3A or HIST1H3B/C) gene mutations associated with a short survival (<1 year). However, within each molecular subgroup (IDH-WT, IDH-mutant, H3-mutant), overall survival varies from patient to patient despite similar histopathological and genetic features. Novel reliable prognostic markers are sorely needed in GBM.

Mitochondria are cytoplasmic organelles energy to the cell through production of ATP. Two major pathways, glycolytic and oxidative, are involved in cell metabolism, the oxidative pathway being driven by mitochondria and requiring oxygen. The “Warburg effect” corresponds to the shift from an oxidative to a glycolytic metabolism in tumor cells despite aerobic conditions. As a result, tumor cells transform glucose into lactate through fermentation.\(^5\) Lactates have a profound impact on tumor microenvironment and display an immunosuppressive effect.\(^6\) Inversely, oxidative metabolism decreases tumor invasiveness and supports cell differentiation.

Mitochondrial DNA (mtDNA) is a 16 569 bp, intronless, circular, and double-stranded molecule that is maternally inherited. It encodes, among others, 13 polypeptide subunits of the electron transport chain (ETC) implicated in the oxidative phosphorylation (OXPHOS).\(^8\) One cell may contain hundreds to thousands of mtDNA copies.\(^6\) mtDNA pathogenic mutations have been described in various cancers, including colorectal and ovarian carcinomas, acute myeloid leukemias, and GBM.\(^7\) mtDNA copy number has been correlated with variable prognosis in cancers.\(^8-10\)

We have previously shown that high mtDNA levels were significantly associated with longer overall survival (31.8 months vs 12.9 months; \(P = .013\)) in young adult GBM patients (\(\geq 18\) years-old and < 40 years-old; \(n = 67\)) strongly suggesting that mtDNA copy number may represent a novel prognostic marker in GBM.\(^11\) We assessed the correlation between mtDNA levels and prognosis in a larger cohort of GBM patients with a wider age range, older patients being the most affected by the disease.

mtDNA copy number is strictly regulated by several nuclear-encoded transcription and replication factors. Among them, mitochondrial transcription factor A (TFAM) plays a key role in activating the transcription of mtDNA and initiating its replication.\(^12,13\) mtDNA replication is driven by mitochondria-specific DNA Polymerase Gamma (POLG) that consists of one catalytic subunit (POLGA), encoded by POLG gene, and a dimeric form of its accessory subunit (POLGB), encoded by POLG2 gene. POLGA acts as a DNA polymerase with exonuclease activity and needs an RNA-DNA hybrid primer transcript encoded by TFAM and recognized by POLGB to replicate mtDNA.\(^14\) Thus, TFAM and POLG are essential nuclear-encoded genes in mtDNA copy number regulation.

Gene expression is strictly regulated by epigenetic changes affecting either histones or DNA. DNA methylation is one of the main epigenetic mechanisms that lead to gene silencing.\(^15\) Epigenetic regulation of nuclear DNA (nDNA) has been linked to mtDNA replication through POLG exon 2 methylation.\(^16\) Whether mtDNA copy number is directly regulated by methylation of CpG islands within TFAM and POLG genes has yet to be assessed in GBM.

In the present study, we investigated the correlation between mtDNA copy number and overall survival in a large cohort of GBM patients \(\geq 18\) years. We demonstrate that high mtDNA copy number is associated with longer survival in young adult GBM and with shorter survival in older patients. We assessed whether tumor mtDNA content was epigenetically regulated through the methylation of two nuclear-encoded genes, TFAM and POLG, by pyrosequencing. We showed POLG exon 2 to be highly methylated in all GBM samples, while TFAM promoter remained unmethylated.
Materials and Methods

