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Original Article

Inhibition of EZH2 reverses chemotherapeutic drug TMZ chemosensitivity in glioblastoma

Tian-Yu Fan[1,2], Hai Wang[1], Peng Xiang[2], Ya-Wei Liu[1], He-Zhen Li[1], Bing-Xi Lei[1], Meng Yu[1], Song-Tao Qi[1]

[1]*Department of Neurosurgery, Nan-Fang Hospital of Southern Medical University, Guangzhou 510515, P.R. China;*
[2]*Department of Neurosurgery, Chang-Sha Central Hospital, Changsha 410004, Hunan, P.R. China*

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Abstract: Glioblastoma remains among the most devastating cancers with a median survival of less than 15 months and virtually no survival beyond five years. Currently, the treatment of glioma includes surgery, radiation therapy, chemotherapy, and comprehensive treatment. Intrinsic or acquired resistance to TMZ, is one of the greatest obstacles in successful GB treatment, and is thought to be influenced by a variety of mechanisms. The EZH2 gene, which is expressed in various solid tumors, can regulate gene transcription and promote the generation and progression of tumors. Our aim was to investigate the relationship between EZH2 and multidrug-resistance of human glioblastoma cells. In this study, we established TMZ-resistant U251 and U87 clones (U251/TMZ and U87/TMZ cells), which expressed high level of EZH2. Using RNA interference, we demonstrated that the downregulation of Ezh2 expression in U251/TMZ and U87/TMZ cells resulted in apoptosis and a cell cycle arrest in the G1/S phase. Furthermore, the reduced expression of Ezh2 altered the MDR, MRP and BCRP mRNA and protein levels. These findings suggest that EZH2 plays an important part in the development of multidrug resistance and may represent a novel therapeutic target for multidrug-resistant glioblastoma.

Keywords: Glioblastoma, EZH2, multidrug-resistance, therapeutic target

Introduction

Glioblastoma is well known as the most common primary aggressive malignant brain tumor of the central nervous system and became one of the most lethal forms of cancer in human. According to the WHO classification standard in 2007, Glioblastoma is divided into four subtype's pathological grade (I-IV) [1]. Glioblastoma multiforme (GBM, WHO IV) is the most malignant form of brain tumor with poor prognosis [2]. Although the treatment strategies have developed dramatically fast during the past decades, its characteristically invasive growth pattern makes surgical resection remains the cornerstone of glioblastoma treatment, therefore requiring the use of adjuvant therapies to prolong survival. In other cancers where radiation can prolong survival or even cure tumors, the addition of chemotherapy to radiation improves survival over radiation treatment alone. In a recent randomized trial, concomitant and adjuvant temozolomide chemotherapy with radiation significantly improves, from 12.1 months to 14.6 months, progression free survival and overall survival in glioblastoma multiforme patients [3]. Temozolomide (TMZ) which seems to work by sensitizing the tumor cells to radiation is an oral chemotherapy with a limited side effect profile that has become the standard of care in glioblastoma treatment [4, 5]. While TMZ has made an impact on survival, tumor recurrence and TMZ resistance remain major challenges. Although the addition of TMZ to radiotherapy has resulted in an overall increase in survival of glioblastoma patients, therapy still fails in almost all glioblastoma patients due to incomplete tumor resection, and/or the apparent resistance of tumor cells to irradiation and TMZ. Some tumors are insensitive to TMZ already at diagnosis, whereas others may develop acquired TMZ-resistance during treatment. Therefore, TMZ-resistance represents a major obstacle in the treatment of this disease.

Histone-lysine N-methyltransferase (EZH2) is an enzyme that in humans encoded by the EZH2 gene, which is located at chromosome 7q35 and contains 20 exons and 19 introns [6, 7]. EZH2 encodes a member of the Polycomb-group (PcG) family, which forms multimeric protein complexes and is involved in maintaining the transcriptional repressive state of genes over successive cell generations. A previous study suggested that the EZH2-exerted transcription repression involves a mechanism that directly controls DNA methylation [8]. With the exception of certain stem cell types, the expression of EZH2 is barely detectable or is suppressed in normal cells [9, 10]. On the other hand, the dysregulated expression of EZH2 has been observed in many types of cancer, including prostate cancer, lymphoma and hepatic cancer [11-13], suggesting a role in cancer malignancy and progression. Despite its well-documented role in cancer malignancy, the involvement of EZH2 in cancer resistance to chemotherapy has not been extensively studied. In the current study, we investigated the role of EZH2 in the chemotherapy drug resistance of GB cells using the U251 and U87 GB cell lines and the U251 and U87- derived multi-drug-resistant cell line, U251/TMZ and U87/TMZ, as the MDR models.

