Med Oncol (2015) 32:69
DOI 10.1007/s12032-015-0517-y

ORIGINAL PAPER

p38 MAPK-dependent Nrf2 induction enhances the resistance of glioma cells against TMZ

Leina Ma · Jia Liu · Xinling Zhang · Jieqiong Qi · Wengong Yu · Yuchao Gu

Received: 30 January 2015 / Accepted: 13 February 2015 / Published online: 19 February 2015

Abstract Temozolomide (TMZ) is an effective agent for clinical glioma treatment, but the innate and acquired resistance of glioma always limits its application. Although some advances have been achieved to elucidate the molecular mechanism underlying TMZ resistance, the role of Nrf2 (a principle regulator of cellular defense against drugs and oxidative stress) has not been well established in the acquisition of this phenotype. Our data showed that TMZ treatment induces the activation of Nrf2 and p38 MAPK signaling in glioma cells, while p38 inhibition abolished the effect of TMZ on Nrf2. Further study revealed that Nrf2 silencing was able to enhance the response of glioma cells to TMZ. Additionally, Nrf2 overexpression overrides the effect of p38 MAPK activation on Temozolomide resistance. In conclusions, we identified a p38 MAPK/Nrf2 signaling as a key molecular network contributing to TMZ resistance of glioma, and provided evidence that suppressing this signaling may be a promising strategy to improve TMZ's therapeutic efficiency.

Keywords Temozolomide · Glioma · Nrf2 · p38 MAPK

L. Ma · X. Zhang · J. Qi · W. Yu · Y. Gu (✉)
Key Laboratory of Marine Drugs, Chinese Ministry of Education; Key Laboratory of Glycoscience and Glycotechnology of Shandong Province; School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao 266003, China
e-mail: guych@126.com

J. Liu
College of Medicine, Qingdao University, Qingdao 266021, China

Introduction

Temozolomide (TMZ) has been widely applied for clinical treatment of malignant glioma. The underlying anti-tumor mechanism involves with forced methylation of DNA in target cells by the active form of TMZ, MITC (5-(3-methyl three nitrene-1-based) imidazole-4-amide). The methylation event can elicit the apoptosis in cancer cells. However, some subpopulation of glioma cells develops the resistance against the action of TMZ, thereby impairing its therapeutic efficiency. The mechanism by which glioma becomes refractory to TMZ treatment has been studied for a long time. *O*-6-alkylguanine DNA alkyltransferase (AGT) encoded by the *O*-6-methylguanine-DNA methyltransferase (MGMT) gene has been reported to repair DNA damage and thus enable glioma cells insensitive to the pro-apoptotic effect of TMZ [1]. An AGT inhibitor, *O*-6-BG, has been shown to increase the efficacy of TMZ on glioma both in vitro and in animal models. However, mixed results from a phase-II clinical trial showed that no significant benefit from the combination of *O*-6-BG and TMZ on the prolonging of glioblastoma patients' survival, although some improvement was achieved by the application of for the treatment of TMZ-resistant anaplastic glioma [2]. This finding suggests that there are still other mechanisms of glioma TMZ resistance, which has not been elucidated yet [3].

Recently, nuclear transcription factor erythroid 2p45 (NF-E2)-related factor 2 (Nrf2), which has been considered as a principle regulator of cellular defense against drugs and oxidative stress [4], was shown to contribute to the resistance of glioblastoma cells to the combined treatment of TMZ and radiotherapy [5]. However, it is still unknown if TMZ alone can increase the activity of Nrf2 and the resulting resistance of glioma against TMZ. In addition, the

molecular mechanism by which TMZ, if so, activates Nrf2 has not been studied yet in glioma.

In this study, we employed U87 MG and U251 MG glioma cells as models to verify the role of Nrf2 activation in single TMZ resistance of glioma and to explore the involved mechanism.