Glioblastoma Tissue Samples

All primary GBM from patients over 18 years-old operated at Angers University Hospital between 2000 and 2018 were reviewed. Only cases with available clinical, histopathological and molecular data were included in the study (n = 192). The histopathological diagnosis was made according to the 2016 WHO classification. Molecular characterization had been previously performed for all cases using pangenomic SNP (Single Nucleotide Polymorphism) arrays (CytoSNP-850K®, Illumina, San Diego, CA, USA) and Sanger sequencing (IDH1/2 and H3F3A/HIST1H3B genes). All patients had provided written informed consent for genetic analyses with the approval of the research ethics committee of Angers University Hospital (Comité de Protection des Personnes, n° CP CB 2015/08). Additionally, 11 GBM samples were provided by the Pitié-Salpêtrière University Hospital (AP-HP, OncoNeuroTek, Paris, France), 21 by the French Glioblastoma Biobank, and 8 were obtained from the Timone University Hospital (AP-HM, AP-HM Tumor Bank, http://doi.org/10.5334/ojb.63, Marseille, France). Of the 232 cases included, 67 have already been reported. The whole cohort is presented in Supplementary Material S1.

Tumor DNA Extraction

Tumor DNA was extracted from fresh-frozen GBM tissue samples using NucleoSpin® Tissue kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer’s recommendations. DNA concentration was evaluated using Spectrophotometer Nanodrop ND 1000 (Thermo Fisher Scientific, Courtaboeuf, France).

Quantitative Polymerase Chain Reaction of Tumor mtDNA

Tumor mtDNA was quantified by quantitative polymerase chain reaction (qPCR) using Chromo4 System (Biorad, Hercules, CA, USA) as previously described. Two mitochondrial-encoded genes, MT-CO1 and MT-ND4, and two nuclear-encoded genes, β2-microglobulin (B2M) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were analyzed using specific primers as described in Supplementary Material S2. Their respective copy numbers were evaluated using a calibration range. mtDNA amount was assessed according to the ratio “mtDNA copy number: nDNA copy number”. Statistical adjustment was performed for chr 12p (harboring GAPDH gene) and 15q (B2M gene) copy number alterations and ploidy status using SNP array data.

High-Throughput Sequencing of Tumor mtDNA

High-throughput mtDNA sequencing (IonTorrent, Thermo Fisher Scientific, Waltham, MA, USA) was performed in 28 GBM as previously described. An in-house bioinformatic pipeline was used to detect mtDNA point mutations, combined with eKLIPse software, designed to detect and quantify mtDNA rearrangements as described elsewhere.

Tumor DNA Bisulfite Conversion, Bisulfite-Specific Polymerase Chain Reaction, and Pyrosequencing

We assessed the DNA methylation status of six CpG sites located in POLG exon 2 and in TFAM promoter in 44 tumor tissue samples (26 young and 18 older patients (see below)) and 51 tumor tissue samples (31 young and 20 older patients (see below)), respectively. Tumor DNA (1 µg) was sodium bisulfite converted using the EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer’s recommendations.

CpG islands of TFAM promoter and POLG exon 2 were located using the website Database of Transcriptional Start Sites (DBTSS). Their genomic sequences were retrieved from Alamut Visual version 2.15 (Interactive Biosoftware, Rouen, France), with the following ID number for TFAM transcript: NM_003201.2 (region 5835067-5835237) and for POLG exon 2: NM_002693.2 (region 8933509-8933745). Bisulfite-specific primers (BSP) were designed using the open access MethPrimer 2.0 program (Supplementary Material S3), with POLG reverse primer modified manually. Reverse primers were labeled with biotin at the 5’ end and purified using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC). PCR products of TFAM and POLG were 171 and 237 base pairs long, respectively. Details of the PCR are provided in the Supplementary Material S4.

Two different pyrosequencing primers were designed for TFAM and one for POLG, using PyroMark® ID Q96 Software (Qiagen, Venlo, Netherlands) (Supplementary Material S5). Pyrosequencing reaction was carried out using Pyromark Gold Q96 Reagents (Qiagen, Hilden, Germany), as described elsewhere, and performed in the Pyrosequencer IDQ96 (Qiagen, Venlo, Netherlands). Only samples with pyrograms displaying peak height > 7 RLU (Relative Light Unit) on at least the first 6 CpG sites were taken into account.