Materials and methods

Cell culture and transfection

Human glioblastoma cell line U251 cells and U87 cells were maintained in DMEM (GIBCO) with 10% heat-inactivated fetal bovine serum, 100 IU penicillin/ml, and 0.1 mg streptomycin/ml in a humidified 5% (v/v) atmosphere of CO_2 at 37°C. The parental U251 cells and U87 cells were exposed to 100 IM of TMZ for 2 weeks to generate TMZ-resistant colonies. The TMZ containing medium was switched every three days. The majority of the cells died, but a small population survived and propagated. The surviving colonies were selected and established as TMZ-resistant U251 clones (U251/TMZ cells) and U87 clones(U87/TMZ cells).

Transfection with Lipofectamine 2000 Reagent followed the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Quantitative RT-PCR

To detect the relative levels of the transcripts, real-time RT-PCR was performed. Briefly, a cDNA library was generated through reverse transcription using M-MLV reverse transcriptase (Promega, Madison, WI) with 2 μg of the large RNA extracted from the cells. The cDNA was used for the amplification of EZH2 gene, and the β-actin gene was used as an endogenous control for the PCR reaction. PCR was performed under the following conditions: 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. PCR primers were as follows: human EZH2 gene forward, 5'-GCC AGA CTG GGA AGA AAT CTG-3' and reverse, 5'-TGT GCT GGA AAA TCC AAG TCA-3'; and β-actin forward, 5'-ACC CCC ACT GAA AAA GAT GA-3' and reverse, 5'-ATC TTC AAA CCT CAT GAT G-3', which was used as the internal control.

MTT assay

Cell survival was determined using the methylthiazolyl-tetrazolium (MTT) assay. Cells were plated in 96-well plates at 5x104 cells/well and treated withTMZ (200 μg/ml) for 24, 48, 72, 96 and 120 h. After a 3-day culture, 20 μl of MTT solution (5 mg/ml; Sigma) was added to each well for 4 h of incubation. The MTT solution was then removed and 200 μl of dimethyl sulfoxide (DMSO; Sigma) were added to dissolve the crystals. Optical density was measured at a wavelength of 570 nm using an ELISA reader.

Western blotting

Cultured cells were lysed by RIPA (0.1% SDS, 1% Triton X-100, 1 mM MgCl 2, 10 mM Tris-HCl, pH 7.4) in 4°C for 30 min. Lysates were collected and cleared by centrifugation, and the protein concentration was determined. Total cell lysates (50 μg) were fractionated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes. Nonspecific binding sites of membranes were saturated with 5% skim milk in TBST solution (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and incubated for 2 hours with antibodies at room temperature. The following antibodies were used: anti-EZH2 and anti-GAPDH. After washing with TBST 4 times, the membranes were incubated with goat anti-rabbit peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO) in 5% skim milk in TBST solution for 1 hour at room temperature; reactions were developed using enhanced chemilu-

