Published online 2023 May 15 **Original Article**

# Facilitation of Temozolomide Resistance of Glioblastoma by Long Noncoding RNA DLK1-35

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**Received** 2022 October 22; **Revised** 2023 March 11; **Accepted** 2023 March 13.

**Abstract**

**Background:** Long noncoding RNAs played critical roles in glioblastoma development.
**Objectives:** This study aimed to examine the impacts of lncRNA DLK1-35 on glioblastoma cells and mice.
**Methods:** Methyl Thiazolyl Tetrazolium (MTT) was applied for examining the viabilities of U87 and U251 cells, as well as $IC_{50}$ values of temozolomide (TMZ). LncRNA DLK1-35 expressions were detected using RT-qPCR. Proliferation and apoptosis of TMZ-resistant U251 (U251 TR) cells were evaluated using colony formation and flow cytometry, respectively. Western blot was applied to analyze O6-methylguanine-DNA methyltransferase (MGMT) protein expressions. The xenograft model was used for detecting the weight and volume of tumors in mice.
**Results:** TMZ treatment suppressed the viabilities of glioblastoma cells dose-dependently. Moreover, TMZ-resistant glioblastoma cells had higher $IC_{50}$ values. lncRNA DLK1-35 was upregulated in TMZ-resistant cells while the suppression of lncRNA DLK1-35 caused low proliferation and a higher apoptosis rate. Moreover, MGMT was also inhibited by lncRNA DLK1-35 downregulation. Additionally, the weight and volume of tumors in mice were also inhibited with the knockdown of lncRNA DLK1-35.
**Conclusion:** Knockdown of lncRNA DLK1-35 inhibited MGMT to decrease the TMZ resistance in vitro and in vivo in glioblastoma.

*Keywords:* Delta-like non-canonical Notch ligand 1-35, Glioma, Temozolomide

## 1. Background

Glioblastoma multiforme (GBM) is an aggressive and common malignancy within the brain (1). Treatments for patients with GBM are surgical resection, radiotherapy, and chemical (2, 3). Only two drugs have been approved for GBM treatment, namely temozolomide (TMZ) for newly diagnosed GBM and bevacizumab for recurrent GBM (4, 5). TMZ, an oral alkylating agent was discovered in 1987 (6). Thousands of studies have verified the impacts of TMZ on eliminating cytotoxic mechanisms in GBM tumors, and TMZ treatment has also been reported to improve the quality of life in GBM patients in comparison with radiation alone [4]. Unfortunately, these approaches have limited effects on the prognosis of GBM patients due to increased resistance (7, 8). Therefore, elucidation about the mechanism of TMZ resistance in GBM might provide a new aspect for GBM treatment.

With the development of genetic technology, noncoding RNAs have attracted increasing attention in many kinds of malignancies. Long noncoding RNAs (lncRNAs) are RNA molecules over 200 nucleotides without protein-coding capacities (9, 10). Dysregulation of lncRNAs has been reported to be associated with GBM development and drug resistance. Long noncoding RNA small nucleolar RNA host gene 12 was promoted in TMZ-resistant GBM cells, which could elevate mitogen-activated protein kinase 1 and E2F transcription factor 7 via sponging miR-129-5p, resulting in TMZ resistance (11).

LncRNA RP3-439F8.1 upregulated nuclear receptor subfamily 5 group A member 2 in GBM cells through sponging miR-139-5p, leading to facilitated proliferative and invasive abilities, which also accelerated tumor growth in mouse models (12). LncRNA Delta-Like Non-Canonical Notch Ligand 1-35 (DLK1-35, Ensembl Transcript, ENST00000556720), a derived from imprinted DLK1-DIO3 domain, was also named lncRNA MEG8 (https://lncipedia.org/), which was demonstrated to be elevated in TMZ-resistant GBM cells (13). Nevertheless, the impacts of lncRNA DLK1-35 on modulating TMZ resistance in GBM were unclear. Hence, exploring the mechanisms of lncRNA DLK1-35 might provide a novel way to modulate TMZ resistance in GBM.