Statistical Analyses

Data have been expressed as medians and averages for continuous variables and as absolute values and frequencies for categorical variables. Comparisons were performed using Chi-Square test or Fisher’s exact test depending on the number of samples to compare categorical variables. P values less than .05 were considered significant. The Kaplan–Meier method was used to compare overall survival rates. Statistical analyses were performed with GraphPad Prism 9.

Results

Clinical and Histomolecular Features of the Cohort

The cohort comprised 232 GBM diagnosed according to the 2016 WHO classification. All cases were primary GBM, with no past history of lower-grade diffuse glioma. The median age at diagnosis was 56.6 years and the sex ratio (M:F) 1.6:1. The median overall survival was
13.3 months: 1) 153 GBM (66 %) were IDH-WT and characterized by chr 7 gain/chr 10 loss and/or EGFR amplification, 2) 23 (9.9 %) were IDH-mutant, 3) 3 (1.3 %) displayed H3F3A/HIST1H3B mutations (H3-mutant), and 4) 53 (22.8 %) had none of the aforementioned alterations (referred to as NEC for Not Elsewhere Classified). The median age at diagnosis was 58.5 years for the IDH-WT group, 34 years for the IDH-mutant group, 39 years for the H3-mutant group, and 55.2 years for the NEC group. The median overall survival was 15.5 months for the IDH-WT group, 34.5 months for the IDH-mutant group, 11.3 months for the H3-mutant group, and 14.1 months for the NEC group.

Quantification of mtDNA copy number

The mtDNA level was assessed using the mtDNA/nDNA ratio that ranged from 28 to 3,882 (median ratio 237.7). Patients were subdivided into two groups according to the median ratio: 1) Low mtDNA level (116 patients) where the mtDNA/nDNA ratio ≤ the median ratio and 2) High mtDNA level (116 patients) where the mtDNA/nDNA ratio > the median ratio. The overall survival of the Low vs High group was not significantly different (14.5 months vs 17 months, respectively; P = .75) (Figure 1).

Since we had shown in a previous work that high mtDNA levels were significantly associated with longer overall survival in young adult GBM patients, we assessed this correlation in a larger cohort of GBM patients with a wider age range. Patients were subdivided into two groups according to the median age at diagnosis (56.6 years) in order to distinguish a young adult group (age < median age, n = 117 patients) and an older adult group (age ≥ median age, n = 115 patients). A correlation between mtDNA level (High or Low) and overall survival was assessed in both (young and older) age groups. Of note, mtDNA amount was not associated with patient age (P = .44, Kruskal-Wallis test).

High mtDNA Copy Number is Associated with Longer Overall Survival in Young Adult GBM

In the young adult group, the overall survival was significantly longer in the High vs Low subgroup (27.3 months vs 15 months; P = .0203) (Figure 2). In this age group (n = 117 patients), 65 GBM (55 %) were IDH-WT, 22 (19 %) IDH-mutant, 3 (3 %) H3-mutant and 27 (23 %) NEC. There was no significant association between the High status and the IDH-mutant status (P = .17, Chi-Square test).

In the NEC group, the overall survival was significantly longer in the High subgroup (n = 13) compared to the Low subgroup (n = 14) (27.8 months vs 12.9 months, respectively; P = .0214). In the IDH-mutant group, there was a trend toward a longer overall survival in the High subgroup (n = 14) compared to the Low subgroup (n = 8) but that did not reach statistical significance (37.6 months vs 15 months, respectively; P = .89). In IDH-WT GBM, no significant difference in survival was found in the High (n = 31) vs Low group (n = 34) (17.6 months vs 17.1 months, respectively; P = .69). In H3-mutant GBM, overall survival was shorter in the High vs Low group (5 months vs 13.7 months, respectively; P = .15) but the very small number of patients in this group (n = 3) does not allow to draw any definitive conclusion (Table 1).

High mtDNA Copy Number is Associated with Shorter Overall Survival in Older Adult GBM

In the older age group, the overall survival was significantly shorter in the High vs Low group (10.2 months vs 14.5 months, respectively; P = .0116) in contrast to what was observed in the young adult group (Figure 3). In the older age group (n = 115 patients), 88 GBM (76 %) were IDH-WT. one (1 %) was IDH-mutant, none was H3-mutant, and 26 (23 %) were NEC.

