Neurosciences

Mitochondrial DNA Content Modulates Chemosensitivity and Cellular Adaptation in Glioblastoma Cells

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ABSTRACT

Background: Mitochondrial DNA (mtDNA) copy number alterations are closely associated with glioblastoma (GBM) progression and therapeutic resistance. This study investigates how changes in mitochondrial content affect the chemotherapeutic response of GBM cells in vitro.

Methods: U87 and LN18 cell lines were first treated with ethidium bromide (EtBr) to deplete mtDNA, followed by withdrawal to allow mtDNA restoration. Cells were subsequently exposed to temozolomide (TMZ) at concentrations determined by preliminary IC50 analysis, with treatment conditions optimized for each experiment. Real-time polymerase chain reaction (PCR) quantified mtDNA levels. Cell viability was evaluated using the CCK-8 assay, whereas wound-healing and Transwell assays assessed migration and invasion. Apoptosis was examined by flow cytometry with Annexin V-FITC/PI kit staining. Reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi$m), and adenosine triphosphate (ATP) levels were also measured using flow cytometry and luminescent assays.

Results: GBM cells with depleted mtDNA exhibited an increase in mtDNA content following TMZ exposure. Despite this, these cells showed reduced viability, migration, and invasion, while also displaying adaptive responses suggestive of emerging resistance. In contrast, cells in which mtDNA content had been restored demonstrated partial recovery across all parameters, indicating minimal sensitivity to TMZ.

Conclusion: This study highlights the essential role of mtDNA content in modulating GBM cell sensitivity to chemotherapy, as variations in mtDNA levels can influence cellular responses to treatment. These findings suggest that mtDNA content could act as a potential biomarker and that its modulation may offer a promising strategy to overcome drug resistance in GBM.

Keywords: Glioblastoma, mitochondrial DNA copy number, mtDNA depletion, temozolomide sensitivity

Introduction

Glioblastoma (GBM) is the most aggressive primary brain tumor, marked by rapid growth, therapy resistance, and poor prognosis. Its global incidence ranges from 0.76 to 4.8 per 100 000 individuals annually, with GBM comprising up to 48% of primary brain tumors in the United States.[1] Despite maximal surgery, radiotherapy, and chemotherapy, median survival remains low, with only 2% of patients surviving beyond 3 years and 0.71% reaching 10-year survival.[2] The World Health Organization classifies GBM as a Grade 4 malignant tumor due to its high malignancy and unfavorable clinical outcomes.[1,2]

Temozolomide (TMZ), an orally bioavailable alkylating agent, is a first-line treatment for GBM.[3] It produces an active metabolite that methylates DNA, inducing DNA damage, cell cycle arrest, and apoptosis, particularly in highly proliferative tumor cells. Temozolomide crosses the blood-brain barrier, achieving 30%-40% effective concentrations within the central nervous system (CNS).[4] While it can delay glioma recurrence, its clinical efficacy is limited by a short half-life, chemical instability, and intrinsic or acquired resistance of GBM cells, which substantially impair long-term therapeutic outcomes.[3]

Mitochondria play a crucial role in modulating chemotherapeutic sensitivity in GBM cells. Beyond energy production, they regulate reactive oxygen species (ROS), calcium homeostasis,

and apoptosis. Mitochondria contain their own 16.5 kb circular genome, mitochondrial DNA (mtDNA), which is independent of nuclear DNA and encodes essential elements for mitochondrial gene expression and replication.

Each cell contains hundreds to thousands of mtDNA copies, depending on its type and metabolic demand. Alterations in mtDNA copy number are linked to mitochondrial myopathies, neurodegenerative disorders, aging, and various cancers.[5,6] The recent analysis revealed elevated mtDNA levels in brain tumors compared to matched blood, correlating with prolonged survival, particularly in high-grade tumors.[7] Hence, mtDNA copy number serves as a promising diagnostic and prognostic indicator of mitochondrial health. Notably, changes in mitochondrial content occur following TMZ treatment, and mtDNA depletion is often achieved using ethidium bromide (EtBr).[8]

Low concentrations of EtBr selectively inhibit mtDNA replication without affecting the nuclear genome, allowing the study of cellular adaptations to mitochondrial dysfunction.[8] Mitochondrial DNA depletion alters cellular responses to TMZ, affecting redox balance, apoptosis, and DNA repair.[4] Additionally, transient mtDNA loss enhances invasiveness and apoptosis resistance, promoting tumor progression and metastasis.[9,10]

Altered mtDNA content contributes to the metabolic adaptability of cancer cells under treatment stress.[8,11] However, the response of mtDNA-depleted GBM cells to TMZ remains unclear. This study examines how EtBr-induced mtDNA depletion affects TMZ efficacy, offering insights into mitochondrial mechanisms underlying treatment response.

Material and Methods

All experimental procedures were conducted in the cell culture and molecular research laboratories of the Department of Neurosciences, Universiti Sains Malaysia. Ethical approval and informed consent were not required for this study, as it did not involve any human participants, human tissues, or animal models. The experiments were performed exclusively using established human-derived cell lines obtained from the American Type Culture Collection (ATCC), a recognized public cell resource. These anonymized cell lines are commercially supplied for research purposes and, in accordance with institutional regulations, do not require ethics committee approval or informed consent.

