KLF8 Promotes Temozolomide Resistance in Glioma Cells via β-Catenin Activation

Guo Yu a Fei Wu b Ersong Wang a

a Department of Neurosurgery, Jinshan Hospital, Fudan University, Shanghai b National Engineering Laboratory for Anti-tumor Protein Therapeutics, Tsinghua University, Beijing People’s Republic of China

Key Words
KLF8 • Temozolomide • Glioma • β-catenin

Abstract
Background/Aims: The transcription factor Krüppel-like factor (KLF) 8 plays important roles in tumorigenesis and tumor metastasis. However, the relationship between KLF8 and glioma cell chemoresistance is not known. Methods: The effects of KLF8 on glioma cell proliferation, apoptosis and chemosensitivity to temozolomide (TMZ) were analyzed by Cell Counting Kit 8 assay and flow cytometry assay. A xenograft model was used to study the effect of KLF8 on tumor growth and sensitivity to TMZ. Results: We found that in the absence of KLF8, glioma cells showed greater sensitivity to TMZ, resulting in the inhibition of cell growth and enhanced apoptosis. KLF8 overexpression had the opposite effect; that is, cell resistance to TMZ was increased, which was associated with β-catenin activation. Conclusion: Taken together, these data suggest that KLF8 modulates glioma cell resistance to TMZ via activation of β-catenin; therefore, therapies that inhibit KLF8 levels in glioma can enhance the efficacy of TMZ treatment.

Introduction
Glioma is the most common type of primary brain tumor in humans, with an incidence of about five cases per 100,000 adults [1, 2]. Although there have been many advances in glioma treatment by surgery, chemotherapy, and radiotherapy, patient prognosis remains poor, with a median survival of 12–15 months [3, 4]. Temozolomide (TMZ), a DNA-alkylating antineoplastic drug, is the most commonly used chemotherapeutic agent in the treatment of glioma [5, 6]. However, its efficacy is limited by the development of chemotherapeutic resistance in tumors [7, 8], the mechanism of which is not well understood. Krüppel-like factor (KLF) 8 is a member of the KLF family of transcription factors that contain three C2-H2 zinc fingers in the C-terminal DNA-binding domain, a nuclear
localization signal, and a distinct N-terminal domain [9, 10]. KLF8 is frequently upregulated in several types of cancer, including hepatocellular carcinoma (HCC), renal carcinoma, and breast, ovarian, and gastric cancers, and plays an important role in a wide range of biological processes, including oncogenic transformation, epithelial-to-mesenchymal transition, and DNA damage response as well as cell growth, invasion, and differentiation [11-15]. Recent studies have found that KLF8 is expressed in different grades of glioma; KLF8 knockdown inhibited glioma cell viability and induced G0/G1 arrest and apoptosis [16, 17]. However, the relationship between KLF8 overexpression and glioma chemoresistance is unclear.

This was investigated in the present study by knocking down or overexpressing KLF8 in glioma cells that were then treated with TMZ. We found that in the absence of KLF8, the chemosensitivity of glioma cells to TMZ was increased, whereas KLF8 overexpression promoted their resistance to the toxic effects of the drug, which was associated with β-catenin activation. These findings suggest that suppressing KLF8 expression levels in glioma may be an effective strategy for preventing the development of resistance to TMZ.

Materials and Methods

Cell culture

Human glioma cell lines U87 and U251 were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA) containing 2 mM glutamine, 10% fetal calf serum (FBS), 100 U/ml penicillin (Sigma, St. Louis, MO, USA), and 100 μg/ml streptomycin (Sigma). Cells were maintained in an incubator at 37°C in a 5% CO₂ atmosphere.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from glioma tissues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and reverse transcribed to cDNA using the Prime Script RT reagent kit (Takara Bio, Dalian, China). qRT-PCR was carried out using SYBR green Premix Ex Taq II (Takara Bio) on a Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA), with β-actin used as the internal control. The following forward and reverse primer sequences were used: KLF8, 5'-TCT GCA GGG ACT ACA GCA AG-3' and 5'-TCA CAT TGG TGA ATC CGT CT-3'; and β-actin, 5'-GGA AAT CGT GCG TGA CAT-3' and 5'-AAG GAA GGC TGG AAG AGT G-3'. Relative expression levels were determined with the 2^−ΔΔCT method. All of the reactions were run in triplicate.