For the IDH-WT cases, the overall survival was significantly shorter in the High vs Low group (11.4 months vs 17.1 months, respectively, P = .0228; 43 and 45 patients in

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**Figure 1.** Overall survival in the young adult group (High vs Low GBM). Kaplan–Meier survival curves in the young adult group stratified according to mtDNA content.

**Figure 2.** Overall survival in the young adult group (High vs Low GBM). Kaplan–Meier survival curves in the young adult group stratified according to mtDNA content.
Each group, respectively). For the NEC cases, there was a trend towards a shorter survival in the High vs Low group but that did not reach statistical significance (7.1 months vs 12.3 months, respectively, \( P = .19 \); 13 patients in each group) (Table 2).

### High-Throughput Sequencing of Tumor mtDNA Reveals No Alteration

mtDNA sequencing was performed in 28 GBM (12 %), from 18 young adults (10 High and 8 Low) and 10 older patients (6 High and 4 Low). Of these cases, 14 were IDH-WT, 7 IDH-mutant, and 7 NEC. Using the eKLiPse software, \(^{17}\) no significant accumulation of mtDNA rearrangements was detected (notably no deletion). No somatic variant was found in young adult GBM and only very few somatic variants were detected in older adult cases (average: 1 variant per tumor).

### POLG Exon 2 is Highly Methylated While TFAM Promoter Remains Unmethylated in all GBM Samples

To highlight an underlying epigenetic mechanism involved in mtDNA levels in tumor cells, we focused on two genes implicated in mtDNA copy number regulation. According to the above-mentioned criteria (see Materials and Methods), 20 GBM samples (13 young and 7 older patients) yielded valid results for POLG exon 2. We found very high methylation levels of POLG exon 2 both in the Low (12 patients) and High (8 patients) groups, with similar median methylation levels of 86.3 % and 82.3 %, respectively (Figure 4A). With regard to TFAM promoter, 34 GBM samples (19 young and 15 older patients) yielded interpretable results. No CpG site was methylated (methylated level of 0 %) in any GBM, whether from the Low (21 cases) or High (13 cases) group (Figure 4B).

### Discussion

We studied 232 primary GBM from patients aged 18 and over. Four molecular subgroups were defined according to the underlying genetic alterations. The aim of the study was to evaluate the prognostic impact of mitochondrial genetics in the whole cohort and within each molecular subgroup—since genetic alterations with known prognostic value may be confounding factors. mtDNA copy number was assessed in all 232 cases and mtDNA was sequenced by NGS in a series of 28 cases (28/232, 12 %). Methylation status of two nuclear-encoded genes implicated in mtDNA copy number regulation (i.e. TFAM and POLG) was assessed.

We distinguished two groups of GBM, High and Low, according to the median mtDNA/nDNA ratio. No significant correlation was found between mtDNA copy number and overall survival in the whole cohort (\( P = .75 \)). These results differ from those of a previous work where a high mtDNA

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**Table 1. Median Overall Survival in Months According to mtDNA Content and Molecular Subgroup in Young Adult GBM**

| Group                  | High group \( (n = 59) \) | Low group \( (n = 58) \) | \( P \) value |
|------------------------|-----------------------------|---------------------------|--------------|
| Young adult group      | 27.3                        | 15                        | .0203        |
| IDH-WT \( (n = 65) \)  | 17.6                        | 12.9                      | .0214        |
| NEC \( (n = 27) \)      | 27.8                        | 13.7                      | .89          |
| IDH-mutant \( (n = 22) \) | 37.6                        | 13.7                      | .15          |
| H3-mutant \( (n = 3) \) | 5                           | 13.7                      |              |

The High group comprised 31 IDH-WT GBM (53 %), 14 IDH-mutant GBM (24 %), one H3-mutant GBM (1 %), and 13 NEC GBM (22 %). The Low group comprised 34 IDH-WT GBM (59 %), 8 IDH-mutant GBM (14 %), two H3-mutant GBM (3 %), and 14 NEC GBM (24 %). Molecular subgroups were equally distributed between both groups (High vs Low). In the NEC subgroup, High GBM displayed a significantly longer overall survival compared to Low GBM (\( P = .0214 \)). In the IDH-mutant subgroup, there was a trend towards longer overall survival in the High vs Low GBM but the difference did not reach statistical significance.