Main Points

- Alterations in mitochondrial DNA (mtDNA) copy number have been strongly associated with glioblastoma (GBM) progression and diminished responsiveness to chemotherapy.
- Mitochondrial DNA–depleted GBM cells exhibited elevated mtDNA levels following temozolomide (TMZ) treatment but showed reduced viability, migration, and invasion, along with adaptive changes indicative of emerging drug resistance.
- Glioblastoma cells with restored mtDNA content displayed partially recovered cellular functions and minimal responsiveness to TMZ, underscoring the pivotal role of mitochondrial integrity in modulating chemotherapeutic sensitivity.

Cell Culture and Ethidium Bromide Treatment

Human GBM cell lines (U87 and LN18) were sourced from the ATCC (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle's Medium with 10% FBS, 1% penicillin-streptomycin, 1% Minimum Essential Medium (MEM) non-essential amino acids, 4.5 g/L glucose, L-glutamine, and sodium pyruvate at 37°C, 5% CO_2. To establish cells with varying mtDNA levels, GBM cells were treated with 50 ng/mL EtBr for 4 days, followed by culturing in EtBr-free medium for another 4 days to allow recovery. Total DNA was then extracted using the Geneaid Isolation Kit (Geneaid Biotech Ltd., Taiwan) and stored at –80°C.

Determination of Half Maximal Inhibitory Concentration (IC_{50})

Cells (5×10^3/well) were seeded in 96-well plates and incubated for 24 hours before treatment with 50-1000 µM TMZ (Sigma-Aldrich, St. Louis, MO, USA) for 48 hours. Cell viability was assessed using Cell Count Reagent SF (CCK-8) (Nacalai Tesque Inc., Kyoto, Japan). Absorbance at 450 nm was measured with a Tecan Sunrise microplate reader (Tecan, Zürich, Switzerland), and IC_{50} values were determined from dose-response curves.

Determination of Mitochondrial DNA Copy Number

The relative mtDNA copy number was measured by quantitative real-time PCR (qPCR) and calculated as the ratio of the mitochondrial gene (mt-ND1) to the nuclear gene (β-actin).[12,13] Quantitative real-time PCR was performed using 2× Brilliant III SYBR Green Master Mix with Low ROX on an Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA). ND1 primers (5′-TCTCACCATCGCTCTTCTAC-3′; 5′-TTGGTCTCTGCTAGTGTGGA-3′) and β-actin primers (5′-CATGTGCAAGGCCGG CTTCG-3′; 5′-CTGGGTCATCTTCTCGCG GT-3′) were used as mitochondrial and nuclear targets, respectively.[14] Quantitative real-time PCR conditions were 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 20 seconds. Samples were run in triplicate, and relative mtDNA content was calculated using the $2 \times 2^{-\Delta CT\ (CtmtDNA\ -\ CtnDNA)}$ and $2^{-\Delta\Delta CT}$ methods.

Cell Viability Assay

Cells with different mtDNA levels (5×10^3/well) were seeded in 96-well plates, allowed to adhere for 24 hours, and treated with TMZ for 24 or 48 hours. Cell viability was then assessed using Cell Count Reagent SF (CCK-8) (Nacalai Tesque Inc., Kyoto, Japan).

Wound-Healing Assay

Cells with different mtDNA levels (1.5×10^5/well) were cultured in 24-well plates for 24 hours to form monolayers. Linear scratches were made using a 100 µL pipette tip, and debris was removed with 1 × PBS. Cells were treated with TMZ in fresh medium, and scratch areas were imaged at 0, 24, and 48 hours under 100× magnification. Wound widths were manually measured using ImageJ software (v1.52a, U.S. National Institutes of Health, Bethesda, Maryland, USA) to evaluate cell migration.

Migration and Invasion Assay

Cell invasion was assessed using Transwell inserts with 8-µm pore membranes (Nest Scientific Inc., Rahway, NJ, USA). The membranes were initially pre-coated with Geltrex basement membrane matrix (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C for 1 hour. Serum-starved U87 (3×10^4) and LN18 (1×10^5) cells were seeded into the upper chamber, with medium containing 10% FBS in the lower chamber. After 24 hours, migrated or invaded cells were fixed with methanol, stained with crystal violet, and quantified microscopically under 100× magnification.

Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential ($\Delta\psi m$) was assessed using the JC-1 fluorescent probe (Elabscience, Wuhan, Hubei, China) according to the manufacturer's protocol. Treated cells (1×10^8) were incubated with JC-1 at 37°C for 20 minutes and analyzed using a BD FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA, USA), where reduced red-to-green ratios indicated mitochondrial depolarization.

Measurement of Cellular ATP Levels

ATP production was measured in cells with different mtDNA levels (1.2×10^4/well) cultured in 96-well white plates and treated with TMZ for 24 hours. Levels were quantified

using the Luminescent ATP Detection Assay Kit (Abcam, Cambridge, MA, USA) and read on a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Intracellular Reactive Oxygen Species Assay

Intracellular ROS levels were assessed using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe (Elabscience, Wuhan, Hubei, China). Treated cells were incubated with 20 μM probe at 37°C for 45 minutes, washed, and analyzed by BD FACSCanto II Flow Cytometry (BD Biosciences, San Jose, CA, USA). Fluorescence intensity was quantified using FlowJo v10.9 (FlowJo LLC, Ashland, OR, USA).

