Hypoxia promotes temozolomide resistance in glioblastoma cells via ROS- mediated up-regulation of TRPM2

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Abstract

Background: Hypoxia, an essential feature of gliomas, is thought to promote chemo-resistance by regulating reactive oxygen species (ROS) levels. Transient receptor potential melastatin 2 (TRPM2) is one of the ion transport proteins and is involved in the regulation of oxidative stress. However, relationship between ROS and TRPM2 expression in hypoxia-induced temozolomide (TMZ) resistance of glioblastoma cells remains unclear.

Methods: U87MG cells were cultured with different concentrations of TMZ for the indicated times under normoxia (21% O_2) or hypoxia (2.5% O_2). Cell viability was detected with WST-1 test and observed by a neurite outgrowth assay. The intracellular ROS scavenging activity was detected according to the H2DCF-DA method. The cells were also treated with the scavenger of ROS NAC and the inhibitor of TRPM2 2-APB. Impaired mitochondrial membrane potential ($\Delta\Psi m$) and intensity of intracellular Ca^{2+} were measured under fluorescence microscope. Online database was used to assess the relationship between MGMT and TRPM2 expression level. Western blot was used to analyze the protein levels of TRPM2, MGMT, MSH3 and APNG.

Results: Compared with the normoxia group, hypoxia significantly promoted glioma cells survival after treatment by TMZ (200μM) for 24 h or 48 h, accompanied with reduction of mitochondrial dysfunction and intracellular ROS. However, the baseline levels of ROS were mildly increased under hypoxia, which had no impact on mitochondrial function in glioma cells. Additional, TRPM2 expression was obviously increased under hypoxia and inhibited by NAC in glioma cells. We found that the expression levels of TRPM2 were positively correlated with MGMT both in online database ($rho=0.165$, $P < 0.05$) and GBM cancer tissues ($r=0.9302$, $P < 0.05$). Over-expression of TRPM2 participated in the up-regulation of APNG and MGMT, but down-regulation of MSH3 in glioma cells under hypoxia. Our findings also demonstrated that the treatment group during NAC or 2-APB add-on could significantly attenuate calcium influx, followed by increasing mitochondrial dysfunction and cytotoxicity in glioma cells, in comparison with TMZ alone.

Conclusion: The hypoxia-induced up-regulation of baseline ROS levels contributes to the decrease in the sensitivity of glioma cells to TMZ via promoting demethylation and inhibiting DNA mismatch repair. Moreover, TRPM2-mediated Ca^{2+} influx attenuates mitochondria dysfunction and then protects glioma cells against TMZ damage. TRPM2 may be a potential target in adjuvant treatment with TMZ for glioblastoma multiforme (GBM) patients.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive intracranial tumor in adults, and the 5-year mortality of that was next only to pancreatic and lung cancer(Louis et al., 2016). The current standard therapies for newly diagnosed GBM patients include surgery, post-operative radiotherapy, and temozolomide (TMZ) treatment (Wu et al., 2021). TMZ belongs to the second generation of an oral

alkylating agent and serves as the first-line chemotherapeutic agent of GBM patients. Unfortunately, more than half of GBM patients have poor prognosis and overall survival due to TMZ resistance, which has became the main obstacle for successful treatment in GBM patients(Fabian et al., 2019). Hence, it is important to investigate the mechanisms of TMZ resistance for developing new therapeutic drugs.

In vivo, TMZ is first converted into the active metabolite MTIC, which is further decomposed into 5-amino-imidazole-4-amide (AIC) and diazomethane. Diazomethane alters DNA or RNA at N^7 and O^6 sites on guanine and the N^3 on adenine through the addition of methyl groups, followed by inducing cell death. DNA mismatch repair (MMR) is helpful for tumor cells sensitive to TMZ via fixation of the methylated sites. In contrast, the demethylation of O^6-methylguanine-DNA methyltransferase (MGMT) and the N^7 methylated sites removed by alkylpurine-DNA-N-glycosylase (APNG) could alleviate the damage of TMZ to tumor cells(Agnihotri et al., 2012;Happold et al., 2012;Nam et al., 2021). Accordingly, over-expression of MGMT and APNG and/or deficiency of MMR were considered to mainly participate in the resistance of tumor cells to TMZ.

Hypoxia occurs not only in natural environments such as high altitudes, underground caves, and deep seas, but also in human pathological conditions, such as hypoxic solid tumors(Jiang et al., 2021). Tumor cells are exposed to hypoxia microenvironment as the insufficient distribution of blood vessels. Hypoxia plays a key role in the resistance to chemotherapy, immunotherapy and radiotherapy through multiple mechanisms such as dysregulation of DNA repair system, anti-apoptosis, and drug efflux(Minassian et al., 2019;Weng et al., 2022). It also potentially results in poor prognosis for cancer patients with chemotherapy. Previous studies have reported that hypoxia was related with resistance to TMZ in glioblastoma(Chen et al., 2015;Gao et al., 2019;Hsieh et al., 2015;Monteiro et al., 2017). However, the exact mechanisms of hypoxia-induced TMZ resistance are incompletely understood.

Hypoxia exhibited a greater level of reactive oxygen species (ROS) mainly due to vigorous metabolism and mitochondrial dysfunction in malignant cells(Prasad et al., 2017). Interestingly, ROS was considered as a double-edged sword. The physiological ROS levels contributed to the proliferation, migration and differentiation of tumor cells, while the lethal ROS levels could lead to DNA damage and cell death (Cheng et al., 2013;Wang et al., 2013). ROS was related to chemo-resistance in some tumor cells(Ledoux et al., 2003;Stieg et al., 2022). Therefore, it is essential for illuminating the effect of ROS on glioma cells and its underlying mechanisms involved in drug resistance.

Transient receptor potential melastatin 2 (TRPM2) belongs to the ion transport protein family, including sodium, potassium and calcium ion channels, and highly expresses in several cancers(Wang et al., 2020). Calcium channels embedded in cell membrane were gated by activation of TRPM2, which could be up-regulated with oxidative stress such as ROS or hydrogen peroxide(Naziroglu, 2012;Wehage et al., 2002;Ye et al., 2023;Zhu et al., 2018). In SH-SY5Y neuronal cells, hypoxia-induced increased levels of ROS also positively motivate the function of TRPM2 channel(Akyuva and Naziroglu, 2020). However, few studies reported the relevance between ROS and TRPM2 in glioma cells.

It is very complicated about the physiologic and pathophysiologic role of Ca^{2+} influx into the cells. Naziroglu M indicated that enhancement of intracellular Ca^{2+} contributed to DNA repair, chem-resistance and cell survival(Naziroglu, 2007). Paradoxically, Zhu J suggested that Ca^{2+} influx could induce apoptosis in hepatocellular carcinoma cells (Zhu et al., 2018). Thus, it is necessary to understand the relationship of Ca^{2+} entry with drug resistance in glioma cells. In the present study, we aimed to analyze the effect of ROS and TRPM2 on TMZ resistance and reveal the underlying mechanisms in glioma cells.

