



Temozolomide Drives Ferroptosis via a DMT1-Dependent Pathway in Glioblastoma Cells

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Purpose: Temozolomide is used in first-line treatment for glioblastoma. However, chemoresistance to temozolomide is common in glioma patients. In addition, mechanisms for the anti-tumor effects of temozolomide are largely unknown. Ferroptosis is a form of programmed cell death triggered by disturbed redox homeostasis, overloaded iron, and increased lipid peroxidation. The present study was performed to elucidate the involvement of ferroptosis in the anti-tumor mechanisms of temozolomide.

Materials and Methods: We utilized the CCK8 assay to evaluate cytotoxicity. Levels of lactate dehydrogenase (LDH), malondialdehyde (MDA), iron, and glutathione (GSH) were measured. Flow cytometry and fluorescence microscope were used to detect the production of reactive oxygen species (ROS). Western blotting, RT-PCR and siRNA transfection were used to investigate molecular mechanisms.

Results: Temozolomide increased the levels of LDH, MDA, and iron and reduced GSH levels in TG905 cells. Furthermore, we found that ROS levels and DMT1 expression were elevated in TG905 cells treated with temozolomide and were accompanied by a decrease in the expression of glutathione peroxidase 4, indicating an iron-dependent cell death, ferroptosis. Our results also showed that temozolomide-induced ferroptosis is associated with regulation of the Nrf2/HO-1 pathway. Conversely, DMT1 knock-down by siRNA evidently blocked temozolomide-induced ferroptosis in TG905 cells.

Conclusion: Taken together, our findings indicate that temozolomide may suppress cell growth partly by inducing ferroptosis by targeting DMT1 expression in glioblastoma cells.

Key Words: Temozolomide, glioblastoma, DMT1, ferroptosis, reactive oxygen species

INTRODUCTION

Glioblastoma (GBM) is one of the most common and lethal cerebral tumors.^{1,2} Currently, the standard procedure for treating GBM consists of a combination of surgical resection, ra-

diotherapy and chemotherapy with temozolomide (TMZ).^{3,4} The median survival rate of GBM patients is 12–15 months; the 5-year survival rate after diagnosis is less than 5%.^{3,5} Temozolomide is an alkylating agent that methylates DNA at the O³ position guanine.⁶ DNA damage, apoptosis, and cell cycle arrest have been suggested as important mechanisms supporting the cytotoxicity of temozolomide to malignant cells.⁷ However, there are likely to be many unknown mechanisms for the anti-tumor effects of temozolomide.

Ferroptosis is a form of programmed cell death different from apoptosis, necrosis, and autophagy.⁸ This process is characterized by an accumulation of lipid hydroperoxides and reactive oxygen species (ROS) derived from iron metabolism.⁹ It can be triggered in cancer cells by depleting glutathione (GSH) or by inhibiting the phospholipid hydroperoxidase glutathione peroxidase 4 (GPX4).^{10,11} The process of ferroptosis involves multiple signaling pathways, including Nrf2/HO-1 pathway.¹² The Nrf2/HO-1 pathway plays an important role in regulating

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antioxidant responses and is involved in autophagy and cell necrosis.¹³ Meanwhile, research has also shown that ferroptosis plays a role in glioblastoma development.^{14,15} Buccarelli, et al.¹⁶ found that a combination of quinacrine and temozolomide inhibited tumorigenesis partially by triggering ferroptosis in glioblastoma stem cells. Therefore, we speculated that temozolomide may suppress tumor growth by regulating ferroptosis in glioblastoma cells.

As an important component of ferroptosis, the divalent metal transporter DMT1 can regulate iron levels and has been found to be essential for maintaining iron homeostasis.^{10,17} Until now, there has been no direct evidence of a correlation between DMT1 expression and glioblastoma. Thus, the present study aimed to explore whether temozolomide induces ferroptosis by targeting DMT1 to inhibit cell growth in glioblastoma cells and to suggest a possibility for better treatment of glioblastoma with temozolomide.