Materials and methods

Cell lines and reagents

U87 and U251 were obtained from ATCC. Cells were cultured in RPMI-1640 supplemented with 10 % FBS (Gibco, Life Technologies) and penicillin–streptomycin, at 37 °C, in 5 % CO_2, and was subcultured every 3 or 4 days. Temozolomide (TMZ), SB203580, anisomycin and tert-butylhydroquinone (tBHQ) were purchased from Sigma-Aldrich. The media supplements for cell culture were purchased from Life Technologies. Antibody of NRF2 was purchased from Abcam. Antibodies of p38, phospho-p38, cleaved caspase-3 and GAPDH were purchased from Cell Signaling Technology.

Plasmids and transfection

NC16 pCDNA3.1-FLAG-NRF2 was a gift from Randall Moon (Addgene plasmid # 36971). pGV113-shNRF2 and pGV113-shControl were purchased from Shanghai Genechem, China. Short hairpin sequence targeting Nrf2 coding region was AGCAAACAAGAGATGGCAA. The construction of pARE-TI-luciferase reporter (ARE-luciferase) was according to previous description [6, 7]. Cells were transfected with plasmid using Attractene transfection reagent (Qiagen) according to the manufacturer's instructions.

Western blot

After treatments, cells were harvested with RIPA lysis buffer containing protease inhibitors (Roche). The samples were then separated in a 7.5 % SDS-PAGE at 120 V and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA) for 1.5 h using a semi-dry transfer system (Bio-Rad). The membranes were blocked for 1 h at room temperature and incubated with primary antibodies overnight at 4 °C. After hybridization with primary antibody, membranes were incubated with secondary antibodies with horseradish peroxidase (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Final detection was performed with SuperSignal West PICO (Pierce, Thermo). Bands were exposed to X-ray film. Exposure scan varied from 5 s to 60 min. Experiments were performed in triplicate.

ARE-driven luciferase activity assay

Cells were transfected with the Renilla (Promega) and ARE-luciferase plasmid using Lipofectamine 2000 (Invitrogen). At 24 h after transfection, the cells were lysed and assayed for luciferase activity with the dual luciferase assay system (Promega), according to the manufacturer's instructions. Relative light units were measured in a SpectraMax M5 (Molecular Devices). Experiments were performed in triplicate.

Quantitative real-time PCR

Total cellular RNA was isolated using the RNeasy Mini Kit (QIAGEN). cDNA was prepared using cDNA Reverse Transcription kit (Invitrogen). Quantitative RT-PCR was performed on ABI 7500 Real-Time PCR System (Applied Biosystems) using FastStart SYBR Green Master (Roche). The qRT-PCR were carried out using the following primers: NRF2-F, AACCAGTGGATCTGCCAACTACTC; NRF2-R, CTGCGCCAAAAGCTGCAT; HO-1-F, TGCTCAACATCCAGCTCTTTGA; HO-1-R, GCAGAATCTTGCACTTTGTTGCT; NQO-1b-F, ATTGAATTCGGGCGTCTGCTG; NQO-1b-R, AGGCTGGTTTGAGCGAGT. All reactions were performed in triplicate, and the experiments were repeated at least twice.

Cell viability

Cells were washed with phosphate-buffered saline (PBS) followed by the addition of MTT (1 mg/ml) and incubation for 4 h at 37 °C. The medium was discarded, formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm. Experiments were performed in triplicate.

Statistical analysis

Each experiment was reproduced at three times with consistent results. Comparisons were made using Student's t test or a nonparametric Mann–Whitney *U* test. A two-tailed p value < 0.05 was considered statistically significant for all analyses.

Results

Temozolomide increases the expression level and transcriptional activity of Nrf2

First of all, we employed immunoblot assays to detect the expression level of Nrf2 in glioma cells treated with TMZ. The results revealed that its expression was significantly

induced in U87 MG and U251 MG cells exposed to TMZ, in a dose-dependent manner (Fig. 1a, b). To confirm the translational activity of Nrf2, luciferase assay was performed with pARE-TI-luciferase reporter plasmid transfected. The luciferase assay indicated that TMZ also increased the transcriptional activity of Nrf2 in glioma cells (Fig. 1c, d).