## 2. Objectives

This study aimed at exploring lncRNA DLK1-35 expressions in TMZ-resistant glioblastoma cells and its effects on modulating proliferation and apoptosis of U251TR cells and tumorigenesis in mouse models.

## 3. Methods

### *3.1. Main Reagents*

The main reagents in this study included

Dulbecco's modified Eagle's medium (Gibco, USA), fetal bovine serum (FBS) (Gibco), penicillin/streptomycin (Gibco), TMZ (Sigma-Aldrich, USA), lipofectamine 3000 (Invitrogen, USA), TRIzol reagent (Invitrogen, USA), PrimeScript™ RT reagent Kit (Takara, Japan), SYBR Green kit (Beyotime, Shanghai, China), MTT (Solarbio, Beijing, China), dimethyl sulfoxide (Beyotime), paraformaldehyde (Beyotime), crystal violet (Beyotime), Annexin V-FITC (Beyotime), anti-MGMT (1:1000; ab39253, Abcam, UK), anti-GAPDH (1:2000; ab9484), Goat Anti-Mouse IgG H&L (HRP) (1:2000; ab205719), and Western LightningTM Chemiluminescence Reagent (PerkinElmer, Wellesley, MA, USA).

### *3.2. Cell culture and establishment of cells with TMZ resistance*

Human glioblastoma cell lines (U87 and U251) were obtained from Saierbio (Tianjin, China) and cultivated by Dulbecco's modified Eagle's medium (Gibco, USA) with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C and 5% $CO_2$. To create TMZ-resistant cells, parental cells in the log phase were incubated with 10μM TMZ (Sigma-Aldrich, USA). During the cell culture process, mediums were replaced every three days. After cell confluence reached 80%, cells were passaged according to 1:2, repeating the above steps twice. Next, the cells were treated with TMZ (50, 100, 200 and 325 μM). TMZ-resistant cells (U87TR and U251TR) were established when cells survived in 325 μM TMZ for 2 months with normal activity.

### *3.3. Transfection*

LncRNA DLK1-35 siRNA (si-DLK1-35-1, -2, and -3) and the negative control (si-NC) were acquired from GenePharma (Shanghai, China). Meanwhile, TMZ-resistant cells were cultivated using a 6-well plate, and transfection was performed using lipofectamine 3000 (Invitrogen, USA) after the confluence of cells reached 70%. After transfection for 24h, lncRNA DLK1-35 expressions were assessed using RT-qPCR. Sequences of si-DLK1-35 were shown in Table 1.

**Table 1.** siRNA sequences

| Name | Sequence (5'→3') |
|---|---|
| **si-DLK1-35-1** | TTT CTG CTC CTT AGT CCA CAA |
| **si-DLK1-35-2** | CAA ATC CAA TCT CGT CTA TTC |
| **si-DLK1-35-3** | TTC ATC AGC AGA GAC TGA TTA |
| **si-NC** | GUG GAU AUU GUU GCC AUC A |

Abbreviations: si-siRNA; NC-negative control

### *3.4. RT-qPCR*

TRIzol reagent (Invitrogen, USA) was first applied for isolating total RNA, followed by synthesis of cDNA using PrimeScript™ RT reagent Kit (Takara, Japan). Thereafter, PCR was performed using the SYBR Green kit (Beyotime, Shanghai, China) and PikoReal 96 System (Thermo Scientific, USA). GAPDH was the normalization of lncRNA ELK1-35 expression, and the $2^{-\Delta\Delta ct}$ method was used for RNA expression calculation. Primers were listed in Table 2.