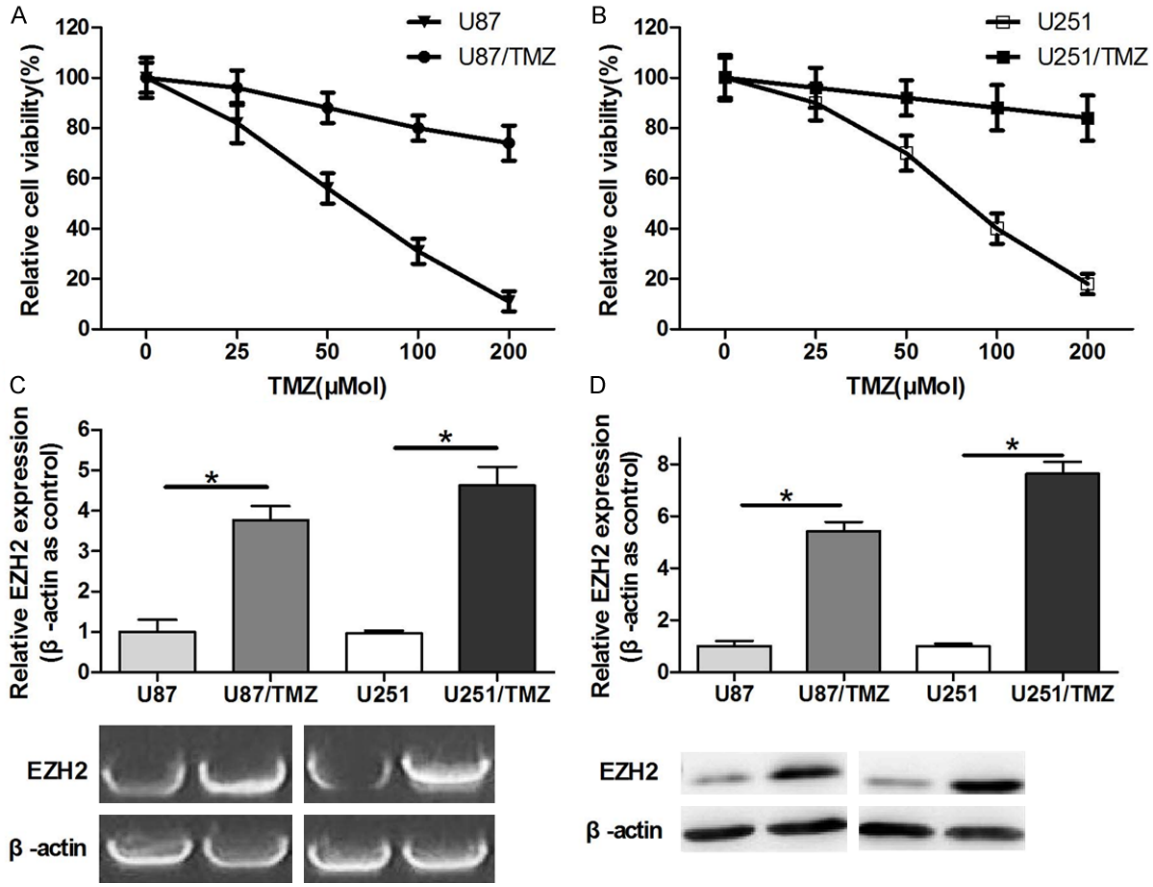

Figure 1. Increased EZH2 expression in TMZ-resistant GB cells. A. The cell viability rate of U87 and U87/TMZ cells. The cells were treated with various doses of TMZ. After 48 h of incubation, the growth inhibition rate of the cells was measured with the MTT assay. *$P < 0.05$. B. The cell viability rate of U251 and U251/TMZ cells. The cells were treated with various doses of TMZ. After 48 h of incubation, the growth inhibition rate of the cells was measured with the MTT assay. *$P < 0.05$. C. Representative images of RT-PCR analysis of EZH2 mRNA in the indicated cell lines. *$P < 0.05$. D. Representative images of western blot analysis of EZH2 protein in the indicated cell lines. *$P < 0.05$.

minescence (Perkin-Elmer Life Sciences, Boston, MA, USA).

SiRNA transfection

EZH2 and control siRNAs were synthesized by Shanghai GenePharma (Shanghai, China). The targeting sequence against EZH2 and that of the control was: 5'-GAC UCU GA A UGC AGU UGC UTT-3' and 5'-AGC A AC UGC AUU CAG AGU CTT-3', respectively. Cells were seeded in a 6-well plate and grown in serum- and antibiotic-free medium. Transfection was performed when the cells reached 60% confluence using Oligofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were refreshed with regular medium 4-6 h after transfection and were subjected to the measurement of knockdown efficiency 48 h later.

Flow cytometry analysis

After 48 hours transfection as earlier described, the cells were harvested and washed twice with PBS. Washed cells were resuspended in 0.6 mL PBS, and fixed by the addition of 1.4 mL 100% ethanol at 4°C overnight. The fixed cells were rinsed twice with PBS, and resuspended in propidium iodine (PI) solution, including 50 mg/mL PI and 50 mg/mL RNase A (Sigma) in PBS without calcium and magnesium, and incubated at 37°C for 30 minutes in the dark. Stained cells were passed through a nylon mesh sieve to remove cell clumps and analyzed by a FACScan flow cytometer and Cell Quest analysis software (Becton Dickinson, San Jose, CA, USA). Flow cytometry analysis was repeated 3 times.