**Table 2. Median Overall Survival in Months According to mtDNA Content and Molecular Subgroup in Older Adult GBM**

| Group                  | High group \( (n = 57) \) | Low group \( (n = 58) \) | \( P \) value |
|------------------------|-----------------------------|---------------------------|--------------|
| Older adult group      | 10.2                        | 14.5                      | .0116        |
| IDH-WT \( (n = 88) \)  | 11.4                        | 17.1                      | .0228        |
| NEC \( (n = 26) \)      | 7.1                         | 12.3                      | .19          |
| IDH-mutant \( (n = 1) \) | -                           | 2.7                       |              |

The High group comprised 43 IDH-WT GBM (75 %), one IDH-mutant GBM (2 %), and 13 NEC GBM (23 %). The Low group comprised 45 IDH-WT GBM (78 %), and 13 NEC GBM (22 %). Molecular subgroups were equally distributed between both groups (High vs Low). In the IDH-WT subgroup, Low GBM displayed a significantly longer overall survival compared to High GBM. In the NEC subgroup, there was a trend towards longer overall survival in the Low vs High GBM but that did not reach statistical significance.
Figure 4. Examples of POLG and TFAM pyrograms. Methylation level (estimated according to the Cytosine/(Cytosine + Thymine) ratio) is calculated by the PyroMark Software at each Cpg site (gray bands on the pyrogram) and indicated in the top blue rectangles. Average methylation level
copy number was correlated with a longer overall survival in a cohort of 67 young adult GBM patients (18–40 years) (31.8 vs 12.9 months; \( P = .013 \)). However, by segregating patients according to the median age at diagnosis (56.6 years), we again demonstrated that a high mtDNA level was associated with a better overall survival in the young adult group (273 months vs 15 months; \( P = .0203 \)). Hence, high mtDNA copy number appears as a positive prognostic factor in the young adult population. Interestingly, the median age of our cohort (hence the cutoff we chose to distinguish young vs older adults) was very close to the age proposed by the 2016 WHO classification (i.e., 55 years-old) to distinguish primary IDH-WT GBM occurring in older patients from presumably secondary IDH-mutant GBM arising in young adults.1 In the young adults from the NEC group, a high mtDNA copy number was associated with a longer overall survival compared to a low mtDNA copy number (278 months vs 12.9 months; \( P = .0214 \)) and a similar trend towards longer survival was observed in the High vs Low subgroup within the IDH-mutant group. The lack of association between High status and IDH-mutant status (test du Chi, \( P = .17 \)) indicates that IDH mutation is not a confounding factor for longer survival. Hence, the prognostic value of mtDNA copy number in GBM may be independent of the underlying genetic alterations.

Mitochondria play a key role in various cellular functions, including energy production, formation of reactive oxygen species, and initiation of apoptosis.21,22 Mitochondria are involved in aging23 and cancers.24 Somatic mtDNA variants have been reported in different cancer types and may play a role in tumorigenesis and tumor progression.25,26 mtDNA levels have been associated with longer or shorter survival depending on cancer types. Our results in young GBM patients concur with those of previously published studies showing high mtDNA levels to be correlated with better prognosis and low levels with shorter overall survival. One meta-analysis on 18 studies found that high mtDNA copy number was associated with a better outcome in various cancers, including digestive tract cancers, breast cancers, and gliomas.2 Two studies demonstrated that a low mtDNA copy number was associated with tumor progression and lymph node metastases in colorectal cancers.3,10 The authors linked this result to the ability of tumor cells with low mtDNA content to survive in hypoxic conditions by shifting their metabolism from mitochondrial OXPHOS to anaerobic glycolysis. Moreover, these tumors may display increased chemoresistance. By contrast, tumors with high mtDNA copy number were described as sensitive to hypoxia, slow-growing, and less invasive.9 Other studies showed that high mtDNA levels correlated with a poor prognosis in some tumor types (e.g., colorectal22 and prostate cancers28). Thus, the prognostic value of mtDNA copy number markedly differs across tumor types.