Western blotting

Western blotting was performed according to a standard method, as described previously [18]. Cells were lysed in radiomuno precipitation assay lysis buffer containing protease inhibitor (Beyotime, Nantong, China). Total protein and nucleoprotein were collected separately as previously described [19]. Protein concentration was determined with a bicinchoninic acid assay kit (Beyotime). Equal amounts of proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane, which was blocked in bovine serum albumin solution for 30 min at room temperature, and then incubated overnight at 4°C with antibodies against KLF8 (1:1000, AB76307; Abcam, Cambridge, UK), β-catenin (1:1000, SC-365062; Santa Cruz Biotechnology, Santa Cruz, CA, USA), glyceraldehyde 3-phosphate dehydrogenase (1:1000, SC-365062; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and histone H3 (1:1000, AB76307; Abcam, Cambridge, UK). After secondary antibody treatment, bands were visualized using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

Lentivirus production and infection

Short hairpin (sh) RNA against KLF8, β-catenin and negative control shRNA were cloned into the pL3.7 lentiviral vector. The KLF8 coding sequence was amplified and cloned into the pWPI-eGFP lentiviral vector to generate the KLF8 overexpression vector, with the empty vector serving as a negative control. The vectors were co-transfected with packaging plasmids into HEK-293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions; viral particles were harvested 48 h later. U87 and U251 cells were infected with lentivirus with 6 μg/ml polybrene (Sigma). The shRNA sequence targeting
KLF8 was 5'-CAG AAG AAC TTT TGG CTA G-3', and the control shRNA sequence was 5'-CAG TCG CGT TTG CGA CTG G-3'.

Cell viability assay

Cell viability was determined with the Cell Counting Kit (CCK)-8 assay. Cells were seeded in 96-well plates at a density of 3 \times 10^4/well. After overnight incubation, the cells were infected with lentivirus, then treated with TMZ (100 µM) for 48 h. After a 2-h incubation with 10 µl CCK-8 solution (Dojindo, Kumamato, Japan), cell viability was detected at 490 nm using a microplate reader (BioTek, Winooski, VT, USA). The survival of untreated cells was set at 100% and was used to calculate the half-maximal inhibitory concentration (IC_{50}) [20].

Flow cytometry assay

Apoptotic cells were detected using an Annexin V/Propidium Iodide (PI) Apoptosis Detection kit (BD Pharmingen, San Jose, CA, USA) as previously described [21]. Infected cells were treated with TMZ (100 µM) for 24 h, then collected, stained with annexin V and PI, and analyzed by flow cytometry on a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA).

Caspase 3/7 activity assay

Caspase-3/7 activity was measured with the Apo-ONE Homogeneous Caspase 3/7 Assay kit (Promega, Madison, WI, USA). Cells were seeded in a 96-well plate and cultured overnight. Caspase 3/7 substrate was then added to each well and cells were incubated for 2 h. Caspase activity was determined by measuring absorbance on a microplate reader at excitation/emission wavelengths of 485/520 nm.

Animal models

Animal experiments were approved by The Animal Care and Use Committee of Fudan University (Shanghai, China). U87 cells (5 \times 10^6 in 50 µl media) infected with lentivirus were subcutaneously injected into 5-week-old nude mice. When tumors reached an average size of 150 mm^3, TMZ was administered daily by intraperitoneal injection at a dose of 50 mg/kg. Tumor size was measured with a caliper every 2 days. Mice were sacrificed and xenografted tumors were excised 21 days after inoculation. Tumor volume (V) was calculated with the formula: V = 0.5 \times A \times B^2, where A and B represent the largest and perpendicular diameters, respectively.

Statistical analysis

Data are expressed as mean ± SD and were analyzed using SPSS v.13.0 software (SPSS Inc., Chicago, IL, USA). Differences between groups were evaluated with the Student’s t test; P < 0.05 was considered as statistically significant.