Materials and methods

Reagents

Temozolomide (TMZ) was purchased from Selleck Chemicals (Shanghai, China). Anti-TRPM2 antibody (#ab11168) and Anti-APNG (#ab55461) were purchased from Abcam Biotechnology (San Francisco, USA). 2-Aminoethyl diphenylborinate (#D9754), Anti-GAPDH (#G2267), Anti-MGMT (#SAB2105451) and Anti-MSH3 (#SAB4501924) antibodies were obtained from Sigma-Aldrich (St.Louis, MO, USA). Olaparid (#HY-10162) and N-acetyl-L-cysteine (NAC) (#HY-B0215) were purchased from MedChemExpress (Monmouth Junction, USA). β-actin antibody (#B2315) and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TEMED (#ST728), 10%SDS (#ST628), WB primary antibody diluent (#P0023A), Cell Lysis buffer (#P0013) and BCA protein assay kit (#P0012) were purchased from Beyotime Biotechnology (Shanghai, China). Immobilon Western Chemilum HRP Substrate was purchased from Millipore (Billerica, USA). All other reagents were from Sigma-Aldrich, unless otherwise indicated.

Cell culture

U87MG cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and incubated in DMEM/HIGH Glucose (HyClone-Pierce, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, USA) and antibiotics (100 U/ mL penicillin and 100 μg/mL streptomycin) in hypoxia (2.5% O_2 + 5% CO_2 + 92.5% N_2) or a 5% CO_2 atmosphere at 37°C with or without TMZ.

Tissues

Glioblastoma multiforme tissues were obtained from the Department Oncology of Daping Hospital of Army Medical University (Chongqing, China). In this study, we analyzed the viable samples of cancer tissues (WHO grade IV) from ten patients treated with TMZ, who signed informed consent forms. The study was approved by the Ethics Committee of Daping Hospital of Army Medical University and conformed to the Declaration of Helsinki.

Assessment of cell proliferation

WST-1 Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, China) was applied to detect the effects of TMZ treatment on the viability of U87MG cells under normoxia (21% O_2) or hypoxia (2.5% O_2). U87MG cells (6×10^3/mL) cells were planted in 96-well plates and treated with different concentrations of TMZ (0,

25, 50, 100, 200, 400, 800 μM) for 24 hours. The culture supernatants were collected, and then 10 μL WST-1 solution was added to each well, followed by incubating for 2–3 hours under normoxia or hypoxia. Optical density (OD) was measured at 450nm on a microplate reader (Gene company limited, Hong Kong China). The cell viability was shown as the percentage of the control.

U87MG cells (6×10^3/mL) were seeded in 96-well plates and incubated for 24 hours under normoxia (21% O_2) and hypoxia (2.5% O_2). The samples were treated with TMZ (200 μM) for different time points (0, 6, 12, 24, 48 and 72 hours). The cell viability was measured and calculated as described above.

Assessment of reactive oxygen species (ROS) level

The intracellular levels of ROS were detected by reactive oxygen species assay kits (ROS Assay Kit) (Beyotime, China). Briefly, U87MG cells were plated in 6-well plates at a density of 1.0×10^4 for the indicated time points under normoxia (21% O_2) or hypoxia (2.5% O_2), and then TMZ (200 μM) was added to treat for 24 and 48 hours at 37˚C. The culture medium was removed and the samples were rinsed with PBS. 10 μM 2',7'-dichlorofluorescein diacetate (H2DCF-DA) was added to co-incubate for 30 minutes, and fluorescence was obtained by Thermo VarioskanFlash (excitation at 488 nm and emission at 525 nm). ImageJ 1.46r software (Wayne Rasband, National Institutes of Health, USA) was used to quantitatively analyze the mean fluorescence intensity (MFI) of DCFH-DA.

Measurement of mitochondrial membrane potential (ΔΨm)

U87MG cells were plated in 6-well plates at a density of 1.0×10^4 for 48 hours and then incubated with TMZ (200 μM) at 37˚C for the indicated time points (24 and 48 hours) under normoxia (21% O_2) or hypoxia (2.5% O_2). According to the operational approaches of the mitochondrial membrane potential assay kit with JC-1 (Beyotime, China), 1 ml JC-1 staining solution was added to each well and incubated with U87MG cells for 20 min at 37˚C. The culture medium was sucked off and the cells were washed for 2 times with JC-1 staining buffer (1X). Afterwards, 2ml of culture medium was added, and the fluorescence of cells was visioned under the fluorescence microscope (IX71, Olympus, Japan) with an imaging system (CellSens Standard, V4.2.1, Japan).

Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

U87MG (6×10^3/mL) were seeded in 12-well plates for 24 hours under normoxia (21% O_2) or hypoxia (2.5% O_2). In normoxia groups, the cells were respectively incubated with PBS and TMZ (200 μM) alone for 48 hours. Under hypoxia condition, the cells were respectively treated with PBS, TMZ (200 μM) alone, TMZ (200 μM) plus 2-APB (100 μM) and TMZ (200 μM) plus NAC (100 μM) for 48 hours. 5 μM Fluo-3 AM (Beyotime, China) was incubated with the samples for 60 minutes at 37˚C, and then cleaned with PBS. Furthermore, the samples were observed under the fluorescence microscope (IX71, Olympus, Japan). The excitation wavelength was 506 nm, and the emission wavelength was 526 nm.

Neurite outgrowth assay

U87MG cells were plated in a 24-well plate at a density of 6×10^3 and exposed to 2.5% O_2 for 24 h. Hypoxic cells were respectively co-incubated with PBS, TMZ (200 μM), NAC(100 μM), 2-APB (100 μM), TMZ (200 μM) plus 2-APB (100 μM) and TMZ (200 μM) plus NAC (100 μM) for additional 24 h. The culture supernatants were absorbed out and 200 μL 4% paraformaldehyde was added to fix cell morphology. Cell images were observed under the inverted microscope (CKX41, Olympus, Japan) and 10 fields (200×) were randomly selected to analyze per group.

Bioinformatics analysis of MGMT and TRPM2 expression

The Tumor Immune Estimation Resource 2.0 (TIMER 2.0) (T. Li et al., 2017) was used to assess the relationship between MGMT and TRPM2 expression level in public databases among different cancer groups. Data from tumor tissues were obtained from The Cancer Genome Atlas (TCGA) Research Network (https://www.cancer.gov/tcga). These datasets are publicly available. All the procedures were performed in accordance with the relevant guidelines and regulations. The correlation analysis included all 40 cancer types available at TIMER 2.0. Statistical analysis was performed with Pearson test (**p < 0.01, ***p < 0.001, ****p < 0.0001).