**Table 2**. Primer sequences

| Name | Sequence (5'→3') |
|---|---|
| **DLK1-35** | F: TGAACGCACTCTACCT |
| | R: ATTGCTCCTTACCACA |
| **GAPDH** | F: ACCACAGTCCATGCCATCAC |
| | R: TCCACCACCCTGTTGCTGTA |

Abbreviations: F-forward; R-reverse

### *3.5. MTT*

Cells were seeded onto a 96-well plate ($2\times10^3$ cells/well), followed by TMZ treatment. Thereafter, 20μl MTT (Solarbio, Beijing, China) was added and cultivated with cells for another 4h. Later, 150μl dimethyl sulfoxide (Beyotime) was added to dissolve crystal substances. Cell viabilities and $IC_{50}$ of TMZ were examined using a PHERAstar FSX microplate reader (BMG LABTECH, Germany).

### *3.6. Colony formation*

After transfection, U251TR cells were planted onto a 6-well plate ($1\times10^3$ cells per well) and incubated for 10 days with or without TMZ. Afterward, the cells were fixed with 4% paraformaldehyde (Beyotime) and stained with 0.1% crystal violet (Beyotime) for 20 min. Colonies were counted by a microscope.

### *3.7. Flow cytometry*

U251TR cells and TMZ-treated U251TR cells were collected after transfection. To measure cell apoptosis, Annexin V-FITC (Beyotime) was applied for detecting the apoptosis rate. After cells were coated by Annexin V-FITC buffer, Annexin V-FITC and propidium iodide were used for staining based on the protocol of the manufacturer. Finally, BD Accuri C6 (BD Biosciences, USA) was used for examining the apoptosis rate.

### *3.8. Western blot*

The cells after transfection and TMZ treatment were lysed using RIPA buffer (Beyotime). Thereafter, the protein was isolated with 10% SDS-PAGE and shifted onto PVDF membranes. Then, membranes were blocked by 5% fat-free milk powder and cultivated with anti-MGMT (1:1000; ab39253, Abcam, UK) and anti-GAPDH (1:2000; ab9484) at 4°C overnight. Next, Goat Anti-Mouse IgG H&L (HRP) (1:2000; ab205719) was applied for incubating with membranes for 2h at 25°C. Finally, blots were visualized by Western LightningTM Chemiluminescence Reagent (PerkinElmer, Wellesley, MA, USA), and image J was used for analyzing gray bands. GAPDH was used for normalization.

*3.9. Xenograft model*

This animal experiment was carried out with the approval of the Animal Welfare Committee of North China University of Science and Technology and all experiments involving animals were in accordance with the regulations of the institution. The 4-week-old female BALB/c nude mice were obtained from the Beijing Experimental Animal Center of the Chinese Academy of Medical Sciences, bred, and maintained in a specific pathogen-free facility. Then, they were subcutaneously injected into the left back with $1\times10^6$ U251TR cells and divided randomly into five groups (n=6) after 2 weeks. 24h post-intratumoral injection of transfection agents (si-NC/si-DLK1-35), 325 μM TMZ was injected. The injections were performed every three days for 25 days. Moreover, tumor volume was examined every 3 days and calculated by the formula: volume=length×(width2)/2. All mice were humanely sacrificed by overdose anesthesia, and the tumors were weighed.

*3.10. Statistical analysis*

All data were shown as mean±SD, and analysis was performed using SPSS software (version 19.0) (USA) and GraphPad 9 (USA). Student's t-test or ANOVA was conducted to detect pairwise comparison or multivariate analysis. A value of $P<0.05$ was considered to have significance.