Results

KLF8 knockdown increases glioma sensitivity to TMZ

To investigate the potential role of KLF8 in the regulation of glioma chemoresistance, KLF8 expression was knocked down in U87 and U251 cells by infection with KLF8 shRNA or negative control lentiviral constructs. KLF8 knockdown was confirmed by qRT-PCR and western blotting (Fig. 1A, B). The sensitivity of KLF8-deficient U87 and U251 cells to various concentrations of TMZ was evaluated following drug treatment for 48 h. The IC_{50} value of TMZ was decreased by about 2 folds in KLF8 knockdown cells (Fig. 1C), and sensitivity to TMZ was significantly increased in KLF8 knockdown as compared to control cells (Fig. 1D).

We also examined the effect of KLF8 knockdown on chemotherapy-induced apoptosis of glioma cells by flow cytometry. Loss of KLF8 increased apoptosis relative to control U87 and U251 cells treated with the same concentrations of TMZ (Fig. 1E). Consistent with this finding, the activity of effector caspases (i.e., caspase 3/7) was increased in TMZ-treated KLF8 knockdown cells as compared to control cells (Fig. 1F). These data indicate that KLF8 knockdown enhances the chemosensitivity of glioma cells to TMZ.
KLF8 overexpression enhances glioma cell resistance to chemotherapy

To further investigate whether KLF8 modulates glioma cell responses to TMZ, U87 and U251 cells were infected with lentivirus encoding KLF8 or negative control shRNA. The overexpression of KLF8 was confirmed by qRT-PCR and western blotting (Fig. 2A, B). The IC₅₀ of TMZ was higher in KLF8-overexpressing than in control cells (Fig. 2C). Moreover, the rate of apoptosis induced by TMZ was significantly reduced by KLF8 overexpression, as revealed by flow cytometry analysis (Fig. 2D, E) and a decrease in caspase-3/7 activity assay (Fig. 2F). These data indicate that high levels of KLF8 protect glioma cells from the toxicity of TMZ.

KLF8 modulates glioma chemosensitivity to TMZ in vivo

To confirm the above findings in vivo, mice were inoculated with U87 cells infected with lentivirus encoding KLF8 or negative control shRNA. Mice were treated with TMZ when the average size of subcutaneous tumors reached 150 mm³. Tumor volumes were lower in mice with tumors formed from KLF8-deficient as compared to control cells; this effect was more pronounced in KLF8-deficient tumors following TMZ treatment (Fig. 3A). Tumor weight was measured 21 days after inoculation; the results showed that KLF8 deficiency significantly reduced tumor weight relative to control tumors after treatment with TMZ (Fig. 3B). Conversely, KLF8 overexpression stimulated tumor growth, with tumor weight increased by
**Fig. 2.** KLF8 overexpression enhances glioma cell resistance to TMZ. (A, B) Relative expression levels of KLF8 mRNA (A) and protein (B) in U87 and U251 cells infected with lentivirus encoding KLF8 (KLF8) or an empty lentiviral vector (Ctrl) were determined by qRT-PCR and western blotting, respectively. (C, D) Cells were treated with indicated concentrations of TMZ and IC_{50} (C) and cell viability (D) were determined with the CCK-8 assay. (E, F) Apoptosis in KLF8-overexpressing or control cells treated with TMZ (100 μM) was assessed by flow cytometry (E) and the caspase 3/7 activity assay (F). * indicates a significant difference from the negative control (*P < 0.05, **P < 0.01).

**Fig. 3.** KLF8 modulates glioma sensitivity to TMZ in vivo. (A) Growth curves of tumors in mice injected with U87 cells infected with sh-KLF8 or NC, or KLF8 or Ctrl, followed by treatment with TMZ. (B, D) Images of xenograft tumors and tumor weight. ** indicates a significant difference from the negative control (**P < 0.05, ***P < 0.01).
TMZ treatment (Fig. 3C, D). These data confirm that KLF8 modulates the chemosensitivity of glioma to TMZ in vivo.