Western blot

Total proteins were extracted and their concentrations were measured by BCA protein assay kit (Beyotime, China). Total proteins (30–50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Inc., USA) and transferred to 0.2 μm PVDF membranes (Millipore, USA). The membranes were blocked for 2 h in 5% in Tris-buffered saline containing a non-fat dry milk solution and then incubated with appropriate primary antibodies overnight at 4°C. Dilutions of the primary antibodies were as follows: 1:300 for anti-TRPM2 antibody, 1:500 for anti-MGMT, anti-APNG and anti-MSH3 antibody, 1:1000 for anti-GADPH and β-Actin antibody. Specific protein bands were visualized with ChemiDoc™ Imaging System (Bio-Rad Laboratories Inc., USA) according to the manufacturer's protocol. The gray density of the protein bands were determined by the Image J software (National Institutes of Health, Bethesda, MD, USA) and data were presented as the percentage of the control.

Statistic analysis

All experiments were performed at least three times, and the results were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA). Quantitative analysis of the length of neurite was measured by ImageJ 1.48u. The results were analyzed by one-way analysis of variance (ANOVA) followed by an SNK-q test for multiple comparisons. P < 0.05 was considered to be statistically significant.

Results

Hypoxia induces the resistance of glioma cells to TMZ

Hypoxia is known to cause resistance to chemotherapy in tumor cells(Yang et al., 2022). Firstly, to observe the effects of TMZ on glioma cells under hypoxia and normoxia, U87MG cells are treated by TMZ for 24h, and the concentration of that ranges from 0 to 800 μM. Our data indicated that hypoxia obviously increased the cell viability of U87MG cells treated with TMZ from 100 to 800 μM, compared with the normoxia group (Fig. 1A). Subsequently, the proliferation rate in the presence of TMZ (200 μM) was detected at different time points, and the results demonstrated that hypoxia significantly decreased glioma cells sensitivity to TMZ from 24 to 72 h (Fig. 1B).

Hypoxia ameliorates the sensitivity of glioma cells to TMZ via inhibition of mitochondrial dysfunction

In addition, we detected the changes of mitochondrial membrane potential (ΔΨm) in U87MG cells treated with TMZ (200 μM) at different time points via JC-1 staining under hypoxia and normoxia. The JC-1 green staining percentage of U87MG cells without TMZ under hypoxia has no significant difference with that of the normoxic group (Fig. 1C and 1D). However, after incubating with TMZ from 24 to 48h, the JC-1 green staining percentage of U87MG cells under hypoxia was higher than that of the normoxia group (0 hour) and obviously decreased in comparison with the normoxia group at the same time points (Fig. 1C and 1D).

Hypoxia increases the baseline ROS levels in glioma cells, but down-regulates ROS generation of that exposed to TMZ

Mitochondria are one of the main organelles to produce ROS in cells(Zelentsova et al., 2022). In order to analyze the effects of hypoxia on ROS generation in glioma cells, the H2DCF-DA method was used to detect intracellular ROS levels. As presented in Fig. 1E, after treatment of TMZ from 24 to 48h under hypoxia and normoxia, the intracellular ROS scavenging activity was significantly enhanced than that of the normoxia group (0 hour), and hypoxia markedly alleviates the intracellular ROS levels, compared with normoxia group at the same time points. These results indicate that although inducing the up-regulation of baseline ROS levels in glioma cells, hypoxia decreases the sensitivity of that to TMZ through attenuating ROS production and mitochondrial damage.

ROS is involved in up-regulation of TRPM2 under hypoxia in glioma cells

To explore the relationship between the up-regulation of baseline ROS levels and hypoxia-induced TMZ resistance in glioma cells, the results of future studies showed the intracellular ROS scavenging activity was significantly elevated in a time-dependent manner from 6 to 72 hours, but it has no obvious difference under normoxia (Fig. 2A). Previous studies have also indicated that TRPM2 was the potential downstream targets of ROS in tumor cells(Hirschler-Laszkiewicz et al., 2018). To explore the relevance of ROS in regulating TRPM2 expression in glioma cells, the ROS and TRPM2 expression levels were respectively measured by WST-1 kit and Western blot. TRPM2 expression was gradually up-regulated in a

time-dependent manner under hypoxia within 24 hours (Fig. 2B and 2C). As presented in Fig. 2D and 2E, in the presence or absence of NAC pretreatment, hypoxia significantly increased TRPM2 expression compared to the normoxia group. However, the expression of TRPM2 was obviously decreased in U87MG cells exposed to NAC (a scavenger of ROS) under hypoxia. The results demonstrated that the baseline intracellular ROS levels were enhanced by hypoxia and positively relevant to the over-expression of TRPM2 in glioma cells.

The up-regulation of TRPM2 induced by hypoxia is vital for TMZ resistance

MGMT was considered to be a key protein for TMZ resistance in GBM patients(Wang et al., 2023). To analyze whether hypoxia promotes TMZ resistance to glioma cells via the TRPM2 pathway, we first analyzed the relationship between MGMT and TRPM2 protein expression in GBM groups in the TIMER 2.0 database, then these samples from cancer tissues of GBM patients and U87MG cells were detected by Western blot. In the TIMER 2.0 database, we examined whether there was a correlation between MGMT and TRPM2 in 40 types of cancer, and found a positive trend in the GBM group (n = 153) (Fig. 3A **-** **B**). Next, we found that TRPM2 and MGMT proteins were expressed in all five specimens of GBM patients (Fig. 4A). As shown in Fig. 4B, the expression level of MGMT was elevated with up-regulation of TRPM2 in specimens ($r = 0.9302$, $P < 0.05$).

To further explore the mechanisms of TRPM2 in hypoxia-induced TMZ resistance, the protein levels of MSH3, APNG and MGMT were detected by Western blot in glioma cells treated with or without 2-APB (inhibitor of TRPM2) pretreatment. The expression levels of APNG and MGMT were obviously enhanced, while that of MSH3 was alleviated under hypoxia, in comparison with the normoxia group (Fig. 4D **-** **F**). Moreover, after treatment with 2-APB, protein levels of APNG and MGMT were reduced, but that of MSH3 was over-expressed in U87MG cells under hypoxia (Fig. 4D **-** **F**). Thus, the results demonstrate that the increased expression of TRPM2 was related to hypoxia-induced TMZ resistance in glioma cells.

Hypoxia protects mitochondrial function via TRPM2-dependent calcium influx induced by ROS

To further analyze the mechanism of TRPM2 in TMZ resistance under hypoxia, the intracellular calcium was observed by fluorescence microscope. The Ca^{2+} concentrations in cells treated with TMZ were significantly increased under hypoxia, compared with the normoxia group (Fig. 5A and 5C). Furthermore, under hypoxia condition, U87MG cells treated with TMZ plus NAC or 2-APB executes less intracellular Ca^{2+} concentrations than TMZ alone, respectively (Fig. 5A and 5C). In addition, the mitochondrial membrane potential ($\Delta\Psi m$) in U87MG cells treated with TMZ plus NAC or 2-APB was significantly alleviated, compared to TMZ alone under hypoxia, respectively (Fig. 5B and 5D). These findings demonstrate that ROS and TRPM2 participated in calcium influx, which contributed to inhibiting the mitochondrial dysfunction induced by TMZ in glioma cells.