MATERIALS AND METHODS

Materials

Temozolomide (purity>99%) and ferrostatin-1 (Fer-1) were purchased from MedChemExpress (Monmouth Junction, NJ, USA). Dichlorofluorescein diacetate (DCFH-DA) was purchased from the Beyotime Institute of Biotechnology (Beijing, China).

Cell culture

The human glioblastoma cell line TG905 was obtained from the Cell Bank of China (Shanghai, China) and maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37°C, 95% humidity, and 5% CO₂.

Cell viability assay

TMZ cytotoxicity was determined using the CCK8 assay kit (Biosharp, Beijing, China) according to the manufacturer's instructions. Cancer cells (5×10³ per well) seeded in 96-well plates were exposed to increasing concentrations of TMZ for the indicated times. The measurement of absorbance for each well was performed in triplicate at a wavelength of 450 nm on a microplate reader (SpectraMax M5, MDC, Sunnyvale, CA, USA).

Determination of lactate dehydrogenase (LDH) release, iron content, malondialdehyde (MDA) activity, and intracellular GSH levels

TG905 cells were treated as indicated and collected. LDH release (Solabio, Beijing, China), iron content (Jiancheng, Jiangsu, China), MDA activity (Beyotime, Shanghai, China), and intracellular GSH levels (Beyotime) were measured according to the manufacturer's instructions. The percentage of LDH release was calculated as [(experimental value LDH activity-released from untreated cells)/(maximum releasable LDH activity in the cells by 1% Triton X-100-LDH activity released from untreated cells)]×100.

Measurement of ROS generation

Generation of ROS was measured by flow cytometry as described previously.¹⁸ Briefly, the TG905 cells were harvested, washed, and suspended in serum-free medium containing 10 µM of DCFH-DA at 37°C for 20 min in the dark. Cells were washed and resuspended in PBS, and the induction of ROS was examined by flow cytometry (FACSCanto, New Jersey, BD, USA) and a fluorescence microscope (Olympus, Tokyo, Japan).

siRNA transfection

TG905 cells were plated in 6-well plates and grown to 40–60% confluence prior to transfection. Before transfection, siRNA-transfection reagent complexes were prepared in Opti-MEM containing negative siRNA or siRNA targeting DMT1 and Lipofectamine 2000 transfecting agent. Afterwards, the complexes were added to the cells and incubated for 6 h. Then, the cells were added to normal growth medium and allowed to grow. The transfected cells were treated with TMZ for 48 h for subsequent studies. The sequence of DMT1 siRNA was 5'-CCUAAAGUGGUCACGCUUUTT-3'.

RNA isolation and quantitative RT-PCR

Total RNA from cultured cells was isolated using TRIzol reagent (Takara, Dalian, China). Before performing quantitative RT-PCR assays (qPCR), RNA was reverse transcribed into total cDNA using and PrimeScript RT reagent kits (Takara). The mRNA expression levels were detected by qPCR with an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) with SYBR Premix Ex Taq (Takara) according to the manufacturer's instructions and were normalized versus GAPDH mRNA. The Ct value of target genes was quantified using the 2^{-ΔΔCt} method. All primer se-

Table 1. Primer Sequences

Gene	Forward	Reverse
DMT1	5'-TGTGTTCTACTGGGTTGGCA-3'	5'-GGCCTTTAGAGATGCTTACCGT-3'
TfR	5'-AACTCAGCAAAGTCTGGCGT-3'	5'-ATACGCCACATAACCCCCAG-3'
FPN1	5'-ACTGTCCTGGGCTTTGACTG-3'	5'-GACCTGTCCGAACCAACCA-3'
FTL	5'-TGGGCTTCTATTCGACCGC-3'	5'-TTTCATGCGCTCTGGGGTTT-3'
GAPDH	5'-GCTCTGCTCCTCCTGTTT-3'	5'-GTTGACTCCGACCTTCACCT-3'

quences are listed in Table 1.