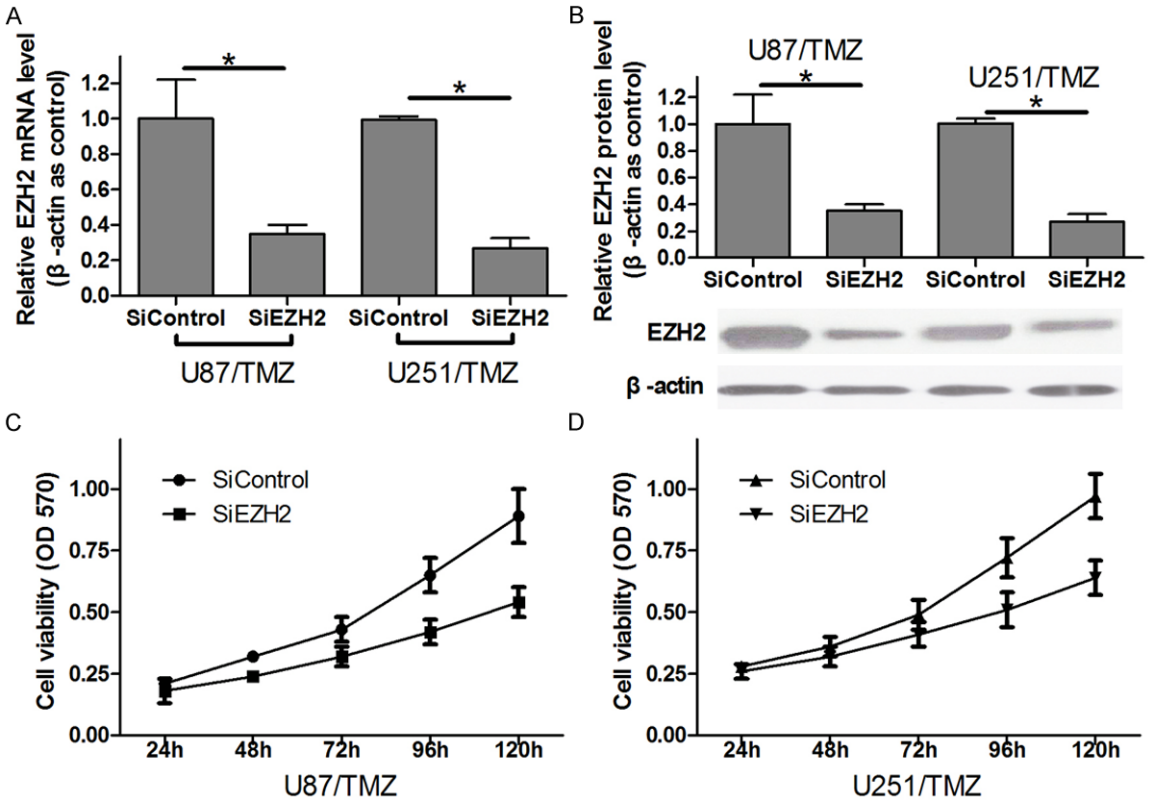

Figure 2. EZH2 siRNA suppresses the growth of TMZ-resistant GB cells. A. qRT-PCR showed that the expression of EZH2 was significantly decreased in the indicated cell lines by the transfection of the siEZH2. *$P < 0.05$. B. Western blot analysis of EZH2 protein levels at 48 h after transfection with siEZH2 in U87/TMZ and U251/TMZ cells. *$P < 0.05$. C. U87/TMZ cells were treated with control or EZH2 siRNA. Cell growth was determined by MTT assay. The experiments were repeated 3 times independently. *$P < 0.05$. D. The cell viability rate of U251/TMZ cells after transfection with siEZH2. *$P < 0.05$.

Statistical analysis

Data are expressed as means ± standard deviation (SD), and $P < 0.05$ is considered statistically significant by the Students-Newman-Keuls test.

Results

EZH2 expression is increased in U251/TMZ and U87/TMZ cells compared to parental glioblastoma cells

We detected the cell growth viability rate of the U251/TMZ, U87/TMZ cell lines and the parental glioblastoma cell lines (**Figure 1A** and **1B**). The results showed that the U251/TMZ and U87/TMZ cell lines were about 5-fold resistant to TMZ compared with the glioblastoma cell lines. We also evaluated the EZH2 expression at the protein level in the glioblastoma parental cells and in the TMZ-induced drug-resistant cells. The results showed that the expression level of EZH2 was higher in the U251/TMZ and U87/TMZ cells than in the parental glioblastoma cells (**Figure 1C** and **1D**), which suggested that EZH2 may be associated with TMZ resistance in glioblastoma cells.