The induction in Nrf2 expression by Temozolomide depends on p38 MAPK activation

Subsequently, we investigated if p38 MAPK, a key kinase during cellular reaction against stress, was able to be activated in glioma cells treated with TMZ. We employed immunoblot assays to detect the expression level of total p38 MAPK and phosphorylated p38 MAPK. The data demonstrated that TMZ induced the phosphorylation of p38 MAPK in both dose- and time-dependent fashions in U87 MG and U251 MG cells (Fig. 2a, b).

Consistently, p38 MAPK inhibition by SB203580 in glioma cells treated with TMZ further confirmed the role of p38 MAPK signaling in Nrf2 overexpression. Immunoblot analysis and luciferase assays revealed that blocking p38 MAPK was able to abolish the effect of TMA treatment on the expression level and transcriptional activity of Nrf2 in U87 MG and U251 MG cells (Fig. 3a, b). Consistently, NQO-1b and HO-1 mRNA, both of which are the downstream targets of Nrf2 [8], were reduced by suppression of p38 MAPK (Fig. 3c).

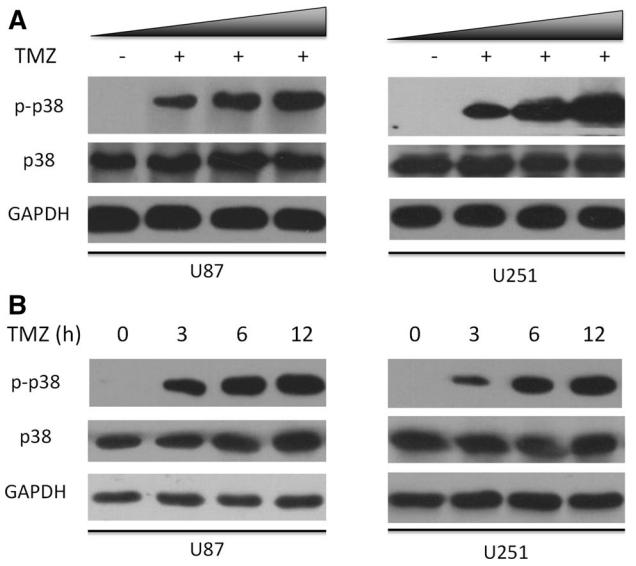

Fig. 2 Temozolomide induces the activation of p38 MAPK signaling in glioma cells. **a** Glioma cells were incubated with TMZ (0, 25, 50 or 100 μM) for 24 h, followed by immunoblot analysis of p38 and phospho-p38 protein level. GAPDH was used as endogenous reference. **b** Glioma cells were incubated with TMZ (100 μM) for 0, 3, 6 and 12 h, followed by immunoblot analysis of p38 and phospho-p38 protein level. GAPDH was used as endogenous reference. Experiments were performed in triplicate

The suppression of p38 MAPK signaling increases the sensitivity of glioma cells to Temozolomide

To confirm the role of p38 MAPK activation in the acquisition of TMZ resistance, we detected the viability of