Western blot analysis

The treated TG905 cells were washed thrice with ice-cold PBS and homogenized with RIPA lysate containing 1 mM phenylmethanesulfonyl fluoride. Samples were centrifuged at 12000 rpm for 10 min, and the supernatant were collected. The total protein concentration was measured using BCA protein assay kits (Solabio). Cell lysates were separated using 10% SDS-PAGE and electro-transferred onto a polyvinylidene difluoride membrane. After incubation in blocking buffer (1×TBS, 0.1% Tween-20 and 5% w/v dry nonfat milk) at 4°C, membranes were incubated with the desired primary antibody, followed by incubation with the appropriate peroxidase-conjugated secondary antibody. The primary antibodies included

those for Nrf2 (16396-1-AP, Proteintech, Wuhan, China), HO-1 (ab189491, Abcam, Cambridge, England), DMT1 (20507-1-AP, Proteintech), GPX4 (ab125006, Abcam), ferritin (ab75973, Abcam), transferrin receptor (ab214039, Abcam), ferroportin-1 (bs-4906R, Bioss, Beijing, China), and GAPDH (ab181602, Abcam). The films were scanned, and the intensity values of each band were determined using an Alphamager HP system (Cell Biosciences Inc., Santa Clara, CA, USA).

Statistical analysis

The data are described as a mean±SD and were analyzed using ANOVA and Student's t-test. Differences with *p* values<0.05 were considered statistically significant. Statistical analysis was carried out with GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA).