EZH2 siRNA reverses the resistance of U251/TMZ and U87/TMZ cells to chemotherapy

To determine whether EZH2 plays a key role in TMZ resistance, we transfected the EZH2 siRNA or control siRNA into the U251/TMZ and U87/TMZ cells. After transfection with EZH2 siRNA and sicontrol vector in U251/TMZ and U87/TMZ cells, the validity of EZH2 ectopic expression was confirmed by quantitative RT-PCR (**Figure 2A**). Western blot results also showed that EZH2 siRNA effectively reduced the protein level of EZH2 (**Figure 2B**). The MTT assay showed that knockdown the expression level of

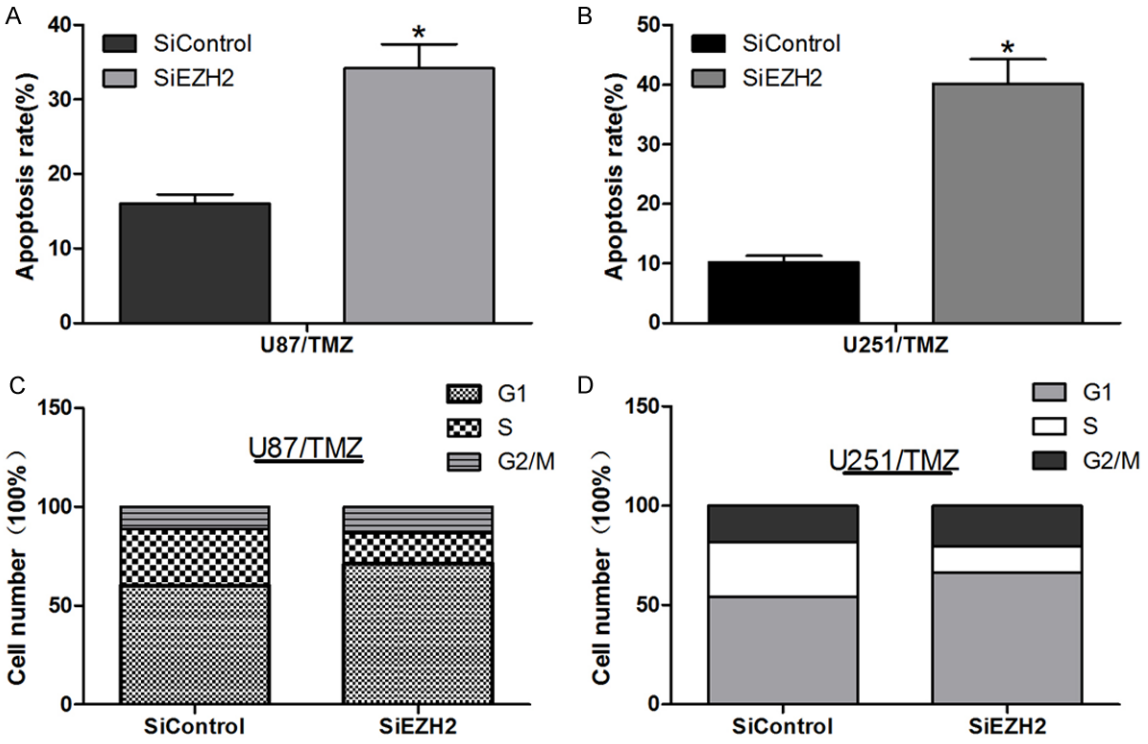

Figure 3. EZH2 siRNA enhances the apoptosis of TMZ-resistant GB cells. A. Apoptosis detected by flow cytometry. U87/TMZ cells were transfected with siEZH2 or sicontrol for 48 h, and then apoptotic cells were detected by flow cytometry. *$P < 0.05$. B. Apoptosis detected by flow cytometry. U251/TMZ cells were transfected with siEZH2 or si-control for 48 h, and then apoptotic cells were detected by flow cytometry. *$P < 0.05$. C. U87/TMZ cells were treated with control or EZH2 siRNA and cell cycle distribution was analysed by flow cytometry. EZH2 siRNA arrests U87/TMZ cells at the G1 phase. D. U251/TMZ cells were treated with control or EZH2 siRNA and cell cycle distribution was analysed by flow cytometry. EZH2 siRNA arrests U251/TMZ cells at the G1 phase.