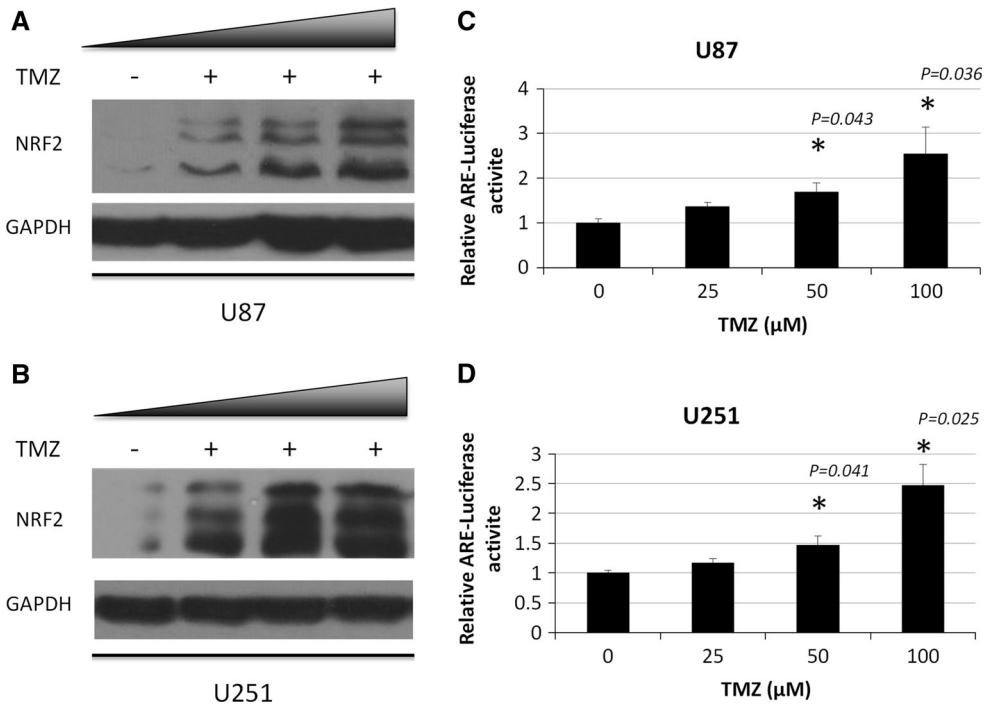

Fig. 1 Temozolomide increases the expression level and transcriptional activity of Nrf2. **a** U87 cells were incubated with TMZ (0, 25, 50 or 100 μM) for 24 h, followed by immunoblot analysis of Nrf2 protein level. GAPDH was used as endogenous reference. **b** Nrf2 expression level was also examined by immunoblot assay in U251 cells. **c** U87 cells were treated with TMZ (0, 25, 50 or 100 μM). 24 h later, ARE-luciferase reporter assay was used to detect the transcriptional activity of Nrf2. **d** The same experiment was also performed in U251 cells. All experiments were performed in triplicate