Hypoxia decreases the TMZ-induced cytotoxicity through ROS-regulated increases of TRPM2 in glioma cells

To further verify the relevance of ROS and TRPM2 in TMZ resistance, neurite outgrowth assay was used to evaluate the effects of NAC and 2-APB on the toxicity of TMZ for neurites in U87MG cells under hypoxia. Our results indicated that the neurite length of U87MG cells treated with TMZ alone was significantly shorter than the control group (Fig. 6A and 6B). Moreover, compared with TMZ alone, the neurite length was markedly decreased in U87MG cells treated with TMZ plus NAC or 2-APB and increased in cells treated with NAC or 2-APB alone, respectively (Fig. 6B). These findings demonstrate that ROS and TRPM2 are implicated in decreasing the cytotoxicity of TMZ to glioma cells.

Discussion

Whether it is in the plateau environment or in the human microenvironment, how to skillfully balance the impact of hypoxia on the body has always been a hot topic of scientific research. Hypoxia is one of the representative features in the tumor microenvironment (TME) and is caused by the inadequate supply of blood and oxygen with the rapid expansion of tumor cells(Shao et al., 2018). The oxygen pressure levels are evidently lower in tumor tissues than in normal tissues. In order to adapt with the anoxic environment, tumor cells could tend to be more aggressive and induce resistant tumor phenotypes (Roma-Rodrigues et al., 2019). Tumor metastasis has remained the primary reason for death in GBM patients.It has been reported that hypoxia was associated with drug resistance in cancer patients and could facilitate invasions of tumor cells into the healthy brain regions(Jensen et al., 2014;Kaur et al., 2005). Consistent with the previous studies (Ge et al., 2018), we also demonstrated that hypoxia exposure (2.5% O_2) contributes to decreasing the sensibility of glioma cells to TMZ, compared with the normoxia group. However, the molecular mechanisms of TMZ resistance to GBM under hypoxia is not entirely clear.

Owing to lack of oxygen in tumor tissues, ROS levels are commonly higher than that of normal tissues (Syu et al., 2016). The mild up-regulation of ROS was beneficial for cell proliferation and promoted drug resistance to cancer cells such as breast cancer and choriocarcinoma cells(Shen et al., 2015;Zhu and Zuo, 2013). Interestingly, excessive ROS could damage cellular biomolecules including proteins, DNA, and RNA, followed by inducing cell apoptosis and death in tumor cells. In this study, we demonstrated that TMZ significantly increased mitochondria dysfunction and intracellular ROS levels under hypoxia or normoxia, while suppressing the viability of glioma cells, suggesting that excessive ROS executes negative impacts on proliferation of glioma cells. In addition, our results also found that hypoxia induced the increasing level of baseline ROS and had no effects on mitochondria functions in glioma cells untreated by TMZ. Therefore, we speculated that hypoxia-induced mild up-regulation of ROS might be associated with the lower sensitivity of glioma cells to TMZ, but the underlying mechanisms remained unclear.

The TRPM2 channel is widely expressed in many different types of cells. In the brain, TRPM2 expression has been reported in hippocampus(X. Li et al., 2017), cortex(Kaneko et al., 2006), striatum (Hill et al., 2006) and astrocytes, especially in microglial cells(Lee et al., 2010;Wang et al., 2016). In our studies, we found that, compared with the normoxia group, hypoxia markedly promoted TRPM2 expression in glioma cells, in a time-dependent manner. Moreover, NAC evidently inhibited TRPM2 expression under hypoxia, indicating that ROS contributed to up-regulation of TRPM2 protein in glioma cells, which was consistent with the results of previous studies (Hiroi et al., 2013;Jiang et al., 2010). In addition, our results also showed that TRPM2 expression was positively related to MGMT in cancer tissues of GBM patients. The previous studies reported that hypoxia induces TMZ resistance via over-expression of ZEB1 and c-MYB, which could lead to up-regulation of MGMT protein in GBM cells in vitro(Joseph et al., 2015;Siebzehnrubl et al., 2013). Consistently, our results demonstrated that TRPM2 promoted the expression of MGMT and APNG, but inhibited the mismatch repair of DNA under hypoxia in glioma cells. Based on these findings, we concluded that ROS-mediated over-expression of TRPM2 should be related to hypoxia-induced TMZ resistance in GBM cells.

TRPM2 belongs to a key protein for Ca^{2+} entry. The changes in intracellular Ca^{2+} levels were vital for the proliferation or death of cancer cells. It has been reported that Ca^{2+} influx induced by T-type or TRP (transient receptor potential) channels was conducive to cell proliferation in breast and prostate cancer (Du J et al., 2009;Panner and Wurster, 2006). In our research, we found that hypoxia contributes to Ca^{2+} influx in glioma cells, in comparison with the normoxia group. Consistent with the previous studies(Bomben and Sontheimer, 2008), our data presented that 2-APB blocked the entry of intracellular Ca^{2+} and increased mitochondria dysfunction induced by TMZ, indicating that TRPM2-regulated Ca^{2+} influx was positively corrected with hypoxia-induced resistance to TMZ. Compared with TMZ alone, the treatment group during NAC add-on decreased significantly the concentration of intracellular Ca^{2+} and mitochondria function, suggesting that ROS is important for TRPM2-regulated Ca^{2+} influx. In addition, our results were also verified by a neurite outgrowth assay that ROS and TRPM2 protein were related with hypoxia-induced TMZ resistance in glioma cells.

In conclusion, our findings suggested that the intracellular levels of ROS and TRPM2 protein were up-regulated in glioma cells under hypoxia, which can further reduce the sensitivity to TMZ via promoting demethylation and inhibiting DNA mismatch repair. Moreover, TRPM2-mediated Ca^{2+} influx attenuated mitochondria dysfunction, and then protected glioma cells against TMZ injury. TRPM2 may be a potential target in adjuvant therapy with TMZ for GBM patients.

Declarations

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Author Contribution

Conceptualization, Peng D. and Zhao Y.B.; methodology, Cai Y.Q.; validation, Li L.; formal analysis, Jiang L.N.; resources, Zhu X.F.; writing—original draft preparation, Peng D. and Zhao Y.B.; writing—review and editing, Cai Y.Q.; visualization, Wang K.Y.; project administration, Li Z.W.; funding acquisition, Chen J.H.. All authors have read and agreed to the published version of the manuscript.