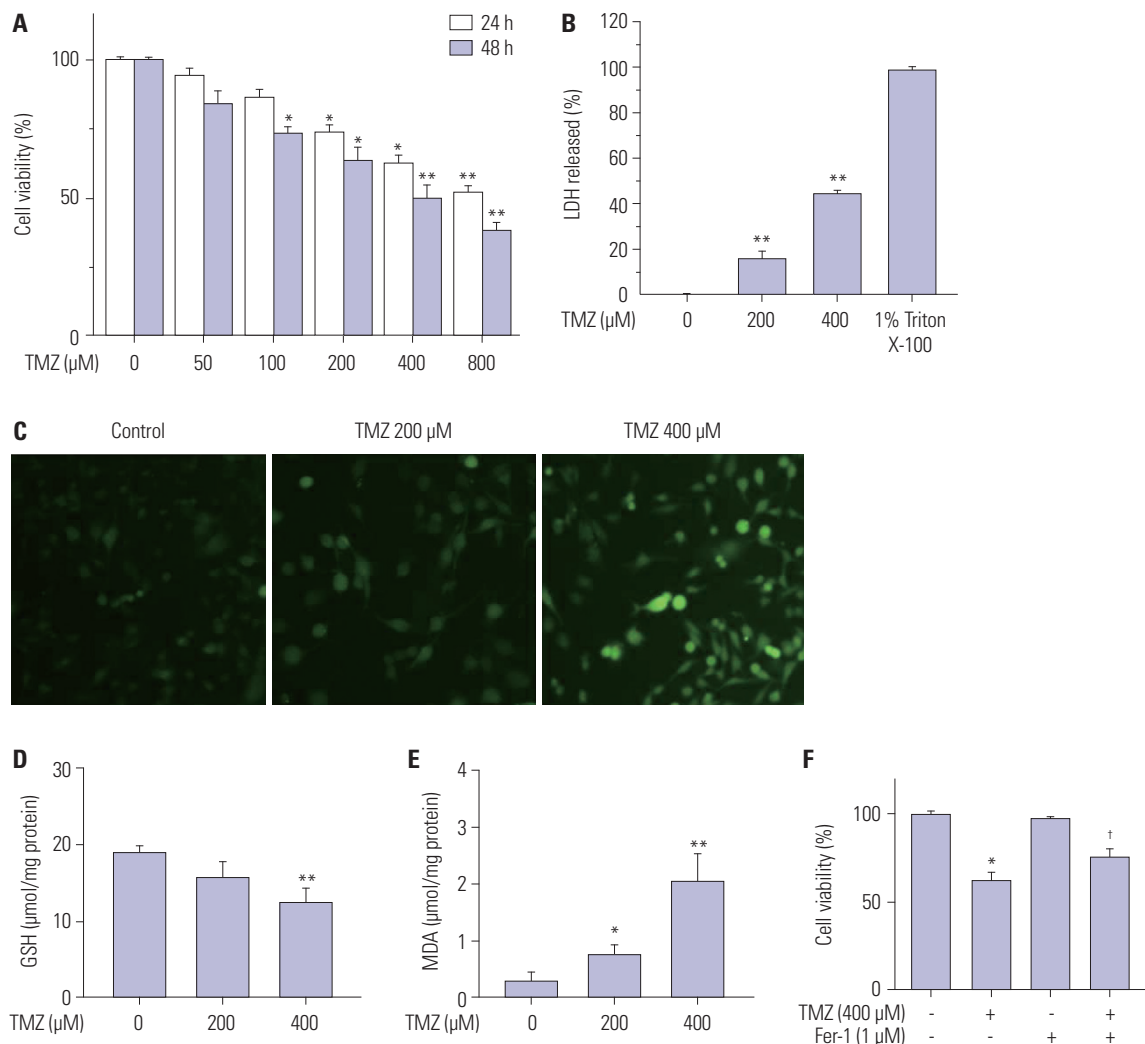


Fig. 1. TMZ induces ferroptosis in TG905 cells. (A) Cell viability was measured using the CCK8. (B) LDH release was measured. Percentage of LDH released was expressed based on maximum releasable LDH in the cells induced by 1% Triton X-100. (C) DCFH-DA staining was used to evaluate ROS production under a fluorescence microscope. GSH (D) and MDA (E) levels were analyzed after treatment with TMZ for 48 h. Data are presented as the mean±SD of three independent experiments. **p*<0.05, ***p*<0.01 compared with the control group (t test). (F) Treatment with Fer-1 mitigated TMZ-induced cell death. Data presented as the mean±SD of three independent experiments. **p*<0.05 compared with the control group, †*p*<0.05 compared with the TMZ-treated group (ANOVA). TMZ, temozolomide; LDH, lactate dehydrogenase; GSH, glutathione; MDA, malondialdehyde; DCFH-DA, dichlorofluorescein diacetate; ROS, reactive oxygen species.

RESULTS

Temozolomide suppresses cell growth in the glioblastoma cell line TG905

To confirm the anti-tumor effects of temozolomide, cell viability was assessed using the CCK8 assay. We found temozolomide induces cell death in TG905 cells in a concentration- and time-dependent manner (Fig. 1A). We used 200 and 400 μM temozolomide for 48 h in subsequent experiments.

Temozolomide induces the production of ROS and lipid peroxidation in TG905 cells

The destruction of cell membrane structures induced by ferroptosis reportedly leads to the release of LDH in the cytoplasm into the culture medium.¹⁹ Therefore, we detected the levels of LDH in the supernatant of cultured cells to determine the degree of cell death. As shown in Fig. 1B, treatment with 200 or 400 μM temozolomide significantly increased the levels of LDH, compared with controls.

Lipid peroxidation is a key driving force of ferroptosis, and ROS accumulation is regarded as one hallmark of ferroptosis.²⁰ To determine whether ROS plays a key role in TMZ-induced cell death, we measured intracellular ROS levels using DCFH-DA fluorescence. Our results showed that temozolomide increases the accumulation of ROS in TG905 cells (Fig. 1C). To investigate whether temozolomide could induce oxidative stress, GSH and MDA levels were measured. Treatment with 400 μM temozolomide remarkably decreased GSH ($p < 0.01$) and increased MDA ($p < 0.01$) levels (Fig. 1D and E). Ferroptosis specific inhibitor Fer-1 could rescue the effect of cell survival inhibition induced by temozolomide (Fig. 1F). Accordingly, these data indicate that ferroptosis may contribute

to temozolomide-induced growth inhibition in TG905 cells.