EZH2 could significantly reduce the viability of U251/TMZ and U87/TMZ cells by about 30-40%, respectively (**Figure 2C** and **2D**), which suggested that EZH2 may modulate TMZ resistance in the U251/TMZ and U87/TMZ cells.

EZH2 siRNA-treated U251/TMZ and U87/TMZ cells undergo apoptosis

To investigate whether the increased apoptosis accounted for the inhibition of cell growth observed in the EZH2 siRNA-treated U251/TMZ and U87/TMZ cells, the apoptotic cells were probed using dual staining with PI and Annexin V. Increased numbers of apoptotic cells were detected in the EZH2 siRNA-treated cells (**Figure 3A**).

EZH2 siRNA treatment leads to the arrest of U251/TMZ and U87/TMZ cells at the G1 phase

Cell cycle progression delay represents another crucial mechanism of the inhibition of the cell growth of cancer cells [14]. The effect of EZH2 siRNA on the cell cycle distribution was examined in the U251/TMZ and U87/TMZ cells. FACS analysis indicated that the ratio of cells at the G1 phase was increased in the EZH2-depleted cells compared with the control groups (**Figure 3C**, **3D**), suggesting that the upregulated EZH2 expression in U251/TMZ and U87/TMZ cells may promote cell cycle progression.

EZH2 siRNA treatment could decrease the expression level of MDR, MRP and BCRP

The development of multi-drug resistance (MDR) by tumor cells is a major obstacle to successful chemotherapy for cancer. One pivotal mechanism by which tumor cells can become resistant to chemotherapy is the increased expression of certain ATP-binding cassette (ABC) transporters which including P-glycoprotein (P-gp, MDR1), multi-drug resistance-associated protein (MRP) and breast cancer resistance protein (BCRP) [3]. These proteins