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Figures

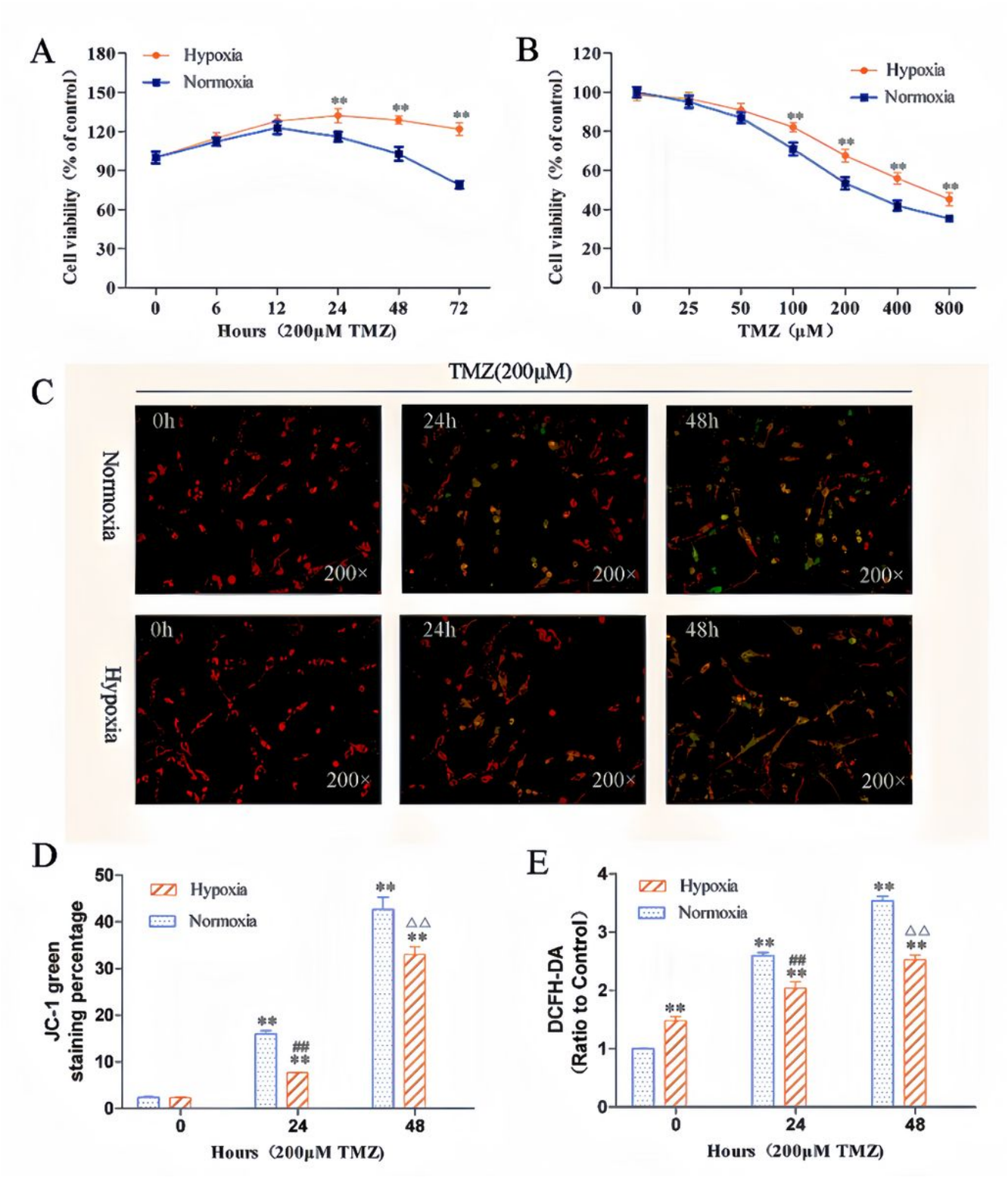

Figure 1

Effects of TMZ on the cell viability, mitochondria function and intracellular ROS levels in U87MG cells under hypoxia and normoxia. (A) U87MG cells were cultured at different concentrations of TMZ for 24 hours under normoxia (21% O_2) or hypoxia (2.5% O_2), and then subjected to WST-1 Assay. **p < 0.01 vs.

normoxia group. (B) After exposition to normoxia (21% O_2) or hypoxia (2.5% O_2) for 24 hours, U87MG cells were treated with TMZ for the indicated time points and measured the cell viability by WST-1 Kit. **p < 0.01 vs. normoxia group. (C) U87MG cells were respectively treated with TMZ for 0, 24 and 48 hours, then stained with JC-1 and observed by fluorescence microscope. Green fluorescence represents JC-1 monomer, indicating impaired mitochondrial membrane potential (ΔΨm). (D) The relative amounts of JC-1 green fluorescence were analyzed. (E) The intracellular ROS scavenging activity was measured according to the H2DCF-DA method. **p < 0.01 vs. normoxia group (0 hour); $^{\#\#}$p<0.01 vs. normoxia group (24 hours) ; $^{\triangle\triangle}$p<0.01 vs. normoxia group (48 hours). Data was shown as mean ± SD, and analyzed by One-way ANOVA.