Temozolomide increases iron content and upregulates DMT1 expression in TG905 cells

Iron is the essential reactive element for ferroptosis. As shown in Fig. 2A, temozolomide treatment triggered an increase in iron content among TG905 cells. We further tested mRNA and protein expression of DMT1, TfR, FPN1, and FTL, which are related to iron uptake and ferroptosis. As the RT-PCR and Western blots showed, DMT1 mRNA and protein expression were increased significantly (Fig. 2B and C).

Temozolomide induces ferroptosis by targeting DMT1 in TG905 cells

To explore the possibility that increased DMT1 expression contributes to temozolomide-induced ferroptosis, TG905 cells were transfected with or without DMT1 siRNA, followed by temozolomide incubation. As shown in Fig. 3A and B, the expression of DMT1 protein and mRNA was decreased in TG905 cells upon DMT1 knockdown by siRNA. CCK8 assay showed that the observed temozolomide-induced decreases in cell viability were inhibited after DMT1 downregulation (Fig. 3C). Similarly, DMT1 inhibition markedly decreased LDH levels induced by temozolomide (Fig. 3D). Moreover, our results showed that 400 μM temozolomide markedly upregulated MDA levels, iron content, and intracellular ROS levels, while silencing DMT1 diminishes these effects (Fig. 3F-I). Interestingly, silencing DMT1 had no significant effect on GSH levels (Fig. 3E). Altogether, these results suggest that temozolomide induces ferroptosis in glioma cells, at least partially, by targeting DMT1.

To further investigate the mechanisms of temozolomide-induced ferroptosis, we examined the expression of DMT1, GPX4,

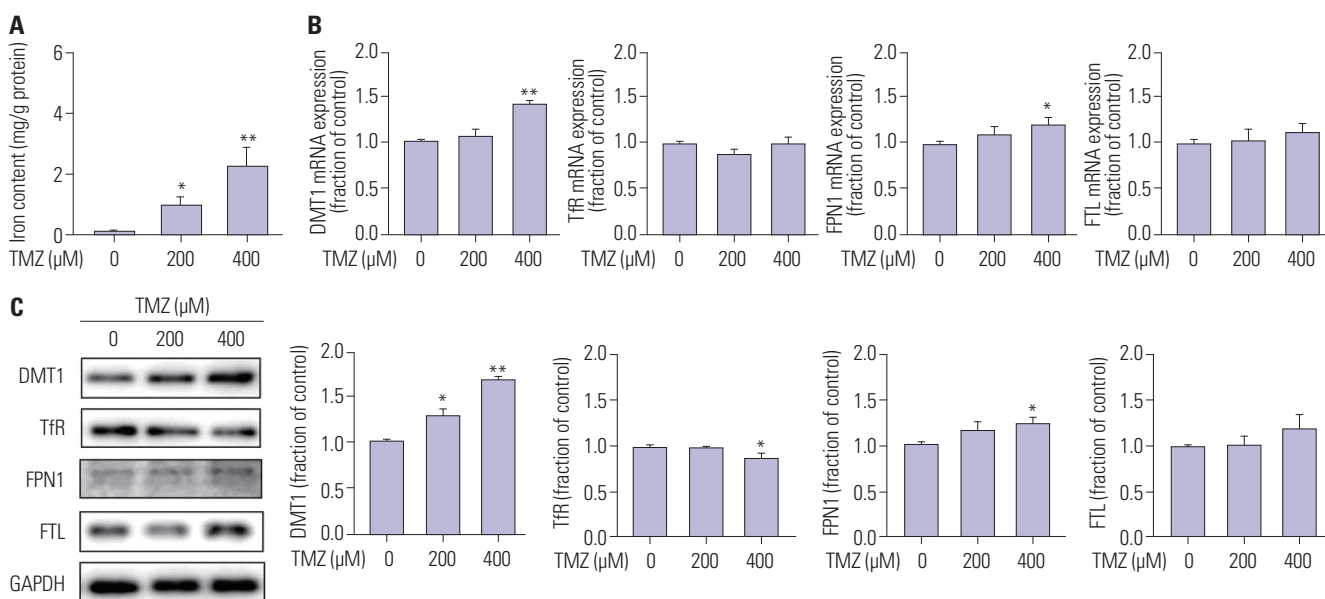


Fig. 2. Effect of TMZ on iron content and iron-related mRNA and protein expression in TG905 cells. Cells were treated with TMZ for 48 h. (A) Iron content, (B) iron-related mRNA, and (C) protein expression were measured. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with the control group (t test). TMZ, temozolomide.