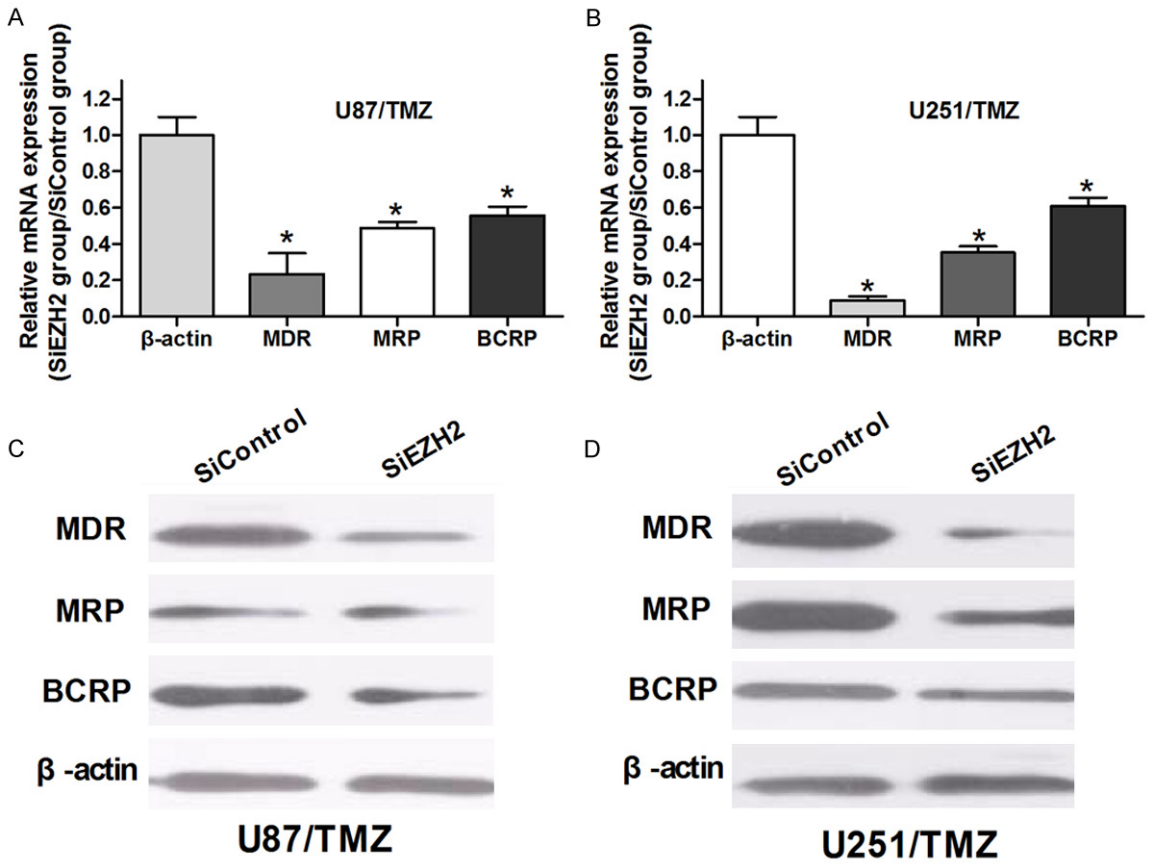

Figure 4. siRNA-mediated depletion of EZH2 down regulates MDR, MRP and BCRP expression in TMZ-resistant GB cells. A. RT-PCR analysis of MDR, MRP and BCRP mRNA in U87/TMZ cells transfected with control or EZH2 siRNA. *$P < 0.05$. B. RT-PCR analysis of MDR, MRP and BCRP mRNA in U251/TMZ cells transfected with control or EZH2 siRNA. *$P < 0.05$. C. Western blot analysis of MDR, MRP and BCRP mRNA in U87/TMZ cells transfected with control or EZH2 siRNA. *$P < 0.05$. D. Western blot analysis of MDR, MRP and BCRP mRNA in U251/TMZ cells transfected with control or EZH2 siRNA. *$P < 0.05$.

are thought to function as energy-dependent efflux pumps of a variety of structurally diverse chemotherapeutic agents, thereby decreasing intracellular drug accumulation. After transfection with EZH2 siRNA, we detected the mRNA and protein expression level of MDR, MRP and BCRP by quantitative RT-PCR and Western Blot (**Figure 4A-D**). The results showed that knockdown the expression level of EZH2 could decrease the expression level of MDR, MRP and BCRP as well.

Discussion

Glioblastoma is the most common malignant primary brain tumor with a median survival rate of 14 months [15]. While adding TMZ to radiotherapy results in an overall increase in survival of glioblastoma patients, almost all patients die. Drug resistance is a multifactorial process which is responsible for the absence of chemoresponse in primary and secondary tumors. Tumor resistance to TMZ has become a serious obstacle. Thus far, enzyme activity of MGMT, MMR, BER repair pathways and drug efflux pumps were considered to be the most important mechanisms underlying resistance of patients with malignant gliomas to TMZ [16]. However, it is unlikely that these mechanisms are responsible for all TMZ-resistance observed. Here we attempted to determine non-canonical factors that may influence the efficacy of TMZ in glioblastoma. Using gene expression analysis of both TMZ-sensitive and TMZ-resistant glioblastoma cell lines, we demonstrated that knockdown the expression level of EZH2 is associated with a TMZ- resistant phenotype in glioblastoma cells.

Enhancer of zeste homolog 2 (EZH2) is a member of the Polycomb Group (PcG) of proteins.

PcG proteins are important epigenetic regulators which can function as transcriptional repressors that silence specific sets of genes through chromatin modification [17]. PcG proteins are grouped in polycomb repressive complexes (PRC). PRC2 includes enhancer of zeste 2 (EZH2), suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED). EZH2 is the catalytically active component of the PRC2 complex that participates in transcriptional repression of specific genes by trimethylation of lysine 27 and, to a lesser extent, lysine 9 of histone H3. Recently, an increasing number of studies linked various oncogenic properties to EZH2, including impaired cellular differentiation and enhanced proliferation and in vivo tumor growth [18-23]. EZH2 is overexpressed in various cancers, such as ovarian and pancreatic cancer, where its overexpression is associated with poor prognosis.