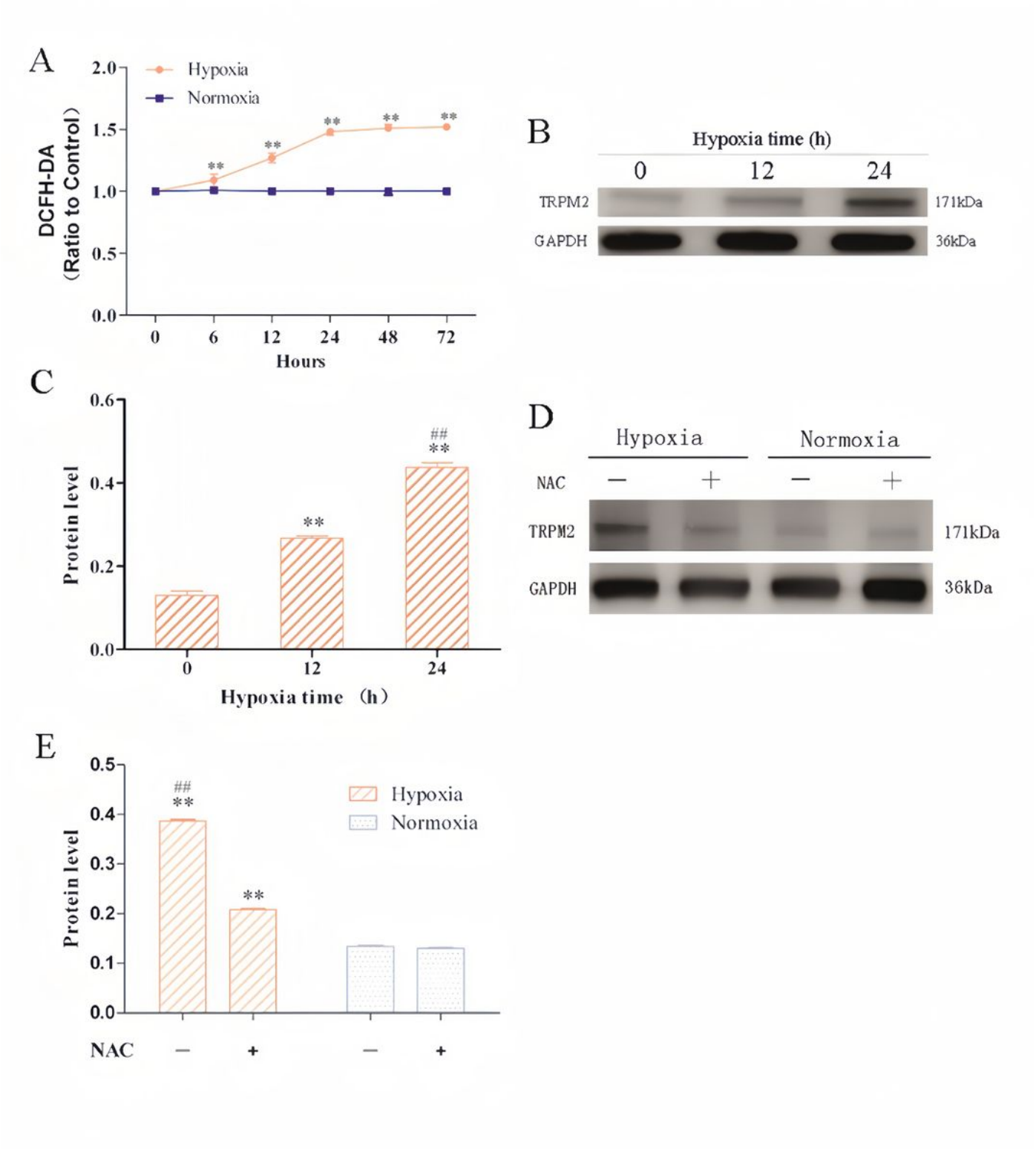

Figure 2

ROS participates in hypoxia-mediated TRPM2 protein expression in U87MG cells. (A) U87MG cells were respectively cultivated at different times under normoxia (21% O_2) or hypoxia (2.5% O_2) and detected for the intracellular ROS levels according to the H2DCF-DA method. (B) U87MG cells were incubated at different times under hypoxia (2.5% O_2), and TRPM2 expression was semi-quantified by western blot analysis. (C) The relative amounts of TRPM2 protein were analyzed by comparing to GAPDH. **p < 0.01

vs normoxia group (0 hour); ##p < 0.01 vs normoxia group (12 hours). (D) U87MG cells were respectively treated with or without 100μM N-acetyl-L-cysteine (NAC) for 24 hours under normoxia (21% O_2) or hypoxia (2.5% O_2), and the protein levels of TRPM2 were semi-quantified by western blot analysis. GAPDH was used as loading control for each lane. (E) Densitometric analysis of TRPM2 expression was executed by comparing with GAPDH. **p < 0.01 vs normoxia group (without NAC); ##p < 0.01 vs hypoxia group (without NAC). Each bar represents the mean ± SD from three independent experiments.

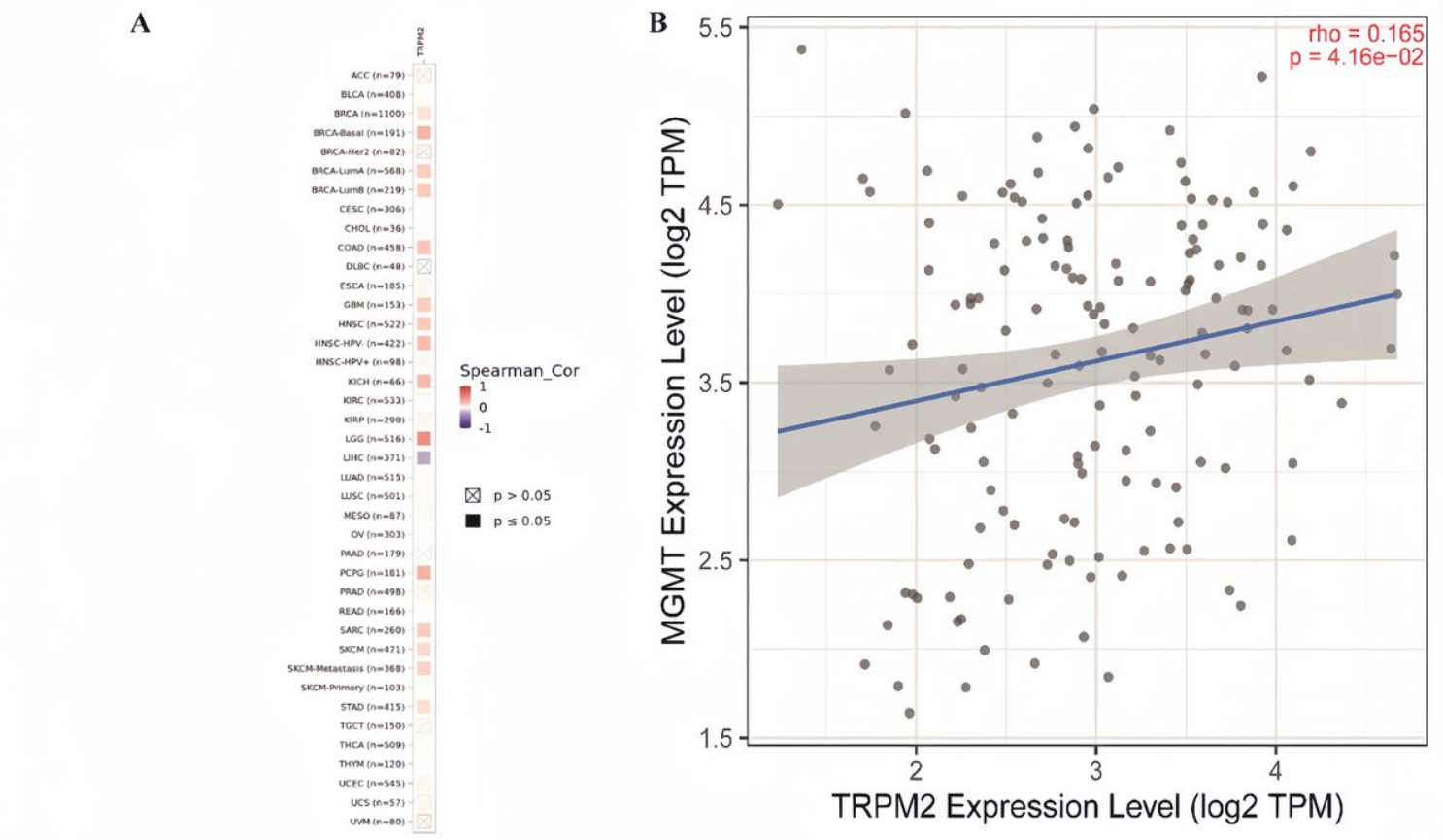

Figure 3

TRPM2 is positive correlation with TMZ resistance-associated protein in glioma cells. (A) Correlation analysis of TRPM2 and MGMT in diverse tumor types. (B) Relationship between TRPM2 and MGMT expression in glioma group used the TIMER 2.0 database.