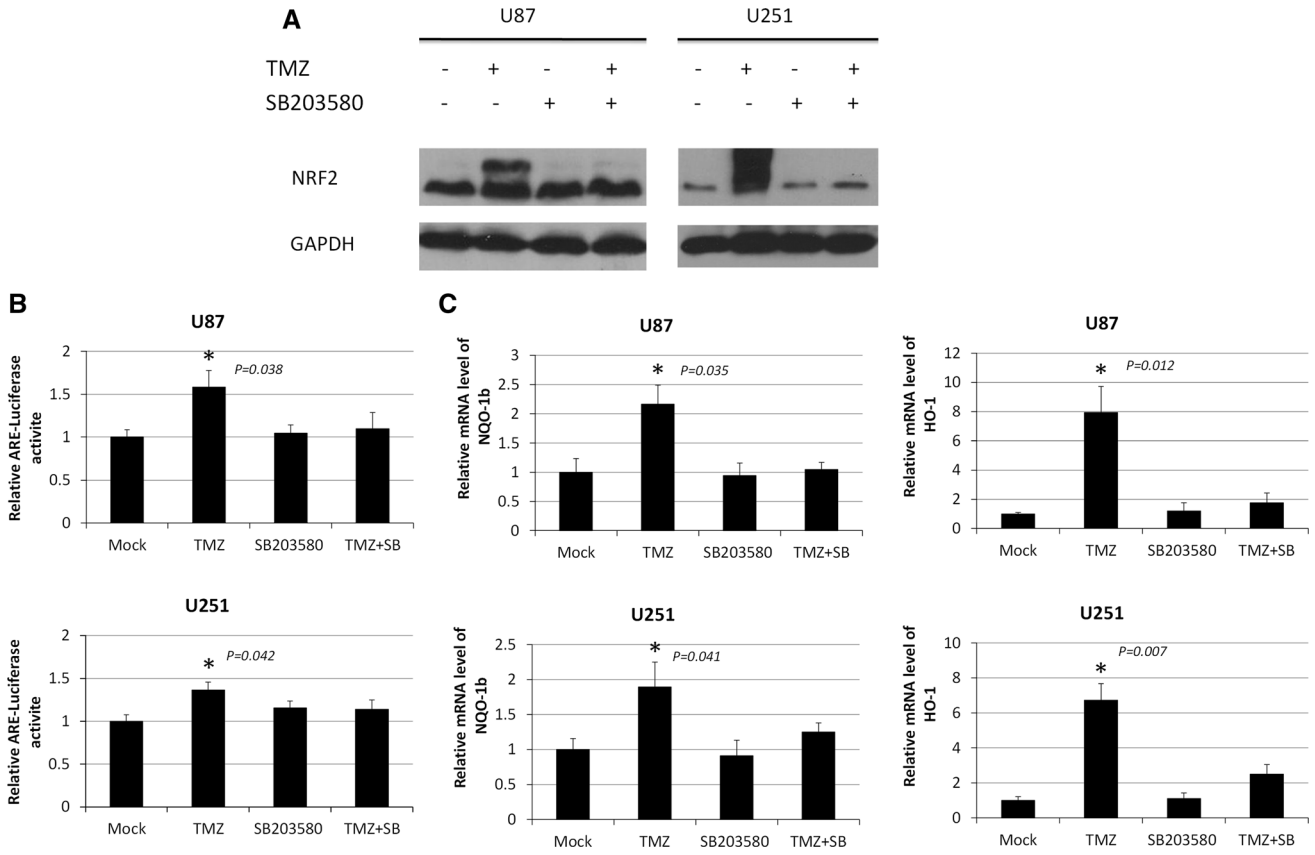

Fig. 3 Inhibiting p38 MAPK activation abolishes Temozolomide's effect on Nrf2. U87 and U251 cells were incubated with 50 μg/ml TMZ and/or 1 μg/ml SB203580 for 24 h, (**a**) followed by immunoblot analysis of Nrf2 protein level. GAPDH was used as endogenous reference. **b** ARE-TI-luciferase reporter assay was used to detect the transcriptional activity of Nrf2. **c** mRNA abundance of NQO-1b and HO-1 was quantified using qPCR. GAPDH was used as endogenous reference. The *bar* represents mean ± SD from three independent experiments. All experiments were performed in triplicate

glioma cells after p38 MAPK blocking. The results revealed that p38 MAPK inhibition was able to increase the sensitivity of glioma cells to TMZ (Fig. 4a, b). This finding suggested that p38 MAPK activation is required for the acquired resistance of glioma cells to TMZ.

Nrf2 silencing abrogates the resistance of glioma cells to Temozolomide

To confirm the importance of Nrf2 for TMZ resistance, we examined the survival of glioma cells after Nrf2 silencing. The data indicated that Nrf2 silencing reversed the resistance of glioma cells against TMZ (Fig. 5a, b), implying that Nrf2 expression plays a role in TMZ resistance.

Nrf2 overexpression overrides the effect of p38 MAPK activation on Temozolomide resistance

Given the fact that TMZ-induced p38 MAPK activation increased the expression of Nrf2, and we subsequently aimed to study the role of Nrf2 overexpression in a p38 MAPK-dependent TMZ resistance. MTT assays showed that Nrf2 overexpression by the transfection of pcDNA3.1-flag-Nrf2 was able to retain the insensitivity of U87 MG glioma cells to TMZ, even when p38 MAPK signaling was suppressed by SB203580 (Fig. 6a, b). These data proved that Nrf2 mediates the effect of TMZ-induced p38 MAPK activation on the response of glioma to TMZ.

Discussion

The innate and acquired resistance of tumor cells is a major obstacle in the application of TMZ for glioma treatment [9]. Many efforts have been made to elucidate the molecular mechanism by which glioma develops resistance against TMZ. Overexpression of Nrf2 in the glioma treated with the combination of TMZ and irradiation was shown to play an important role in the acquisition of drug resistance [5]. However, it is unknown if TMZ or radiotherapy leads to the induction in Nrf2, and the resulting drug resistance in glioma. In this study, our data demonstrated that TMZ