Apoptosis Assay

Apoptotic cell death was evaluated using the Annexin V-FITC/PI Apoptosis Kit (Elabscience, Wuhan, Hubei, China). Treated cells were harvested, stained with Annexin V-FITC and PI, and analyzed with a BD FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA, USA) to distinguish viable, early apoptotic, and late apoptotic or necrotic cells.

Statistical Analysis

Data were analyzed using SPSS v27.0 (IBM Corp., Armonk, NY, USA). Data distribution was assessed for normality using the Kolmogorov–Smirnov test, and the homogeneity of variances was evaluated using Levene's test. Parametric analyses were applied only when these assumptions were satisfied. For non-normally distributed data, differences in mtDNA content were assessed using the Kruskal–Wallis test, followed by Dunn's post-hoc comparisons with Bonferroni adjustment. For normally distributed data, 1-way analysis of variance (ANOVA) with Dunnett's post hoc test was applied for comparisons relative to the control group. Wound healing progression was analyzed using 2-way repeated measures ANOVA to evaluate the effects of treatment and time, with independent *t*-tests applied for pairwise group comparisons at each time point. Results are presented as mean ± SD, with significance at $P < .05$.

Results

Higher Half Maximal Inhibitory Concentration in Glioblastoma Cells

In this study, the cytotoxicity of TMZ in GBM cells was evaluated using a CCK-8 assay after 48 hours. U87 cells exhibited a higher IC_{50} (930 μM) than LN18 cells (620 μM), indicating lower sensitivity to TMZ, likely due to inherent molecular differences. These IC_{50} values were used as standardized doses for subsequent experiments (Figure 1A).

Restoration of Mitochondrial DNA Content in Mitochondrial DNA-Depleted Glioblastoma Cells After Temozolomide Treatment

Mitochondrial DNA depletion in GBM cell lines was successfully induced by treating the cells with EtBr for 4 days, as it intercalates between mtDNA base pairs. Lacking protective histones, mtDNA is structurally disrupted by EtBr, which impairs replication and transcription and progressively reduces mtDNA content over time.

Mitochondrial DNA depletion was confirmed by qPCR using mtDNA-specific primers, with nuclear genes serving as internal controls. Previous studies have shown that prolonged low-dose treatment with EtBr selectively depletes mtDNA, and this effect is reversible upon EtBr withdrawal.[15] In contrast, nuclear DNA, protected by histones and robust repair mechanisms, is minimally affected, with nuclear gene levels remaining consistent between parental and EtBr-treated cells, indicating selective mtDNA depletion without disrupting nuclear DNA replication.

To examine the effect of mtDNA depletion on drug sensitivity, LN18 and U87 cells were co-treated with EtBr and TMZ, followed by measurement of mtDNA levels (Figure 1B). In U87 cells, TMZ treatment increased mtDNA copy number to 2.70-fold relative to untreated controls. In the mtDNA-depleted group, mtDNA content decreased to 0.51-fold but rose to 1.97 following TMZ exposure. Similarly, the mtDNA recovery group exhibited partial restoration of mtDNA content (0.74-fold), which markedly increased to 5.43-fold after TMZ treatment. In contrast, LN18 cells displayed a more pronounced reduction in mtDNA content. TMZ treatment alone decreased mtDNA copy number to 0.26-fold relative to control. mtDNA-depleted LN18 cells showed a dramatic reduction to 0.02-fold, with a modest increase to 0.09-fold following TMZ exposure. The mtDNA recovery group demonstrated minimal restoration to 0.09-fold, which further declined to 0.07-fold after TMZ treatment.

Intriguingly, TMZ exposure increased mtDNA copy number in mtDNA-depleted cells in both U87 and LN18 cells, suggesting a potential adaptive mechanism to preserve mitochondrial function under chemotherapy-induced stress. Overall, these findings indicate that TMZ modulates mtDNA content differently across GBM cell lines, revealing distinct cellular responses to treatment.

Reduced Mitochondrial DNA Content Diminishes Cell Viability in Glioblastoma Cells

The functional impact of mtDNA depletion on GBM cell viability was assessed using the CCK-8 assay in U87 and LN18 cells treated with TMZ (Figure 2). TMZ reduced viability in both U87 (72.0% ± 2.54%) and LN18 (68.7% ± 1.72%) cells, with greater reduction in mtDNA-depleted cells (U87: 62.1% ± 1.61%; LN18: 64.5% ± 0.50%). Cells with restored mtDNA content showed increased viability (U87: 70.6% ± 1.46%; LN18: 73.6% ± 0.26%), suggesting a partial reversal of mitochondrial dysfunction resulting from EtBr-induced mtDNA depletion.

At 48 hours, TMZ-treated parental cells showed reduced viability (LN18: 53% ± 0.72%; U87: 52% ± 0.39%), with mtDNA-depleted cells exhibiting further decline (LN18: 49.3% ± 0.05%; U87: 47.3% ± 1.84%). In contrast, cells with restored mtDNA maintained higher viability (LN18: 67.2% ± 0.21%; U87: 61.6% ± 0.29%), consistent with the pattern observed at 24 hours.