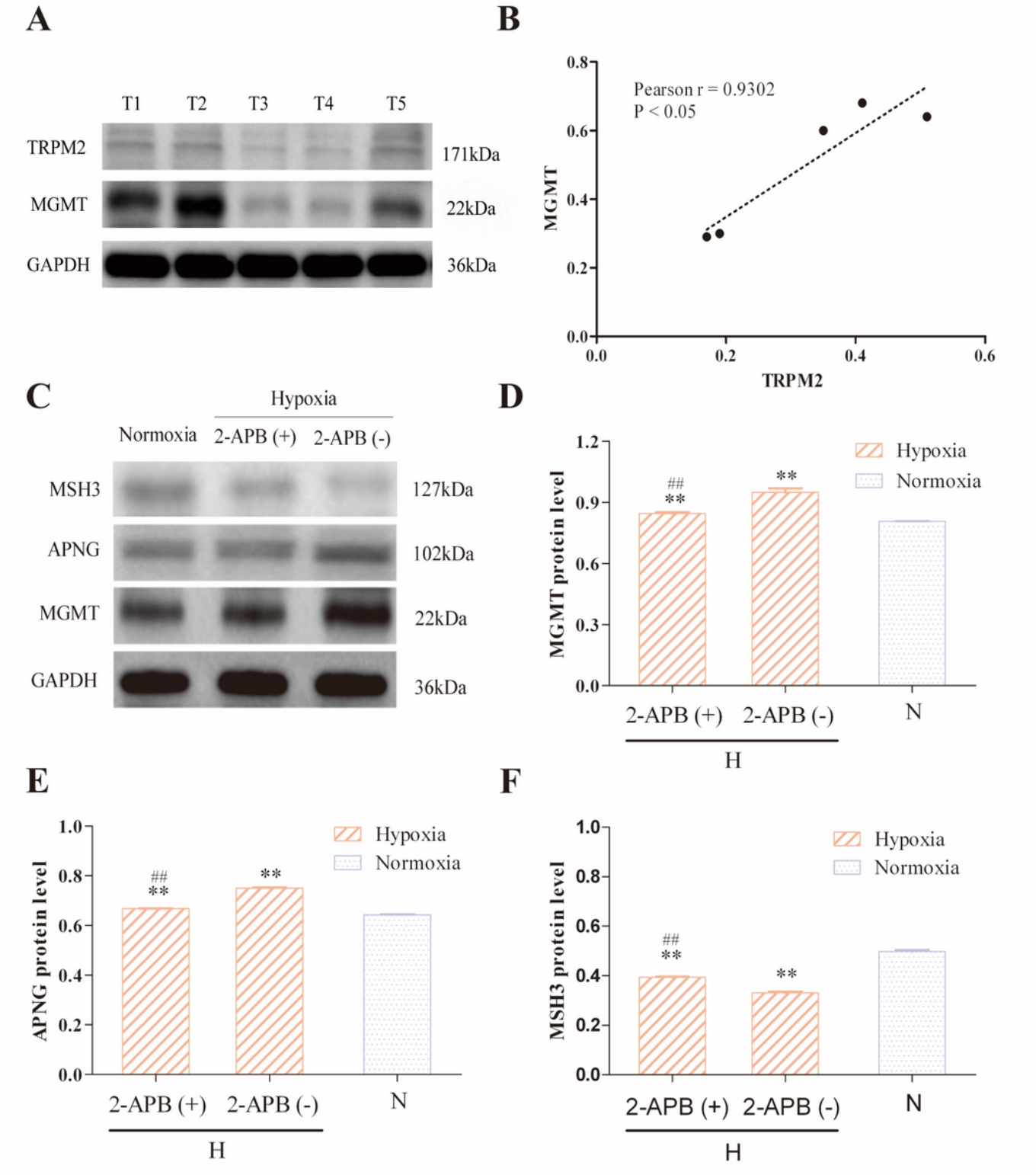

Figure 4

TRPM2 is linked to TMZ resistance in glioma cells. (A) The protein levels of TRPM2 and MGMT were detected by western blot in five glioblastoma tissues. (B) The relevance between TRPM2 and MGMT in human glioblastoma tissues was analyzed. (C) After incubation for 24 hours under normoxia (21% O_2) or hypoxia (2.5% O_2), hypoxic cells were treated with or without 2-APB (inhibitor of TRPM2) for 24 hours, and the equal volume of PBS was added in normoxia group. The protein levels of MSH3, APNG and

MGMT were semi-quantified by western blot analysis. GAPDH was used as loading control for each lane. (D, E and F) Densitometric analysis of MSH3, APNG and MGMT protein levels was performed by comparing with GAPDH. **p < 0.01 vs normoxia group; ##p < 0.01 vs hypoxia group (without 2-APB). Each bar represents the mean ± SD from three independent experiments.