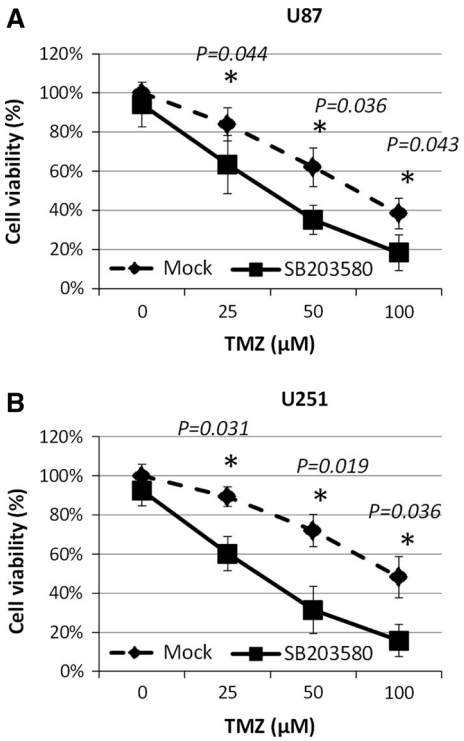

Fig. 4 Suppression of p38 MAPK signaling increases the sensitivity of glioma cells to Temozolomide. **a** U87 cells were treated with TMZ (0, 25, 50 or 100 μM) and/or 1 μg/ml SB203580, and 48 h later, MTT assays were used to detect the viability of the cells. **b** The above experiment was also conducted in U251 cells. Experiments were performed in triplicate

alone is sufficient to induce the expression of Nrf2, and this event renders the insensitivity of glioma to TMZ.

Another unaddressed issue is what is the pathway mediating the effect of TMZ on Nrf2. Our data indicated that p38 MAPK pathway can be induced by TMZ treatment. In lines with our findings, previous publications have shown that p38 MAPK activation is triggered by many natural and synthetic compounds, such as oleanolic acids [10], aplysin [11] and doxorubicin [12]. For TMZ, p38 MAPK signaling has also been verified to be activated by TMZ treatment [13]. However, the role of p38 MAPK activation in the Nrf2 induction by TMZ is unexplored yet. Our work confirmed that p38 MAPK signaling mediates the promoting effect of TMA on Nrf2 abundance in glioma cells, evidenced by the fact that inhibition of p38 MAPK abolished the expression of Nrf2 caused by TMZ.

p38 activation by TMZ has been shown to contribute to the resistance of TMZ in glioma cells [13], and our data further confirmed this finding. However, the involved molecular mechanism has been completely elucidated, although cdc25C and cdc2 may mediate this effect as the downstream effectors. Our work identified that Nrf2 induction is responsible for the effect of p38 MAPK activation on drug resistance in glioma. Our finding not only facilitates our understanding of the implication of p38 MAPK in TMZ resistance, but also verifies that Nrf2 suppression potentiates the anti-tumor activity of TMZ.