KLF8 activates β-catenin

KLF8 has been shown to activate canonical Wnt/β-catenin signaling in HCC [22]. We therefore investigated the effects of KLF8 on β-catenin levels in glioma cells treated with TMZ. KLF8 knockdown combined with TMZ treatment inhibited total and nuclear β-catenin levels relative to cells treated with TMZ only (Fig. 4A); conversely, β-catenin levels were significantly increased in TMZ-treated cells overexpressing KLF8 as compared to the control vector (Fig. 4B). Moreover, silencing of β-catenin reversed KLF8-induced protection against chemotherapy accompanied by decreased cell viability and increased apoptosis (Fig. 4C and D). These data suggest that the modulation of glioma sensitivity to TMZ by KLF8 involves the regulation of Wnt/β-catenin signaling.

Discussion

KLF8 is a transcription factor that plays a critical role in tumor development [23, 24]. It was previously reported that KLF8 upregulation was correlated with tumor cell growth and...
metastasis [11, 14, 17]; for instance, increased levels of KLF8 in HCC were associated with enhanced cell proliferation and metastasis in vitro and in vivo [11]. In glioblastoma cells, KLF8 knockdown suppressed cell proliferation and cell cycle progression and increased apoptosis [17]. KLF8 has also been implicated in the development of chemoresistance in cancer cells [25-27]; for instance, it was shown to inhibit doxorubicin-induced DNA damage and cell death in breast cancer cells [25] while promoting the hypoxia-induced multidrug resistance phenotype, which was abolished by KLF8 inhibition [26]. KLF8 knockdown was also found to suppress cell growth, promote apoptosis, and sensitize cells to 5-fluorouracil in colorectal cancer [27]. Consistent with these findings, the present study demonstrated that loss of KLF8 decreased cell viability and increased apoptosis following TMZ treatment, while the opposite effect was observed upon KLF8 overexpression. The modulation of glioma sensitivity to TMZ by KLF8 was confirmed in a xenograft mouse model.

KLF8 is a target of Wnt/β-catenin signaling that stabilizes β-catenin by binding to the β-catenin/T cell factor 4 complex, which leads to Wnt-induced transcriptional activation in HCC cells [22]. The Wnt/β-catenin signaling regulates cell viability, growth, apoptosis, and invasion in various types of cancer, including glioma [28-30], and inhibiting this pathway decreased viability and increased apoptosis of adrenocortical tumor cells [28]. Pharmacological or genetic inhibition of Wnt activity augmented the effects of alkylating drugs and restored chemosensitivity in different cancers. For instance, Wnt inhibitors augmented the cytotoxic effects of TMZ in colon carcinoma, medulloblastoma, neuroblastoma and glioma cell lines. Wnt/β-catenin pathway regulated MGMT gene expression in cancer and targeting β-catenin restored TMZ chemosensitivity in cancer cells expressing MGMT [31]. In addition, inhibition of Wnt/β-catenin signaling reduced glioma cell proliferation and migration both in vitro and in vivo [30] and enhanced TMZ-induced apoptosis of glioblastoma cells [32] while sensitizing germline stem cells to TMZ-induced proliferation and invasiveness [33]. Based on these observations, we propose that the resistance of glioma to TMZ treatment caused by KLF8 upregulation involves activation of Wnt/β-catenin signaling. Our results showed that KLF8 knockdown combined with TMZ treatment reduced total and nuclear β-catenin levels relative to cells treated with TMZ only. Conversely, KLF8 overexpression combined with TMZ treatment enhanced total and nuclear β-catenin levels. Importantly, silencing of β-catenin reversed KLF8-induced protection against chemotherapy.

There are likely multiple mechanisms that ensure constant activation of β-catenin. A recent study has demonstrated that E-cadherin binding can prevent β-catenin nuclear localization. While overexpression of KLF8 represses the membrane expression of E-cadherin and subsequently promotes the nuclear translocation of β-catenin [14]. However, the exact mechanism underlying the role of KLF8 in chemoresistance of glioma cells still needs further investigation.

In conclusion, we showed that KLF8 reduces TMZ-induced growth inhibition and apoptosis by activating β-catenin. Thus, we believe that targeting KLF8 is a promising strategy to defeat TMZ resistance for glioma therapy.

Disclosure Statement

No potential conflicts of interest were disclosed.

References


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