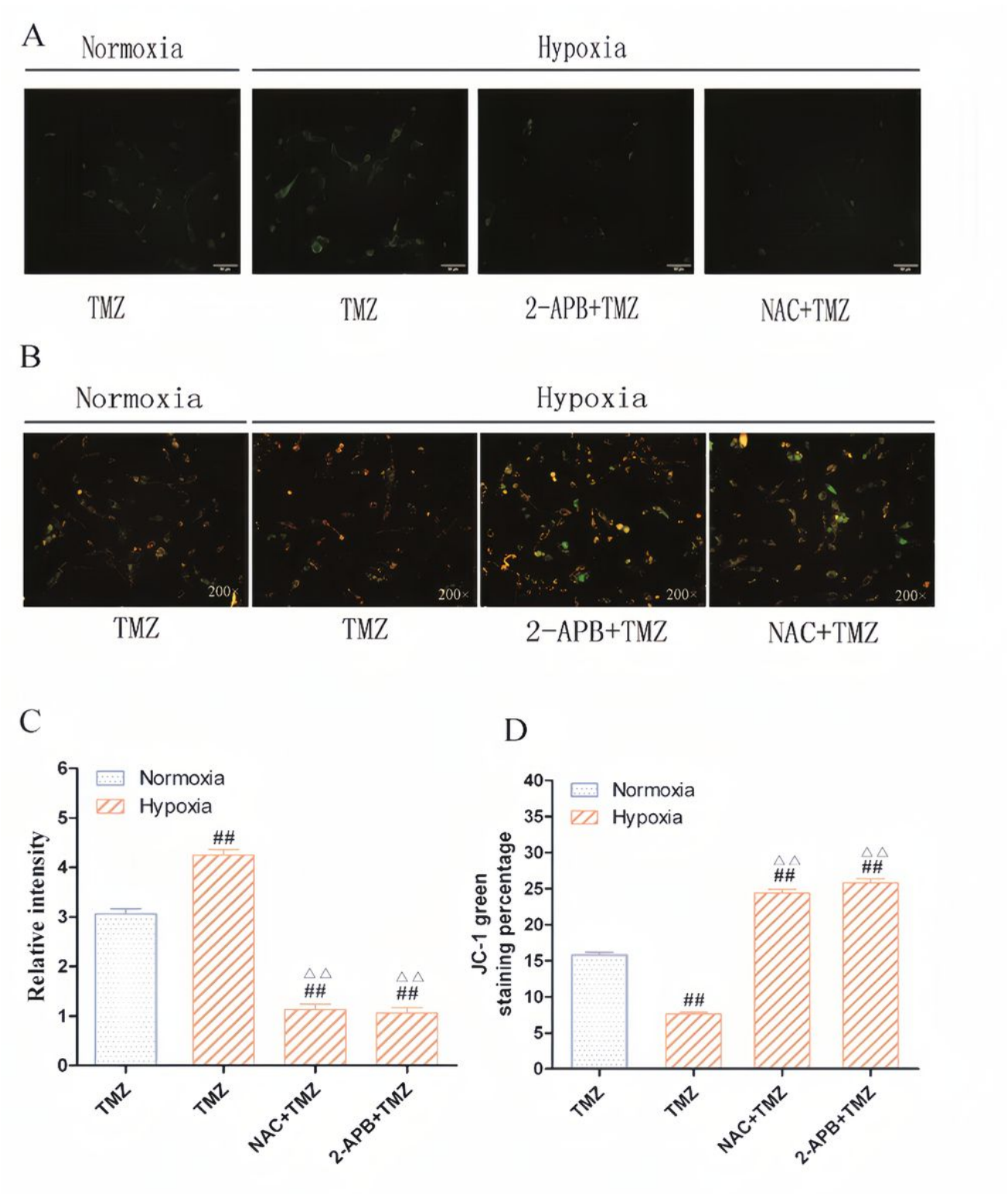

Figure 5

TRPM2-dependent increase of intracellular calcium is implicated in protection of mitochondrial function under Hypoxia. After incubation for 24 hours under normoxia (21% O_2) or hypoxia (2.5% O_2), U87MG cells were respectively treated with PBS, TMZ (200 μM) alone, TMZ (200 μM) plus 2-APB (100 μM) for 24 hours. (A) The cells were stained with Fluo-3 AM (2 μM for 60 min), and the representative images were observed in the fluorescence microscope. (B) U87MG cells were stained with JC-1 and then examined by fluorescence microscope. (C) Changes of the intensity of $[Ca^{2+}]i$ were shown by columns. (D) The relative amounts of JC-1 green fluorescence were measured. Te scale bar = 50 μm. Objective: 20x oil. Each bar represents the mean ± SD from three independent experiments. **p < 0.01 vs. PBS (normoxia group); $^{\#\#}$p < 0.01 vs. TMZ alone (normoxia group); $^{\triangle\triangle}$p < 0.01 vs. TMZ alone (hypoxia group).

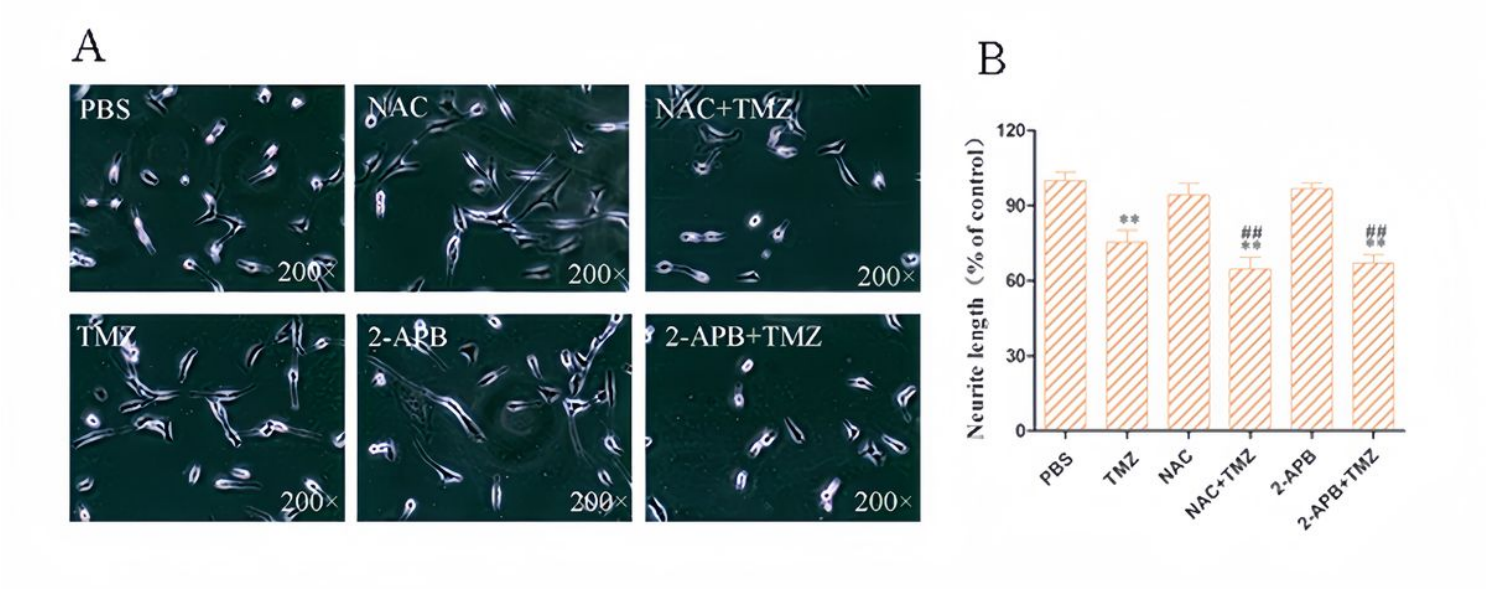

Figure 6

Hypoxia improves TMZ resistance to U87MG cells through ROS - mediated increase of TRPM2 expression. After incubation for 24 hours under hypoxia (2.5% O_2), U87MG cells were respectively treated with PBS, TMZ (200 μM) alone, NAC (100 μM) alone, 2-APB (100 μM) alone, TMZ (200 μM) plus 2-APB (100 μM) and TMZ (200 μM) plus NAC (100 μM) for 24 hours. (A) The representative images were observed by the inverted microscope. (B) Quantification of neurite length was executed. Te scale bar = 50 μm. Objective: 20x oil. Each experiment was performed for three times. Data was shown as mean ± SD. ** p<0.01 vs Control; $^{\#\#}$p<0.01 vs TMZ alone group.