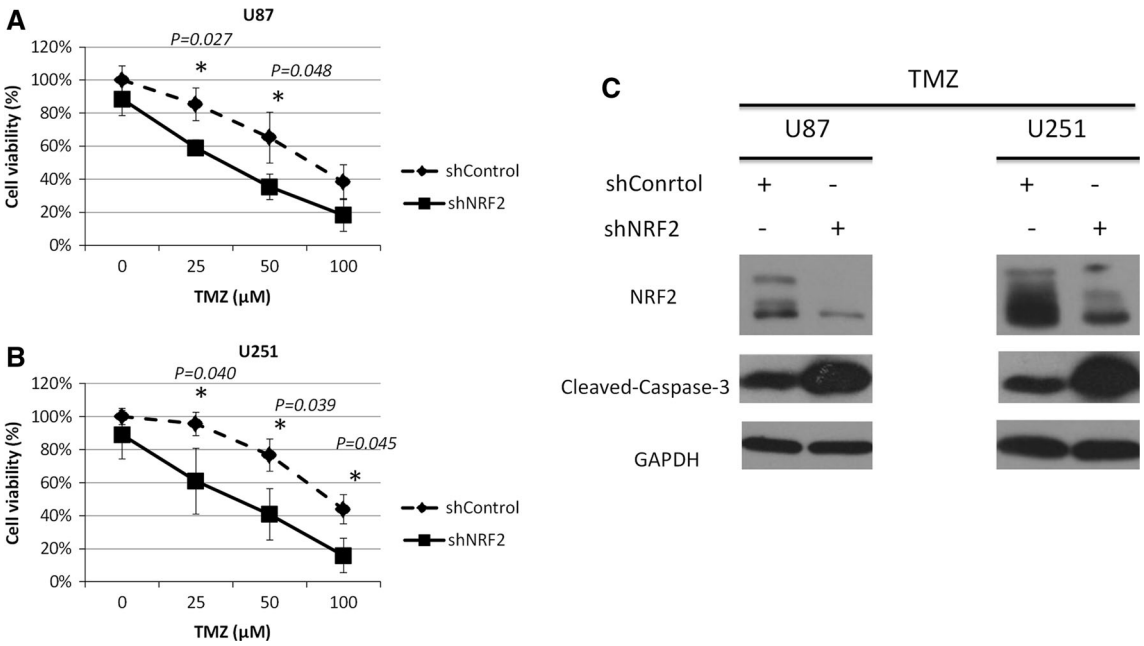

Fig. 5 Nrf2 silencing abrogates the resistance of glioma cells to Temozolomide. **a** Glioma cells were transfected with shNRF2 or shControl plasmid. After 24 h recovery, cells were treated with TMZ (0, 25, 50 or 100 μM), and 48 h later, MTT assays were used to detect the viability of the cells. **b** The above experiment was followed by immunoblot assays which were performed to detect Nrf2 protein level. GAPDH was used as endogenous reference. All experiments were performed in triplicate

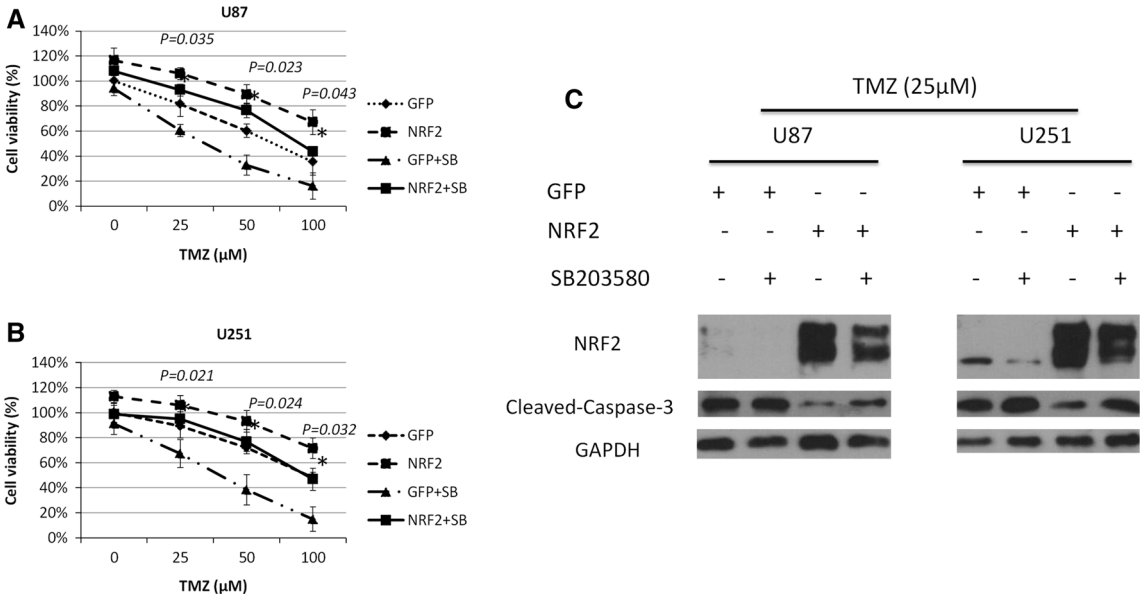

Fig. 6 Nrf2 overexpression overrides the effect of p38 MAPK activation on Temozolomide resistance. **a** Glioma cells were transfected with pcDNA-flag-NRF2 or pcDNA-GFP plasmid. After 24 h recovery, cells were treated with TMZ (0, 25, 50 or 100 μM) and/or 1 μg/ml SB203580, and 48 h later, MTT assays were used to detect the viability of the cells. **b** The above experiment was followed by immunoblot assays which were performed to detect Nrf2 protein level. GAPDH was used as endogenous reference. All experiments were performed in triplicate