Very few studies have focused on mtDNA copy number in GBM. In diffuse gliomas, decreased mtDNA copy number was correlated with increased malignancy according to WHO grade, with lowest mtDNA amounts in GBM (WHO grade IV) suggesting a likely role of mtDNA depletion in tumor progression.29 mtDNA copy number was significantly reduced in all tumors as compared to non-neoplastic tissue. In a similar way and in accordance with our results in young adults, low mtDNA copy number was recently associated with poor outcome and treatment resistance in GBM.30 GBM cancer cells maintain low copy number leading to decreased mitochondrial function and promotion of anaerobic glycolysis and cell proliferation.31 A very recent study identified four distinct GBM subtypes based on RNA sequencing data and key biological functions.32 Mitochondrial GBM relying on OXPHOS were associated with a better prognosis compared to glycolytic/plurimetabolic GBM depending on glycolysis/hypoxia-related functions.

Other works showed that varying mtDNA levels in vitro and in vivo in human GBM cell lines had an impact on tumorigenesis and cell differentiation.33 mtDNA copy number is strictly regulated during cell differentiation, with mtDNA levels depending on cell type-specific energy needs. While embryonic stem cells, which are characterized by high proliferation rates, maintain low mtDNA levels, mtDNA copy number increases during cell differentiation to meet specific functional requirements.34 Dickinson et al. demonstrated that during astrocytic differentiation, human neural stem cells displayed very high mtDNA levels contrary to human GBM cell lines, that are unable to increase their respiratory capability and differentiate.35 However, GBM cell lines highly depleted in mtDNA displayed reduced cell proliferation in vitro and increased expression of early developmental genes (OCT4, SHH). Interestingly, the most aggressive tumors (fast growth and large volume) were observed with partial mtDNA deletions. This study underlines the importance (and complexity of the regulation) of mtDNA copy number in cell differentiation and tumor growth. Similarly, one study showed that mtDNA copy number might regulate methylation levels of nDNA in GBM cell lines in vitro and in vivo in a mouse model.36 mtDNA depletion led to nDNA demethylation and subsequently to differentiation of tumor cells into mature astrocytes by de-repression of differentiation genes via nDNA demethylation. High mtDNA levels may lead to promoter methylation (inactivation) of some oncogenes and/or promoter demethylation (activation) of some tumor suppressor genes. This study highlights the bidirectional control of nuclear and mitochondrial genomes.

Gene expression is highly regulated by DNA methylation occurring at CpG islands. One study investigated the impact of nDNA methylation on mtDNA copy number in cancer cells from GBM cell lines, demonstrating that mtDNA is epigenetically regulated.36 Demethylation agents (such as 5-azacytidine and vitamin C) promoted a > 3-fold increase in mtDNA copy number, pointing out that DNA demethylation of key genomic regions is associated with increased mtDNA replication. Interestingly, POLG was
found upregulated by demethylation, whereas TFAM was downregulated, suggesting their likely divergent roles in mtDNA replication.

In the present study, we found high methylation levels of POLG regardless of mtDNA content. To our knowledge, these results are the first to confirm on tumor tissue samples obtained on cancer cell lines by Lee et al., highlighting that POLG exon 2 is highly methylated in human adult GBM. Of note, high levels of gene body methylation have been found in active genes, precluding any conclusion on POLG transcriptional activity in GBM. POLG methylation levels do not appear to be correlated to mtDNA amount, in opposition to our original hypothesis, suggesting that other mechanisms are implicated in the regulation of mtDNA copy number in GBM. However, bisulfite sequencing did not allow us to evaluate de novo DNA demethylation occurring through hydroxylation of 5-methylcytosine by ten-eleven translocation (TET) family demethylases, that could be involved in mtDNA copy number increase in some GBM. Besides, the methylation analysis performed herein was done on a small number of tumors.