## 4. Results

*4.1. Establishment of TMZ-resistant Glioblastoma Cells*

To investigate the effects of TMZ, different concentrations of TMZ were applied to treat U87 and U251 cells. MTT results indicated that the viabilities of these two were decreased dose-dependently (Figure. 1A, $^{**}P<0.05$). Thereafter, TMZ-resistant cells were created, showing that U87TR and U251TR cells had irregular polygons with unclear boundaries and cell surface protrusion reduction (Figure. 1B, $^{**}P<0.05$). Then, TMZ (325μM) was applied for treating parental cells and resistant cells, indicating that U87TR and U251TR cells had higher cell viability than their parental cells, respectively (Figure. 1C, $^{**}P<0.05$). Beyond that, different concentrations of TMZ were used to treat parental and resistant cells, revealing that U87TR and U251TR cell viabilities were higher than their parental cells while the inhibition rate was lower (Figure. 1D, E, $^{**}P<0.05$). Moreover, the $IC_{50}$ value was also higher in U87TR and U251TR cells (Figure. 1F, $^{**}P<0.05$). These results demonstrated that TMZ-resistant cells were established successfully.

*4.2. Downregulation of lncRNA DLK1-35 suppressed TMZ resistance in U251TR cells*

To explore whether lncRNA DLK1-35 modulated TMZ resistance in glioblastoma cells, its RNA expressions were examined, indicating that U87TR and U251TR cells had elevated lncRNA DLK1-35 RNA expressions (Figure. 2A, $^{**}P<0.05$). However, U87TR cells were polluted during cell cultivation. Therefore, U251TR cells were used for detecting the impacts of lncRNA DLK1-35. According to the knockdown efficiency, si-DLK1-35-2 (si-DLK1-35) was selected for the following experiments (Figure. 2B, $^{**}P<0.05$).

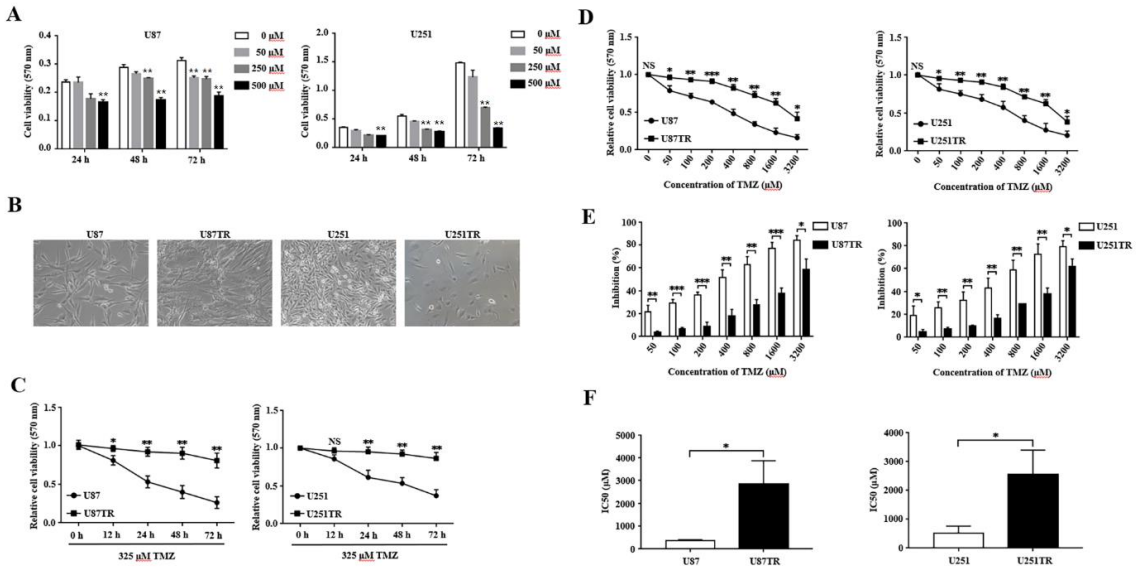


**Figure 1.** Establishment of TMZ-resistant human glioblastoma cell lines U87TR and U251TR. (A) The temozolomide (TMZ) sensitivity of U87 and U251 cells was measured by MTT assay at 24, 48, and 72 h. (B) Morphological characters of the indicated s cells (magnification 100×). (C) The cell viabilities were evaluated by MTT assay after treatment with 325 μM TMZ for 0, 12, 24, 48, and 72 h. (D) The cell survival rates treated with TMZ (0-3200 μM) for 24 h were detected by MTT. (E and F) The inhibition rates and $IC_{50}$ values of the cells. Data were shown using MTT, $^{NS}P>0.05$, $^{*}P<0.05$, $^{**}P<0.01$