In our study, we initially confirmed that EZH2 is highly expressed in multidrug-resistant human glioblastoma cells U251/TMZ and U87/TMZ. EZH2 is capable of methyltransferase activity. The overexpressed EZH2 can regulate transcription of downstream genes by DNA methylation. To interpret the mechanism of action of EZH2, we used RNA interference technology to silence EZH2 expression. We found that EZH2 siRNA was delivered into the cells effectively and quickly silenced EZH2, the levels of mRNA and protein were decreased by more than 70%, indicating the effective inhibitory effects of EZH2 siRNA. We calculated the cell growth viability through the MTT assay to detect the relationship between EZH2 and the growth capacity of TMZ resistant cell lines U251/TMZ and U87/TMZ. Down expressed EZH2 could significantly reduce the cell growth viability of U251/TMZ and U87/TMZ cells by about 30-40%, respectively suggesting that EZH2 might regulate cellular proliferation, which is consistent with findings for breast and prostate cancer. The lethality of TMZ on U251/TMZ and U87/TMZ cells was markedly strengthened after EZH2 silencing.

Cell cycle can be used to evaluate sensitivity to chemotherapy. We analysed the cell cycle distribution of each group, finding that in the control and siEZH2 group, the proportion of cells in the G1 phase followed a gradual increasing trend, while the proportion of cells in the S, G2, and M phases decreased concordantly, indicating G1 arrest. This finding demonstrated that treatment with EZH2 siRNA could inhibit the progression of the cell cycle by blocking the transition from the G1 phase to the S and G2 phases. It was found in colon cancer that silencing EZH2 decreased the expression of cyclin D1, causing cell cycle arrest at G1/S [24]. In addition, in pancreatic cancer, the genetic deletion of EZH2 results in re-expression of p27-Kip1, which also inhibits the G1/S transition [25]. In our experiment, what we found was consistent with the previous reports.

The mechanism of multidrug resistance is very complex and is closely associated with genes, cytokines and transporters. ATP-binding cassette transporters (ABC transporters) are known to play a crucial role in the development of multidrug resistance (MDR). In MDR, patients that are on medication eventually develop resistance not only to the drug they are taking but also to several different types of drugs. This is caused by several factors, one of which is increased excretion of the drug from the cell by ABC transporters. For example, the ABCB1 protein (P-glycoprotein) functions in pumping tumor suppression drugs out of the cell. P-gp also called MDR1, ABCB1, is the prototype of ABC transporters and also the most extensively-studied gene. P-gp is known to transport organic cationic or neutral compounds. A few ABCC family members, also known as MRP, have also been demonstrated to confer MDR to organic anion compounds. The most-studied member in ABCG family is ABCG2, also known as BCRP (breast cancer resistance protein) confer resistance to most of topoisomerase I or II inhibitors such as topotecan, irinotecan, and doxorubicin. Our study confirmed this conclusion at the mRNA and protein levels. To investigate the underlying mechanism of increased sensitivity to chemotherapy after EZH2 depletion, we also checked MDR, MRP and BCRP expression, finding that MDR, MRP and BCRP expression was markedly reduced after EZH2 silencing. As noted above, MDR, MRP and BCRP, which belong to the ABC transporter family, functions as a pump to export drugs out of cells. When MDR, MRP and BCRP expression is upregulated, the drug efflux pump could reduce intracellular concentrations of chemotherapeutic agents and eliminate the agents from cancer cells, which decreases the tumor-cell-killing effect of TMZ and weaken the chemosensitivity of the tumor by disrupting its drug efflux activi-

ty. EZH2 silencing decreases the MDR, MRP and BCRP mRNA and protein levels, which would lead to reduce efflux pump activity, and in turn increased sensitivity to chemotherapy in glioblastoma cells. So, the anti-MDR effect of the EZH2 deletion is likely mediated by MDR, MRP and BCRP.

Overall, our study reveals a role of EZH2 in the glioblastoma cells and demonstrates that the intervention of EZH2 reverses the MDR and inhibits the growth of TMZ-resistant glioblastoma cells. At present, finding drug-resistance-associated genes that can be regarded as therapeutic targets is a hot research field; people hope to reverse drug-resistance in this way. Our experiment studied may provide a novel therapeutic approach for multidrug-resistant glioblastoma and demonstrate the relationship between EZH2 and multidrug-resistance in glioblastoma for the first time. While the RNAi approach was used in vitro to validate the role of EZH2 in this study, the utilization of RNAi as clinical therapy requires further investigation and is technically challenging [28]. The data presented in the current study may aid future studies exploring alternative approaches for modulating EZH2 function.

Disclosure of conflict of interest

None.

Address correspondence to: Song-Tao Qi, Department of Neurosurgery, Nan-Fang Hospital of Southern Medical University, Guangzhou 510515, P.R. China. Tel: +86 020-61641114; Fax: +86 20-6164-1114; E-mail: qisongtao510515@163.com

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