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Depletion of Kruppel-like factor 15 sensitized gliomas to temozolomide cytotoxicity through O^6-methylguanine-DNA methyl-transferase

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ABSTRACT

Temozolomide (TMZ)-based chemotherapy is a standard strategy for gliomas, although chemoresistance remains a major therapeutic challenge. The chemical mechanism by which TMZ induces cell death is DNA methylation, leading to double-stranded breaks (DSBs) and thus to apoptosis. However, TMZ-induced N6-meG sites are efficiently repaired and mediated by the DNA repair protein O^6-methylguanine-DNA methyl-transferase (MGMT), leading to TMZ resistance. KLF15, a member of the Kruppel-like factors family, mainly functions as transcription factor and potential suppressor gene by inhibiting proliferation, migration, and inducing apoptosis. However, the roles and regulatory mechanisms of KLF15 in glioma tumorigenesis and chemoresistance are poorly understood. In this study, KLF15 expression was upregulated in glioma tissues and cell lines upon TMZ treatment. Knockdown of KLF15 amplified TMZ-induced repression of cell proliferation, while KLF15 overexpression reversed this process. Mechanistically, KLF15 functioned as a transcriptional activator of MGMT. Moreover, KLF15 knockdown sensitized tumors to TMZ treatment in vivo. Taken together, these results suggested that KLF15 upregulated MGMT through direct binding to the promoter of MGMT, which plays an important role in glioma resistance to TMZ, and which may be a potential target for cancer diagnosis and treatment.

1. Introduction

Glioma is a highly malignant phenotype characterized by rapid progression, early metastasis, and a limited response to radiotherapy and chemotherapy [1,2]. Temozolomide (TMZ) is one of the most widely used chemotherapy drugs for glioma treatment [3]. However, intrinsic and/or acquired resistance to TMZ-based chemotherapy has increasingly become a limiting factor in the treatment of many patients [4]. Therefore, a detailed investigation into the in-depth molecular mechanisms of drug resistance is essential and may translate into effective therapies that can alter drug resistance to susceptibility.

TMZ treatment always induces DNA methylation, followed by double-strand breaks (DSBs) and thus leading to cell death [5,6]. Most of TMZ-induced methylation sites are N7-meG (>70%) and N3-meA (9.2%), which can be repaired by the alkylpurine-DNA-N-glycosylase enzyme [7]. However, although O6-meG accounts for less than 10% of DNA adducts formed by TMZ, O6-meG lesions are highly cytotoxic to the cell and are repaired via the methylguanine-DNA-methyltransferase (MGMT) pathway, mediated by the O^6-methylguanine-DNA methyl-transferase (MGMT) enzyme [8]. MGMT activity in tumors is crucial to cytotoxic effects of TMZ [9]. Tumor cells lacking MGMT activity are more sensitive to the cytotoxic effects of TMZ [10]. MGMT expression is usually suppressed in tumors by CpG methylation within the *MGMT* promoter, conferring TMZ resistance in patients with glioblastoma multiforme (GBM) [11,12]. Therefore, MGMT expression has been used as a biomarker for glioma response to TMZ treatment.

The Kruppel-like factors (KLFs), an important family of transcription factors, can bind various GC-rich DNA elements and regulate transcription as activators and suppressors [13]. KLFs mainly function as tumor suppressors by inhibiting cell growth, cell migration and

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invasion. Conversely, several KLFs function as oncogenes to promote tumor progression [14–16]. KLF1 promotes metastasis and invasion via the PI3K/Akt signaling pathway in cervical cancer cells [17]. KLF2 mediates colorectal cancer cell biological processes including cell growth and apoptosis via regulating the HIF-1α/Notch-1 signal pathway [18]. In tumors, KLF4 and KLF5 play key roles in tumor cell fate, regulating cell proliferation, cell survival, and the tumor-initiating properties of cancer stem–like cells [19]. KLF8 is required for v-Src-induced transformation and may play a role in tumor progression of human cancer [20]. Disruption of the KLF14 in mice causes centrosome amplification, aneuploidy and spontaneous tumorigenesis [21]. Recently, KLF15 was reported to exert anti-proliferation effects in pancreatic carcinomas, endometrium carcinomas and breast carcinomas [16,22,23]. But so far, the expression and function of KLF15 in gliomas have not been reported. In the present study, we therefore characterized the expression and function of KLF15 in glioma tissues and cell lines as well as the chemoresistance of gliomas.