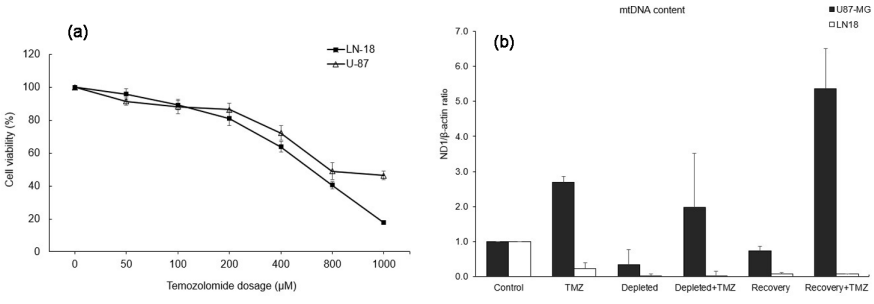

Figure 1. Effects of ethidium bromide (EtBr) and temozolomide (TMZ) on mitochondrial DNA (mtDNA) content in glioblastoma (GBM) cell lines. A. Determination of the half-maximal inhibitory concentration (IC_{50}) for U87 and LN18 cell lines. Dose-response curves indicated a higher IC_{50} for TMZ in U87 cells (930 μM) compared to LN18 cells (620 μM). B. Comparison of mtDNA content between U87 and LN18 cell lines, and the effect of TMZ on mtDNA levels in EtBr-induced GBM cells. Low-dose EtBr treatment reduced mtDNA content in U87 and LN18 cells, whereas TMZ increased mtDNA levels in mtDNA-depleted U87 and LN18 cells. Data are presented as mean ± SD (n = 3) (***$P < .001$ vs. control).

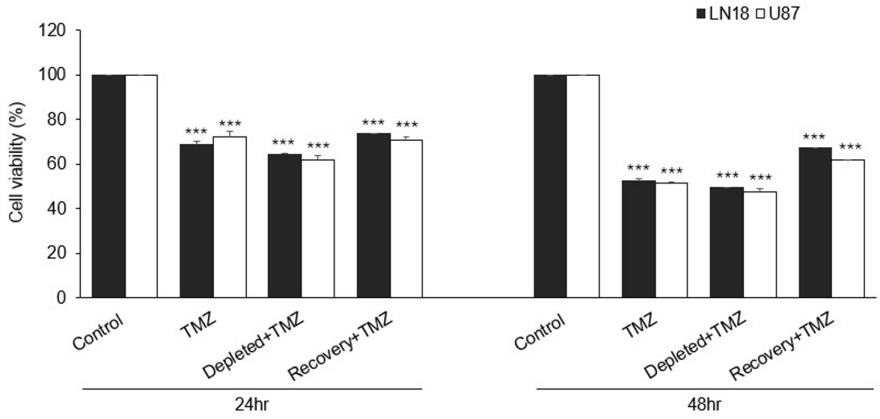

Figure 2. Cell viability assay of glioblastoma (GBM) cell groups following temozolomide (TMZ) treatment. TMZ reduced cell viability in all GBM groups, compared with untreated controls, with a more pronounced reduction observed in the mtDNA-depleted groups. Restoration of mtDNA content in the recovery-phase LN18 and U87 cells significantly improved cell viability. Data are presented as mean ± SD (n = 3) (*** $P < .001$ vs. control).

Temozolomide Inhibits Migration and Invasion of Glioblastoma Cells with Depleted Mitochondrial DNA Content

Highly invasive cells drive aggressive GBM progression, and mitochondrial dysfunction can impair energy-dependent processes such as cell motility. To assess this, a wound healing assay was performed on mtDNA-depleted U87 and LN18 cells following TMZ exposure (Figure 3A). After 24 hours under untreated conditions, wound closure increased from parental cells (LN18: 33.4% ± 3.97%; U87: 48.5% ± 8.17%) to mtDNA-depleted cells (LN18: 39.9% ± 3.23%; U87: 52.1% ± 8.08%) and recovery cells (LN18: 24.8% ± 5.40%; U87: 59.3% ± 7.36%). By 48 hours, closure further increased: parental (LN18: 64.0% ± 1.87%; U87: 73.7% ± 2.41%), depleted (LN18: 82.9% ± 0.57%; U87: 78.5% ± 2.99%), and recovery cells (LN18: 74.0% ± 9.17%; U87: 77.6% ± 3.02%), highlighting the influence of mtDNA content on migratory capacity.

TMZ treatment significantly impaired migration in parental GBM cells, reducing wound closure to 28.3% ± 5.90% in LN18 and 31.1% ± 3.90% in U87, indicating suppression of metastatic behavior. Mitochondrial DNA-depleted cells also showed reduced migration (LN18: 25.5% ± 1.40%; U87: 42.2% ± 5.07%). In contrast, mtDNA-recovered U87 cells exhibited modestly increased migration (46.5% ± 5.38%), while recovered LN18 cells remained relatively stable (24.2% ± 3.92%), suggesting limited restoration of motility in LN18.

At 48 hours, TMZ-treated U87 and LN18 cells showed further wound closure, indicating sustained migratory activity. Despite overall increases over time, the relative differences between parental, mtDNA-depleted, and mtDNA-recovered groups remained consistent, highlighting the persistent impact of mtDNA status on cell migration under TMZ treatment.