As already mentioned, TFAM is a key factor in mtDNA regulation, mtDNA transcription, and replication initiation. While the vast majority of CpG dinucleotides are methylated throughout the genome (around 80 %), most promoter-associated CpG islands appear to be largely unmethylated. To the best of our knowledge, few studies investigated TFAM promoter methylation status in cancers. One study found that in vitro methylation of the nuclear respiratory factor-1 (NRF-1) binding site of the TFAM promoter could inhibit its downstream transcriptional activity. In the present study, we first hypothesized that mtDNA copy number might be regulated by TFAM promoter methylation. The TFAM promoter sequence analyzed in our study was not methylated at any CpG site in any tumor tested, suggesting that the CpG island located within TFAM promoter remains unmethylated in GBM. These results have to be interpreted carefully as we did not investigate TFAM gene expression in GBM. Moreover, alternative mechanisms have been implicated in TFAM regulation, including post-transcriptional changes involving micro-RNA, interactions with key regulating factors (such as NRF-1), and post-translational modifications (such as acetylation, phosphorylation, ubiquitination). Interestingly, TFAM has been found upregulated and overexpressed in glioma cell lines and glioma tissue specimens. The role of TFAM in mtDNA regulation has yet to be fully understood.

RNA sequencing was performed in a series of 43 GBM samples and differential expression levels of POLG and TFAM were not statistically different between the High and Low groups. We did not investigate other nuclear genes encoding key factors implicated in mtDNA replication (e.g. mtDNA helicase TWINKLE, mitochondrial single-stranded binding protein (mtSSB), or mitochondrial RNA polymerase POLRMT). Since POLG and TFAM methylation levels do not correlate with mtDNA levels in the present study, investigating the involvement of other key players in mtDNA replication is necessary and will be part of future experiments. Besides, mtDNA replication (hence mtDNA copy number) has been found to differ according to the underlying mitochondrial haplogroups. mtDNA sequencing on a larger cohort is needed to assess the potential contribution of these haplogroups to the differences in mtDNA copy number in GBM.

In the older adult group, low mtDNA copy number was associated with longer overall survival compared to high mtDNA copy number (14.5 vs 10.2 months; \( P = 0.0116 \)). This result may be explained by profound tumor metabolism changes during aging. The latter has been shown to alter mitochondria and cell metabolism. Numerous studies reported accumulation of mtDNA deletions and mutations during the aging process, especially in tissues characterized by high energy demand, such as the brain. Age-dependent mtDNA alterations are associated with decreased mitochondrial function and decreased oxidative metabolism. Given the differences in metabolism observed between cells from the same tissue according to age, one tumor arising at two different time points will depend on the metabolic characteristics of the tissue of origin and its microenvironment. It can thus be hypothesized that high mtDNA copy number and mitochondria-related metabolism may not be as advantageous for tumors arising in older patients compared to young ones.

High-throughput (NGS) sequencing of mtDNA in 28 (12 %) GBM did not show any large mtDNA rearrangement and showed very few somatic variants in older adult GBM. Larman et al. reported the presence of mtDNA pathogenic mutations in only 13 % of primary GBM. The extremely rapid tumor growth in primary GBM may not allow the accumulation of somatic mtDNA mutations in opposition to slow-growing tumors such as colorectal or prostate cancers. However, the limited number of GBM cases sequenced in our study may have hindered the detection of mtDNA alterations.

In conclusion, high mtDNA copy number is an independent prognostic factor associated with longer overall survival in young GBM patients. The prognostic value of mtDNA copy number may be independent from the underlying genetic alterations. Low mtDNA copy number was associated with better outcome in older patients, suggesting age-related mitochondrial/metabolic changes. These results need to be validated in larger cohorts. Comprehensive characterization of the methylation patterns of mtDNA copy number regulatory genes using high-throughput methyl-sequencing would help assess the impact of epigenetics on mtDNA content. GBM metabolism remains to be thoroughly evaluated to determine whether mtDNA copy number reflects metabolic reprogramming during tumorigenesis or rather regulates tumor metabolism.

**Supplementary material**

Supplementary data are available at Neuro-Oncology Advances online.

**Keywords**

glioblastoma | metabolism | methylation | mitochondrial DNA | prognosis
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