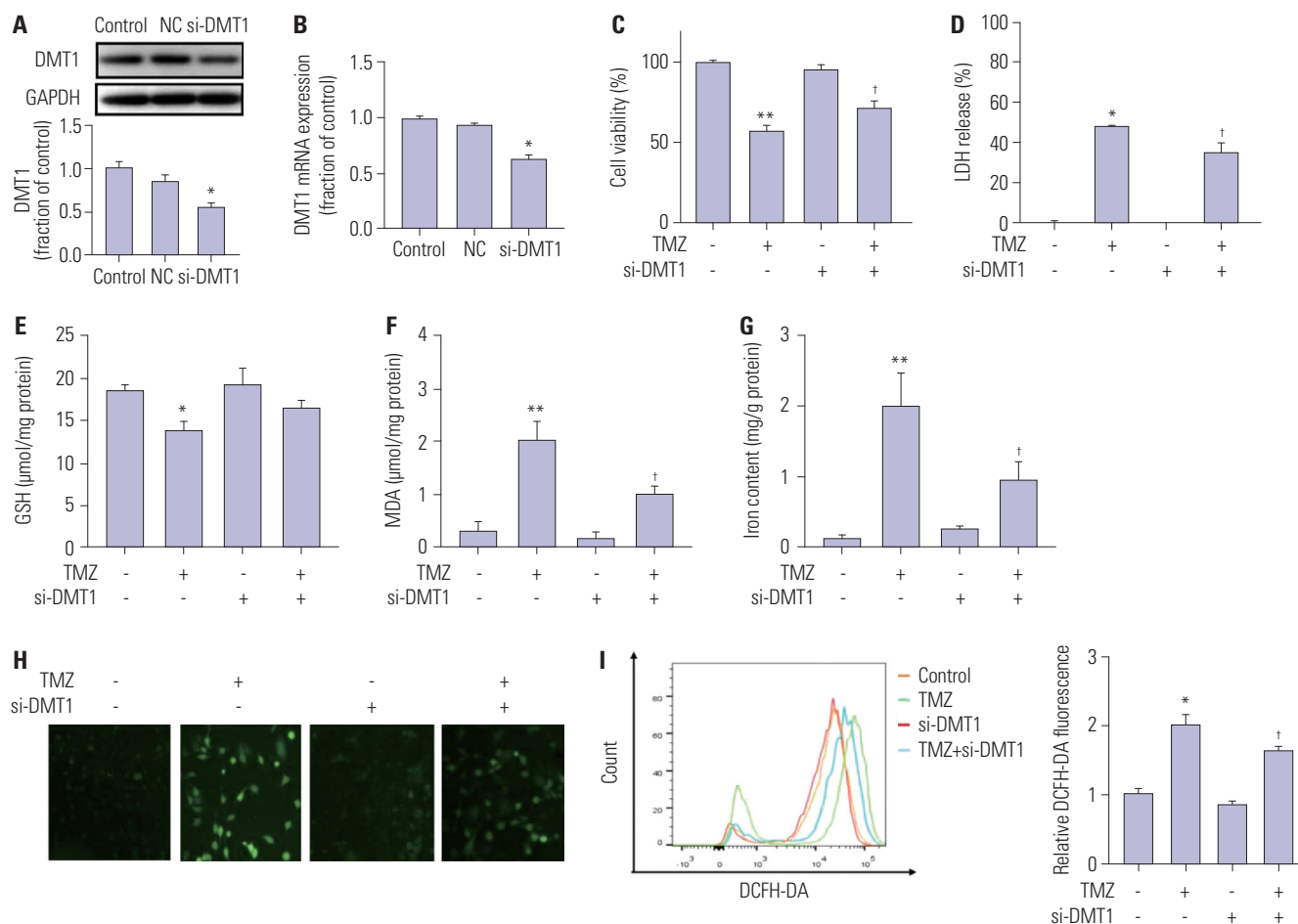


Fig. 3. Effect of DMT1 silencing on TMZ-induced ferroptosis in TG905 cells. (A and B) The expression of DMT1 protein and mRNA was decreased upon DMT1 knockdown by siRNA. Data are presented as the mean \pm SD of three independent experiments. * p <0.05 compared with the control group (t test). (C-G) Cells were transfected with or without si-DMT1 for 6 h and then treated with TMZ (400 μ M) for 48 h. Cell viability, LDH levels, GSH levels, MDA levels, and iron content were analyzed. (H and I) DCFH-DA staining was used to evaluate ROS production under a fluorescence microscope and flow cytometry. Data are presented as the mean \pm SD of three independent experiments. * p <0.05 compared with the control group, † p <0.05 compared with the TMZ-treated group (ANOVA). TMZ, temozolomide; LDH, lactate dehydrogenase; GSH, glutathione; MDA, malondialdehyde; DCFH-DA, dichlorofluorescein diacetate; ROS, reactive oxygen species.

Nrf2, and HO-1 protein. GPX4 is a master regulator of ferroptotic signaling, as it can convert lipid hydroperoxides to lipid alcohols. We found that temozolomide lowered the expression of GPX4 in TG905 cells. On the other hand, silencing DMT1 suppressed the down-regulation of GPX4 by temozolomide, as revealed in Fig. 4. Nrf2 plays a critical role in regulating ferroptosis.²¹ As shown in Fig. 4, temozolomide treatment markedly decreased Nrf2 and HO-1 expression, and the decrease was suppressed in TG905 cells transfected with DMT1 siRNA. Collectively, these results suggest that temozolomide induces ferroptosis by upregulating DMT1.

DISCUSSION

Temozolomide has been shown to be a highly effective and a promising chemotherapeutic agent in treating aggressive GBM. The detailed mechanisms for the anti-tumor effects of temo-

zolomide have not been fully elucidated. In the present study, we noted that temozolomide suppresses cell growth in TG905 cells. Moreover, we found that temozolomide induces ferroptosis in glioblastoma cells and that temozolomide-induced ferroptosis is associated with upregulation of DMT1.

Ferroptosis is a form of cell death characterized by an accumulation of iron dependent lipid peroxides in cells.²² Triggering ferroptosis has emerged to be a promising strategy for cancer treatment, including glioma.^{15,23} Studies have found that GSH loss in cells can lead to ROS accumulation and inhibit the activity of GPX4. As a powerful antioxidant enzyme, GPX4 results in a decrease of cell antioxidant capacity and lipid peroxidation, eventually causing ferroptosis.^{8,22} In this study, we found that ROS and MDA levels increased, while GSH levels and GPX4 expression both decreased in temozolomide-treated TG905 cells, suggesting that temozolomide induces ferroptosis in glioma cells. Nrf2 is an important regulatory factor for ferroptosis.^{21,24} One study has pointed out that ibuprofen can