Transwell invasion assays (Figure 3B) confirmed that TMZ significantly reduced the migratory and invasive capacities of U87 and LN18 cells. Mitochondrial DNA-depleted cells treated with TMZ exhibited marked decreases in both behaviors, while mtDNA-recovered cells gradually regained migratory and invasive activity, consistent with wound healing assay results.

Temozolomide-Induced Alterations in Mitochondrial Function in Mitochondrial DNA-Depleted Glioblastoma Cells

Mitochondrial $\Delta\psi m$ is a key indicator of oxidative phosphorylation and cellular energy status.[16] Since mtDNA-depleted cells are expected to have impaired respiration, $\Delta\psi m$ and ATP production following TMZ treatment were assessed. JC-1 staining revealed distinct $\Delta\psi m$ loss in TMZ-treated LN18 and U87 cells, indicating differential mitochondrial dysfunction between the 2 lines (Figure 4A).

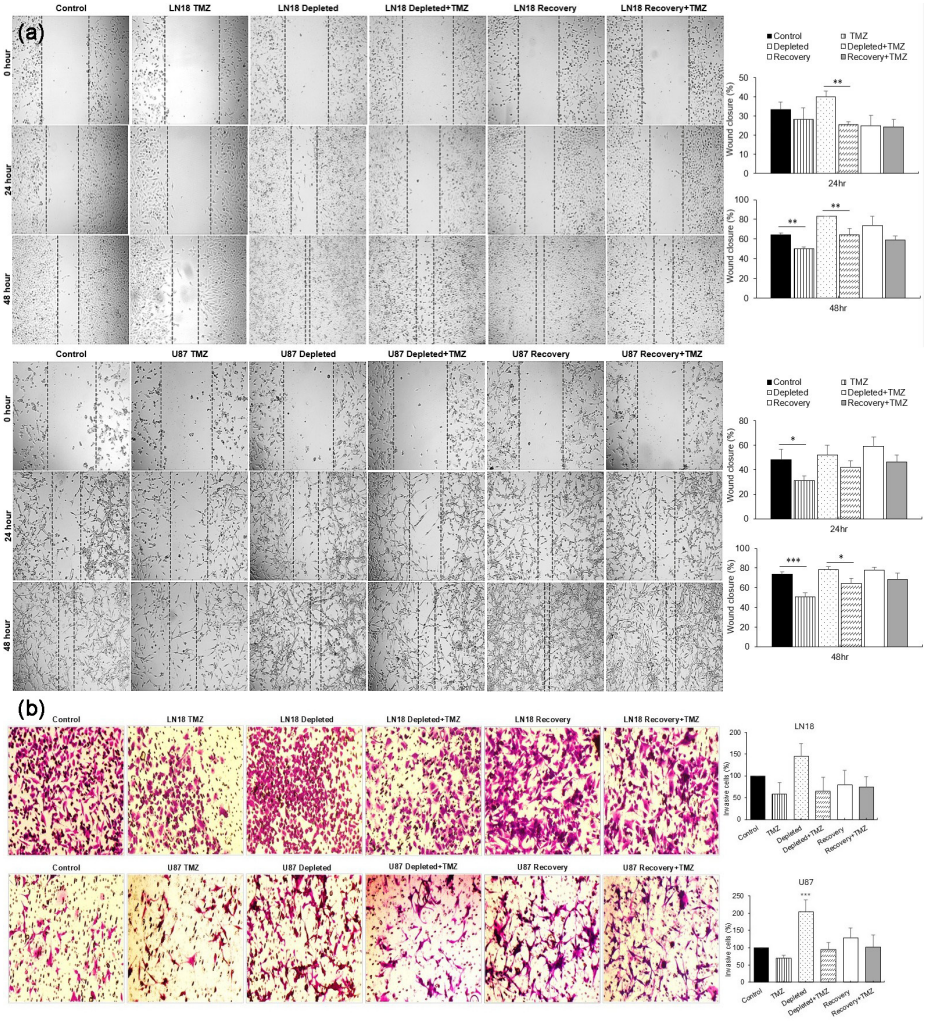

Figure 3. Effects of temozolomide (TMZ) treatment on cell migration and invasion in glioblastoma (GBM) cells with varying mitochondrial DNA (mtDNA) content. A) Representative images and bar graph of the scratch wound healing assay in LN18 and U87 cells. B) Representative images and bar graph of LN18 and U87 cells that migrated or invaded into the lower chamber of the Transwell. Data are presented as mean ± SD (n = 2) (*** $P < .001$ vs. control).