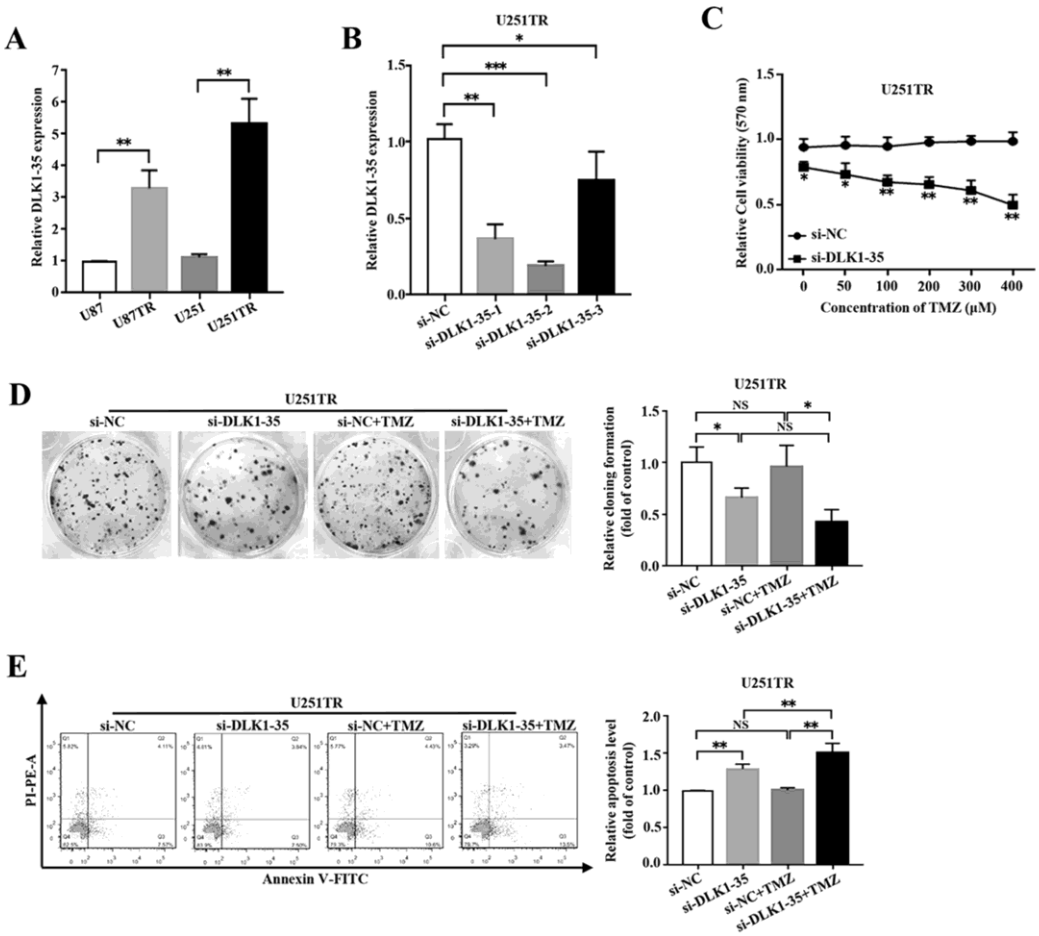


**Figure 2.** DLK1-35 downregulation suppressed TMZ resistance in U251TR cells. (A) The DLK1-35 expression was analyzed by RT-qPCR. (B) The DLK1-35 expression U251TR cells transfected with si-NC, si-DLK1-35-1, -2 and -3 were tested by RT-qPCR. (C) The cell viabilities of transfected U251TR cells with TMZ (0-400 μM) treatments were measured by MTT assay. (D) Colony formation of U251TR cells transfected with si-NC or si-DLK1-35 was detected after treatment with 300 μM TMZ or not. (E) Flow cytometry was applied for evaluating the apoptosis rate of U251TR cells after si-DLK1-35 with or without TMZ, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