Collectively, we provided evidence that p38 MAPK/Nrf2 pathway is associated with the resistance of glioma against TMZ. Simultaneous suppression of p38 MAPK activation and Nrf2 expression may be a promising strategy to abrogate drug insensitivity of glioma, and enhance the therapeutic effect of TMZ on glioma.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Grant Nos. 81272264, 81172013, 81101505). The funding agency had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to submit the article for publication.

Conflict of interest None.

References

1. Jacinto FV, Esteller M. MGMT hypermethylation: a prognostic foe, a predictive friend. DNA Repair. 2007;6:1155–60.
2. Ueno T, Ko SH, Grubbs E, Yoshimoto Y, Augustine C, et al. Modulation of chemotherapy resistance in regional therapy: a novel therapeutic approach to advanced extremity melanoma using intra-arterial temozolomide in combination with systemic *O*-6-benzylguanine. Mol Cancer Ther. 2006;5:732–8.
3. Quinn JA, Jiang SX, Reardon DA, Desjardins A, Vredenburgh JJ, et al. Phase II trial of temozolomide plus o6-benzylguanine in adults with recurrent, temozolomide-resistant malignant glioma. J Clin Oncol. 2009;27:1262–7.
4. Martin-Montalvo A, Villalba JM, Navas P, de Cabo R. NRF2, cancer and calorie restriction. Oncogene. 2011;30:505–20.
5. Cong ZX, Wang HD, Zhou Y, Wang JW, Pan H, et al. Temozolomide and irradiation combined treatment-induced Nrf2 activation increases chemoradiation sensitivity in human glioblastoma cells. J Neurooncol. 2014;116:41–8.
6. Miltenberger RJ, Cortner J, Farnham PJ. An inhibitory Raf-1 mutant suppresses expression of a subset of v-raf-activated genes. J Biol Chem. 1993;268:15674–80.
7. Wasserman WW, Fahl WE. Functional antioxidant responsive elements. Proc Natl Acad Sci U S A. 1997;94:5361–6.
8. Salazar M, Rojo AI, Velasco D, de Sagarra RM, Cuadrado A. Glycogen synthase kinase-3beta inhibits the xenobiotic and antioxidant cell response by direct phosphorylation and nuclear exclusion of the transcription factor Nrf2. J Biol Chem. 2006;281:14841–51.
9. Johannessen TC, Bjerkvig R. Molecular mechanisms of temozolomide resistance in glioblastoma multiforme. Expert Rev Anticancer Ther. 2012;12:635–42.
10. Liu J, Wu N, Ma LN, Zhong JT, Liu G, et al. p38 MAPK signaling mediates mitochondrial apoptosis in cancer cells induced by oleanolic acid. Asian Pac J Cancer Prev. 2014;15:4519–25.
11. Liu J, Ma L, Wu N, Liu G, Zheng L, et al. Aplysin sensitizes cancer cells to TRAIL by suppressing P38 MAPK/survivin pathway. Mar Drugs. 2014;12:5072–88.
12. Chen Y, Zhao Y, Wang C, Xiao X, Zhou X, et al. Inhibition of p38 MAPK diminishes doxorubicin-induced drug resistance associated with P-glycoprotein in human leukemia K562 cells. Med Sci Monit. 2012;18:BR383–8.
13. Hirose Y, Katayama M, Berger MS, Pieper RO. Cooperative function of Chk1 and p38 pathways in activating G2 arrest following exposure to temozolomide. J Neurosurg. 2004;100:1060–5.