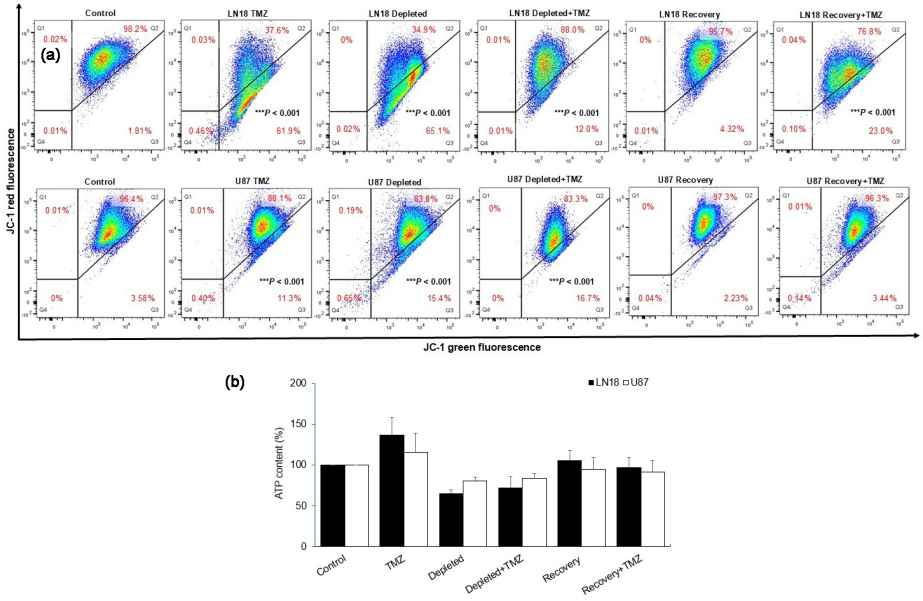

Figure 4. Effects of temozolomide (TMZ) treatment on mitochondrial function in glioblastoma cells with varying mitochondrial DNA content. A) A loss of mitochondrial ΔΨm was indicated by a shift to green fluorescence. Representative data are shown for LN18 and U87 cells (n = 3). B) Intracellular ATP levels in LN18 and U87 cells are presented, illustrating the differences among the experimental groups (n = 5). Data are expressed as mean ± SD (*** $P < .001$ vs. control).

Under untreated conditions, LN18 cells exhibited minimal mitochondrial Δψm depolarization (1.81% ± 0.70%). Ethidium bromide-induced mtDNA depletion caused substantial Δψm loss (65.13% ± 2.45%), whereas mtDNA-recovered cells showed improved mitochondrial function, with depolarization reduced to 4.32% ± 1.30%. Following TMZ treatment, LN18 cells showed marked mitochondrial Δψm loss (61.9% ± 1.10%). Mitochondrial DNA-depleted cells exhibited hyperpolarization with minimal depolarization (12.0% ± 2.10%), suggesting an adaptive response, while mtDNA-recovered cells showed a moderate Δψm decline (23.0% ± 3.91%), indicating partial restoration of mitochondrial function.

In U87 cells, mitochondrial Δψm remained largely intact. Untreated cells showed minimal depolarization (3.58% ± 1.88%), while mtDNA-depleted cells exhibited mild depolarization (15.4% ± 1.60%), indicating limited EtBr impact. Mitochondrial membrane potential in recovery cells remained stable, comparable to untreated cells, suggesting effective restoration of mitochondrial function.

Following TMZ treatment, U87 cells exhibited mild mitochondrial depolarization (11.3% ± 1.60%), indicating a less responsive phenotype. Mitochondrial DNA-depleted cells treated with TMZ showed similar depolarization (16.7% ± 1.35%), suggesting no further mitochondrial impairment, while mtDNA-recovered cells displayed a slight reduction in depolarization (3.44% ± 0.64%).

Disruption of mitochondrial Δψm negatively impacted ATP production in both GBM cell lines, with ATP levels reflecting Δψm changes. The reduction was more pronounced in LN18 cells, consistent with their greater mitochondrial depolarization (Figure 4B).

ROS regulate both cell survival and death, with elevated ROS damaging mtDNA and mitochondrial function, creating a cycle that promotes apoptosis and chemotherapy resistance.[17] To assess mtDNA depletion effects under TMZ, ROS levels were measured in GBM cells. Mitochondrial DNA-depleted LN18 and U87 cells showed increased ROS (LN18: 111.2% ± 20.54%; U87: 116.2% ± 19.39%). In recovery groups, ROS further increased in LN18 cells (136.2% ± 24.17%) but decreased in U87 cells (92.5% ± 8.50%), highlighting differential oxidative responses based on mtDNA content (Figure 5A).

TMZ treatment increased ROS levels in LN18 cells (110% ± 19.31%), indicating oxidative stress, whereas ROS decreased in U87 cells (91.4% ± 15.66%), suggesting differential TMZ responses. Mitochondrial DNA-depleted cells showed reduced ROS (LN18: 93.9% ± 8.52%; U87: 88.5% ± 8.12%), indicating protective mechanisms, while recovery-phase cells exhibited significant ROS elevation (LN18: 149.0% ± 25.54%; U87: 111.8% ± 10.98%) post treatment.

Elevated ROS levels corresponded with increased apoptosis in TMZ-treated GBM cells (Figure 5B). Mitochondrial DNA-depleted LN18 and U87 cells showed higher apoptosis (LN18: 25.9% ± 3.63%; U87: 14.7% ± 2.39%) vs. untreated controls. Recovery-phase cells exhibited reduced apoptosis (LN18: 16.0% ± 3.46%; U87: 11.0% ± 1.09%). Temozolomide increased apoptosis in parental cells (LN18: 19.5% ± 2.95%; U87: 12.4% ± 1.01%), whereas mtDNA-depleted cells showed lower rates (LN18: 18.3% ± 2.10%; U87: 10.2% ± 0.76%). Mitochondrial DNA-recovered LN18 cells maintained consistent apoptosis (17.7% ± 3.60%), while U87 recovery cells showed increased apoptosis (14.6% ± 2.01%) post treatment.