Results of the MTT assay showed that the si-DLK1-35 suppressed the U251TR cell's viabilities (Figure. 2C, $^{**}P<0.05$). Additionally, the colony formation of U251TRells was also inhibited after the transfection of si-DLK1-35 with or without TMZ treatment (Figure. 2D, $^{**}P<0.05$). U251TR cell apoptosis with or without TMZ treatment was increased by the knockdown of lncRNA DLK1-35 (Figure. 2E, $^{**}P<0.05$). Results showed that siDLK1-35 suppressed U251TR cell viabilities and facilitated cell apoptosis.

*4.3. Knockdown of lncRNA DLK1-35 enhanced TMZ efficacy in vivo via suppressing MGMT*

After the effects of lncRNA DLK1-35 on modulating glioblastoma cell progression were explored, its downstream factors were detected. Results of western blot showed that MGMT protein expressions were decreased with the knockdown of lncRNA DLK1-35, and the knockdown of lncRNA DLK1-35 also reduced the MGMT protein expression after cells were treated with TMZ (Figure. 3A & B, $^{**}P<0.05$). The effect of knocking down lncRNA DLK1-35 in TMZ-resistant mouse models was examined after TMZ treatment (325μM), and results showed that tumor weight in the siDLK1-35 group was lower than that in the siNC group (Figure. 3C & D, $^{**}P<0.05$). In addition, tumor volumes were also decreased in the si-DLK1-35 group (Figure. 3E). Results suggested that downregulation of lncRNA DLK1-35 could decrease TMZ resistance in vivo.

## 5. Discussion

In this study, lncRNA DLK1-35 was discovered to be elevated in U251TR and U87TR cells. LncRNA DLK1-35 downregulation has been demonstrated to restrain U251TR cell viability and colony formation but accelerate cell apoptosis. Additionally, lncRNA DLK1-35 also restrained tumorigenesis in vivo by suppressing MGMT protein expressions.

Studies have indicated that lncRNAs are critical in GBM heterogeneity and treatment resistance (14). Hyper-expressed lnc-FOXD2-AS1 enhanced TMZ resistance and facilitated GBM cell's invasiveness and migration by modulating the MGMT promoter methylation (15). The lncRNA TALC acted as a

competitive endogenous RNA of miR-20b-3p and provoked MGMT upregulation by regulating the c-