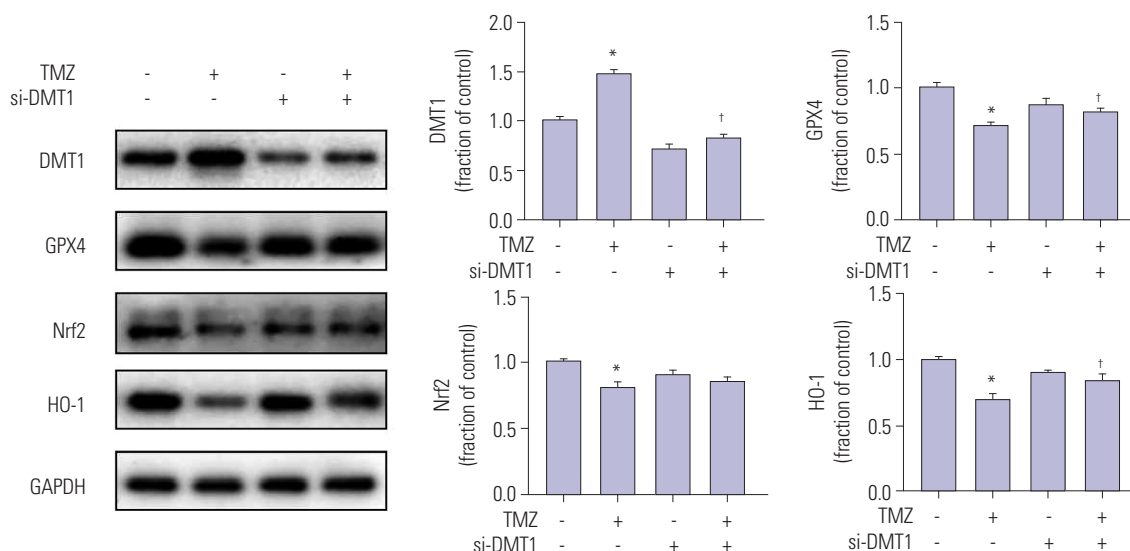


Fig. 4. Effect of DMT1 silencing on protein expression in TG905 cells. Cells were transfected with or without si-DMT1 for 6 h and then treated with TMZ (400 μM) for 48 h. Protein expression was measured by Western blot assay. Data are presented as the mean ± SD of three independent experiments. **p* < 0.05 compared with the control group, †*p* < 0.05 compared with the TMZ-treated group (ANOVA). TMZ, temozolomide; GPX4, glutathione peroxidase 4.

induce ferroptosis of glioblastoma cells via downregulation of Nrf2 signaling.²⁵ Consistent with previous reports, the current study demonstrated that temozolomide induced ferroptosis by inhibiting the Nrf2/HO-1 pathway in glioma cells.

Iron is involved in various metabolic processes, and ferroptosis is dependent upon it. As a proton-coupled metal-ion transport protein, DMT1 is a critical modulator of iron homeostasis and plays a key role in iron uptake.²⁶ Studies have reported that upregulation of DMT1 is associated with ferroptosis.²⁷⁻²⁹ In this study, DMT1 was found to be up-regulated and to increase iron uptake. To further confirm the function of DMT1, we treated TG905 cells with siRNA, and knockdown of DMT1 reduced ROS levels, iron deposition, and MDA levels induced by temozolomide. Moreover, DMT1 downregulation increased the expression of GPX4, Nrf2, and HO-1. These results suggest that temozolomide induces ferroptosis, at least partially, in a DMT1-dependent manner.

In conclusion, the present study demonstrated that temozolomide inhibits tumor growth, at least in part, by inducing ferroptosis by targeting DMT1 expression. Our results provide a greater understanding of the mechanism by which temozolomide affects glioblastoma cells and may be of benefit in the development of methods using temozolomide for the treatment of glioma.

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AUTHOR CONTRIBUTIONS

Conceptualization: Xiaosong Zhu and Xueyuan Heng. **Data curation:** Qingxin Song and Shanxin Peng. **Formal analysis:** Shanxin Peng and Zhiqing Sun. **Funding acquisition:** Qingxin Song. **Investigation:** Xiaosong Zhu. **Methodology:** Xueyuan Heng and Zhiqing Sun. **Project administration:** Xiaosong Zhu and Xueyuan Heng. **Resources:** Xueyuan Heng and Zhiqing Sun. **Software:** Qingxin Song and Shanxin Peng. **Supervision:** Zhiqing Sun. **Validation:** Qingxin Song and Shanxin Peng. **Visualization:** Zhiqing Sun. **Writing—original draft:** Qingxin Song, Shanxin Peng, and Zhiqing Sun. **Writing—review & editing:** Xiaosong Zhu and Xueyuan Heng. **Approval of final manuscript:** all authors.

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