Discussion

In recent years, mtDNA content has been increasingly recognized as a key determinant of cancer cell sensitivity to multiple chemotherapeutic agents.[5,18] Accordingly, this study examined how variations in mtDNA levels influence the responsiveness of GBM cells to these treatments. The major finding of this study is that EtBr-induced mtDNA depletion predominantly conferred TMZ resistance in both U87 and LN18 cell lines, accompanied by reduced proliferation and altered mitochondrial function.

It is worth noting that most high-grade gliomas harbor lower mtDNA content compared with non-cancerous brain tissues,[8,19] with the lowest levels observed in GBM.[20,21] In this study, U87 and LN18 cells exhibited distinct alterations in mtDNA content following TMZ treatment, indicating that the response is governed by cell type-specific regulatory mechanisms. Both the parental and recovery groups of LN18 cells displayed a reduction in mtDNA levels after TMZ exposure, suggesting that LN18 cells are more susceptible to the chemotherapeutic treatment than U87 cells. These observations are consistent with earlier reports showing that LN18 cells possess greater sensitivity to TMZ compared with U87 cells.[22] Recent studies have reported that U87 cells are partially resistant to TMZ rather than strictly sensitive, due to increased expression of cyclooxygenase-2, a known mediator of chemoresistance.[23] Additionally, TMZ has been reported to elevate mtDNA copy number in SHG44 GBM cells, consistent with the increased mtDNA levels observed in U87 cells.[24]

Interestingly, exposure to TMZ led to an elevation in mtDNA levels in EtBr-depleted U87 and LN18 cells. These observations suggest that treatment-induced acute stress may regulate mtDNA biogenesis as part of a cellular defense or compensatory response.[25] Notably, increased mtDNA content has previously been linked to an adaptive mechanism aimed at

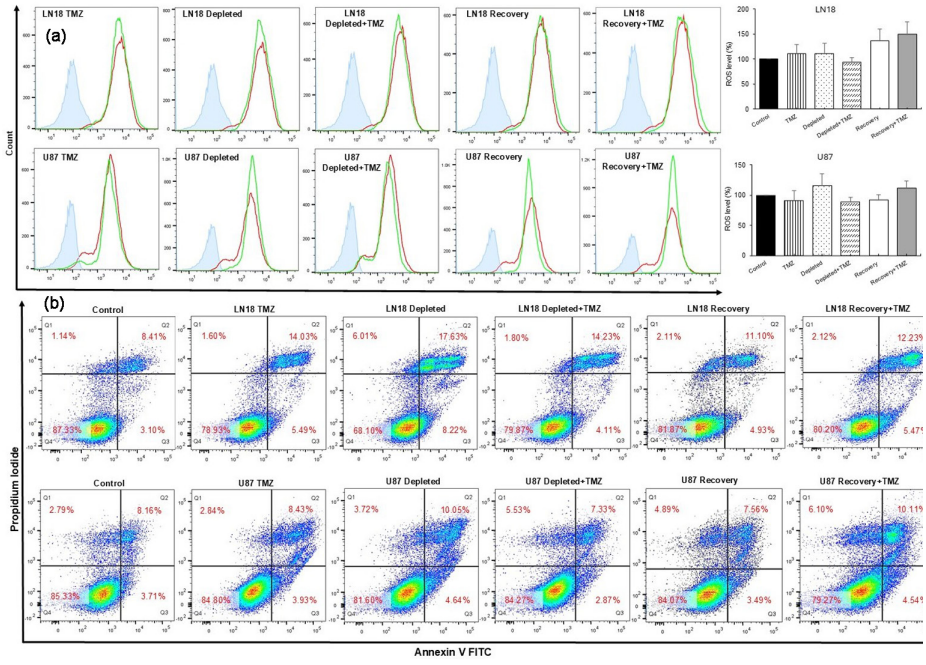

Figure 5. Detection of reactive oxygen species (ROS) and apoptosis in glioblastoma cells with varying mitochondrial DNA content following 24-hour temozolomide (TMZ) treatment. A) The ROS levels in LN18 and U87 cell groups are illustrated, with the red line indicating untreated controls and the green line representing TMZ-treated cells. B) The numbers in each quadrant indicate the percentages of viable (Q4: Annexin$^-$/PI$^-$), early apoptotic (Q3: Annexin$^+$/PI$^-$), late apoptotic (Q2: Annexin$^+$/PI$^+$), and necrotic (Q1: Annexin$^-$/PI$^+$) cells, respectively. The total apoptotic cells were calculated by combining the percentages from the lower right (early apoptotic, Q3) and upper right (late apoptotic, Q2) quadrants. Data are presented as mean ± SD values from 3 independent experiments (n=3).

preserving mitochondrial function.[25] However, chemically induced mtDNA depletion has been proposed not to produce a uniform reduction across all cells within a population.[26] In this context, although the overall mtDNA levels in U87 and LN18 cells were reduced, a subset of cells may have retained relatively higher mtDNA content, potentially conferring a survival advantage under stress conditions such as chemotherapy.