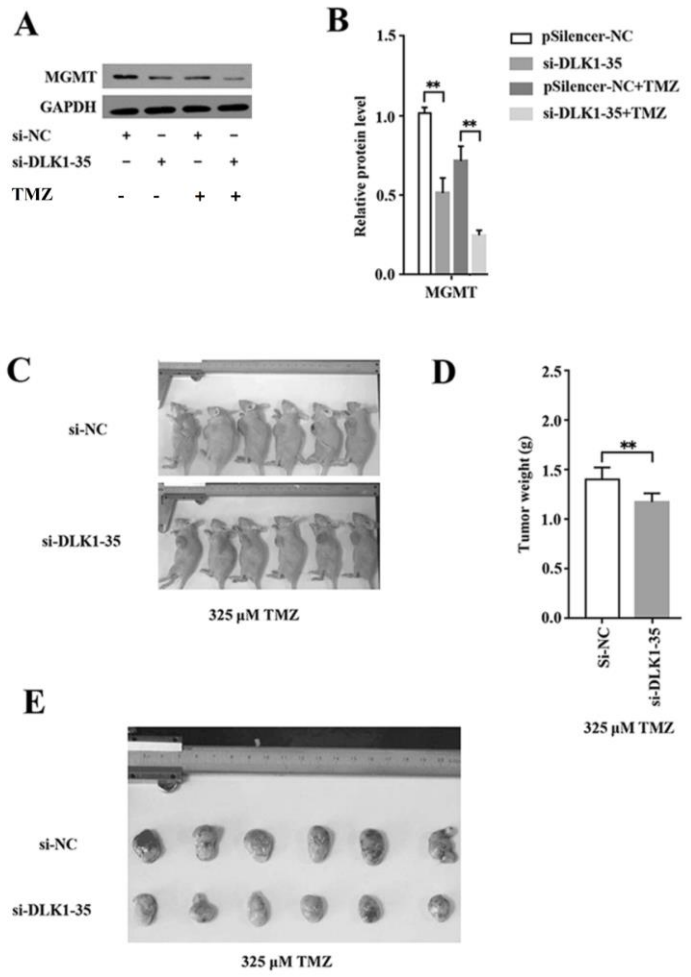


**Figure 3.** LncRNA DLK1-35 facilitated TMZ resistance in vivo via interplaying with MGMT. (A and B) MGMT protein expressions in U251TR cells with si-DLK1-35 and TMZ treatment were detected using a western blot. (C-E) Photographs of tumors on each group of xenograft-transplanted nude mice, weights of tumor xenografts (C-D), and tumor volume (E) were displayed, n=6, $^{**}P<0.01$, $^{***}P<0.001$

Met signaling pathway, resulting in TMZ resistance, tumor development, and GBM progression (16).

LncRNA MALAT1 was upregulated in TMZ-resistant GBM cells facilitating proliferation but hindering cell apoptosis, which also enhances TMZ resistance by suppressing miR-101 (17). LncRNA DLK1-35, also named lncRNA MEG8, has been discovered to act as an oncogene in several kinds of tumors. LncRNA DLK1-35 has been demonstrated to be upregulated in hepatocellular carcinoma cells by promoting proliferation, invasiveness, and migration (18). This research has revealed that lncRNA DLK1-35 was elevated in TMZ-resistant GBM cells while the downregulation accelerated cell apoptosis and inhibited proliferation. Compared to previous studies, this research has investigated the roles of lncRNA DLK1-35 in modulating TMZ resistance in GBM cells. In addition, this study further revealed the modulatory role of lncRNA DLK1-35 on MGMT protein in TMZ-resistant GBM cells.

MGMT has been extensively analyzed as a biomarker in the prediction of prognosis in GBM (19). Moreover, overexpression of MGMT has also been verified to have the capacity in increasing TMZ resistance in GBM (20). In this study, MGMT is decreased with the knockdown of lncRNA DLK1-35. Furthermore, the increase of tumor weight and volume in mouse models was also lower with lncRNA DLK1-35 downregulation. Hence, the impacts of lncRNA DLK1-35 on promoting GBM progression and increasing TMZ resistance have been verified in this study.

## 6. Conclusion

In summary, lncRNA DLK1-35 was promoted in TMZ-resistant GBM cells, while its downregulation restrained TMZ-resistant GBM cell proliferation but promoted apoptosis via suppressing MGMT. Moreover, suppressed lncRNA DLK1-35 also inhibited the increase of tumor weight and volume in mouse models, suggesting that lncRNA DLK1-35 could increase TMZ resistance in GBM.

## Acknowledgments

None.

## Footnotes

**Conflicts of Interest:** The authors declare no conflict of interest.
**Authors' contribution:** YL, XZ, MS, and DL wrote the main manuscript text. ZW, JL, and YL prepared figures 1-3. All authors reviewed the manuscript.
**Ethical approval:** This animal experiment was carried out with the approval of the Animal Welfare Committee of North China University of Science and Technology, and all experiments involving animals were in accordance with the regulations of the institution.
**Financial Disclosure**: None declared.
**Informed Consent:** Not applicable

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