Furthermore, the divergent mtDNA content observed in these GBM cells may have a substantial impact on their behavioral and metabolic activity. Indeed, TMZ has been proposed to induce phenotypic shifts in differentiated GBM toward therapy-adapted states that share features commonly reported in cancer stem-like cells (CSCs), particularly chemoresistance and metabolic reprogramming, which have been associated with altered mtDNA content.[27,28] In the present study, mtDNA-depleted cells exhibited a significant decline in viability following TMZ exposure. Notably, although these cells displayed aggressive migratory and invasive behaviors under baseline conditions, such behaviors were markedly attenuated under TMZ-induced stress. In line with these findings, several studies have reported that EtBr-induced mtDNA depletion renders GBM cells more resistant to TMZ, accompanied by a substantial reduction in proliferation rate.[8,11,29] Moreover, cancer cells have been shown to exhibit reduced proliferation, migration, and invasion following TMZ treatment.[30,31] Together, these observations suggest a shift toward a stress-adapted and less proliferative cellular state in response to chemotherapy, potentially contributing to altered treatment outcomes.

In this study, TMZ treatment induced significant changes in mitochondrial functions in GBM cells, with the effect being most pronounced in the LN18 cell line. These findings revealed that GBM cells with markedly depleted mtDNA initially exhibited reduced $\Delta\psi$m and ATP levels. However, both parameters increased following TMZ exposure, suggesting a potential metabolic adaptation involving upregulated oxidative phosphorylation (OXPHOS) to counter cellular stress. Supporting this, He et al[32] demonstrated that TMZ-resistant glioma cells preserve mitochondrial membrane stability by minimizing depolarization, whereas Oliva et al[11] observed that cells with reduced mtDNA copy numbers displayed suppressed glycolytic activity accompanied by increased ATP synthesis. Moreover, these cells also exhibited reduced ROS levels accompanied by a lower rate of apoptosis compared with untreated mtDNA-depleted cells, suggesting the activation of a cellular survival mechanism that confers resistance to therapeutic stress. Chen et al[33] also reported decreased ROS production and diminished apoptosis in mtDNA-deficient human osteosarcoma cell lines, indicating a survival advantage following exposure to UV irradiation. Therefore, GBM cells with depleted mtDNA appear to survive by limiting oxidative damage, depending on altered mitochondrial function to generate lower ROS levels while maintaining adequate ATP synthesis. This metabolic adaptation consequently appears to reduce their susceptibility to apoptosis. Nevertheless, restoration of mtDNA content appears to partially reverse the resistant phenotype observed in mtDNA-depleted cells. In mtDNA recovery cells, cellular responses shifted toward patterns closely resembling those of parental cells across multiple parameters, although full TMZ sensitivity was not completely achieved. Several similar studies have also reported comparable expression profiles between mtDNA-restored cells and untreated controls, suggesting a functional link between mtDNA integrity and the modulation of chemotherapeutic response.[13,16]

Alterations in mtDNA copy number have been associated with variable prognostic outcomes in GBM patients. Previous studies have shown that reduced mtDNA content correlates with poorer survival and increased treatment resistance in both clinical specimens and experimental models,[8] whereas elevated mtDNA content has been reported to predict longer survival in subsets of glioma patients.[21] These variations suggest that mtDNA copy number may serve as a valuable biomarker for treatment response. Accordingly, assessing mtDNA content dynamics could be leveraged to stratify patients or guide therapeutic decisions. Specifically, strategies aimed at modulating mitochondrial function or biogenesis, rather than directly increasing mtDNA content, may enhance the efficacy of standard chemotherapy. Although further validation is needed, the present findings highlight the regulation of mtDNA content as a promising avenue for improving GBM treatment outcomes.

Limitation and Future Directions

In the present study, the TMZ concentrations used in the CCK-8 viability assays exceeded physiologically achievable levels. Although high concentrations are commonly employed in vitro due to drug instability and the intrinsic resistance of established GBM cell lines, such dosing does not fully reflect clinical pharmacokinetics. Moreover, the CCK-8 assay primarily measures short-term changes in cell viability and may not accurately represent long-term clonogenic

survival following TMZ exposure. Thus, the observed TMZ responses should be interpreted as preliminary and comparative, reflecting relative differences in chemosensitivity associated with mtDNA copy number rather than direct translational efficacy. Future studies incorporating clonogenic assays and pharmacokinetic-compatible treatment regimens are warranted to validate and extend these findings. Furthermore, this study employed JC-1 dye to assess mitochondrial $\Delta\psi m$; however, potential interference from overlapping red fluorescence between JC-1 and EtBr might have influenced the accuracy in GBM cells. Nevertheless, a distinct difference in $\Delta\psi m$ responses between treated and untreated groups was evident, consistent with previous findings.[10] Future research should confirm these observations using alternative $\Delta\psi m$ probes that do not rely on red fluorescence, such as TMRE/TMRM or MitoTracker Green, to strengthen the results. Finally, mtDNA replenishment was achieved through recovery from EtBr-induced depletion rather than direct mitochondrial augmentation, resulting in only partial restoration. Future studies employing mitochondrial transfer approaches, such as mitoception or mitochondrial transplantation, are needed to elucidate the causal role of increased mtDNA content on TMZ sensitivity.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Artificial Intelligence Usage Statement: The authors declared that no Artificial Intelligence tool was used in the preparation of the manuscript.

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