In our study, we found that KLF15 was upregulated in glioma tissue and glioma cells after TMZ treatment. Furthermore, knockdown of KLF15 caused cells to be sensitive to TMZ treatment. Mechanistically, KLF15 promoted MGMT transcription and expression. Knockdown of KLF15 showed drug sensitivity through MGMT. Our results demonstrate the role of KLF15 in drug resistance in glioma cancer through MGMT induction, suggesting that KLF15 is a potential therapeutic target for glioma tumor therapy.

2. Methods

2.1. Cell culture and materials

U87G cells were obtained from the American Type Culture Collection. Cells were maintained in DMEM with 10% (v/v) fetal bovine serum. KLF15-Si (ShRNA) was constructed using the following primers: F′ CCCAATGCCGCCAAACCTAT and R’ GAGGTGGCTGCTCTTGGTGTACATC. Flag-KLF15 expression plasmid was constructed by inserting the coding regions into pCDNA3.0 vector. TMZ were purchased from Sigma-Aldrich. Antibodies were purchased from Sigma-Aldrich (KLF15, MGMT and actin).

2.2. Western blotting

Cells were lysed in lysis buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF), centrifuged for 10min at 12,000 g, and the insoluble debris was discarded. Cell lysates were further analyzed with SDS-PAGE and western blotting using specific antibodies.

2.3. Tissue microarray and IHC staining

Human tissue microarrays of glioma cancer (Shanghai Superbiotek Pharmaceutical Technology Co., Ltd.) were purchased. The clinical characteristics of all samples were downloaded from the Web sites of companies. Antibody against KLF15 was used for immunohistochemistry staining. The intensity of KLF15 staining was quantified, scored and graded (low, 0–4 points; medium, 5–8 points; and high, 9–12 points). To ensure an unbiased result, data were collected in a double-blinded manner.

2.4. Cell death analyses

Cell survival was determined by the MTT assay.

2.5. Luciferase reporter assay

To generate MGMT promoter-activated luciferase reporter, −200 to 1000 bp of MGMT promoter was subcloned into PGL4.17. The luciferase reporter assay was then performed. Briefly, cells in 24-well plates were co-transfected with indicated plasmids and the MGMT-promoter luciferase reporter plasmid for 24 h. Luciferase was measured using the Dual-Luciferase assay kit (Promega). The pRL-TK was co-transfected to normalize transfection efficiency.

2.6. KLF15 expression analysis

KLF15 levels were measured by qRT-PCR analysis and normalized against the expression levels of actin (internal control).

2.7. Tumor growth analysis in mice

Cells were subcutaneously injected into both flanks of male BALB/c nude mice (~5 weeks of age). At 14 days after injection, mice were treated with PBS or TMZ (10 mg/kg) every day by intraperitoneal injection. At day 30, animals were treated according to high ethical and scientific standards with oversight by the animal center at Linyi Normal University.

2.8. Statistical analyses

The results are expressed as the mean ± standard deviation, as indicated in the figure legends. Statistical significance was assessed by two-tailed Student t-tests. Values of $P < 0.05$ were considered significant.

2.9. Ethics statement

The animal study was reviewed and approved by the Institutional Ethics Committee for Clinical Research and Animal Trials of the Linyi Normal University, and written informed consent was obtained from all participants or their appropriate surrogates.

3. Results

3.1. KLF15 was upregulated in glioma tissues and glioma cells upon TMZ treatment

To investigate whether KLF15 was involved in drug resistance in gliomas, we performed immunohistochemistry staining of KLF15 protein on tissue chips of human glioma cancers with or without TMZ treatment. The results showed that KLF15 was dramatically upregulated in glioma tissues after TMZ treatment (Fig. 1A and B). Furthermore, KLF15 expression was detected in U87 cells treated with TMZ for the indicated times, and KLF15 mRNA levels were upregulated followed by TMZ treatment (Fig. 1C). Consistently, KLF15 protein levels were also increased upon TMZ treatment (Fig. 1D). Together, these results showed that KLF15 was upregulated after TMZ treatment.

3.2. KLF15 knockdown sensitized cells to TMZ treatment

To determine the role of KLF15 protein in drug resistance, a KLF15 overexpression stable cell line (KLF15-OE) and a KLF15 knockdown stable cell line (KLF15-Si) were established (Fig. 2A and B). KLF15-Si cells were more susceptible to death in response to TMZ treatment (Fig. 2C). In contrast, cell death was dramatically reduced in KLF15-OE cells upon TMZ treatment (Fig. 2D). Colony formation was decreased dramatically in KLF15-Si cells upon TMZ treatment (Fig. 2E), but colony formation was significantly increased in KLF15-OE cells upon TMZ treatment (Fig. 2F). These results showed that KLF15-Si cells were sensitized to TMZ treatment, whereas, KLF15-OE cells showed resistance to TMZ treatment.

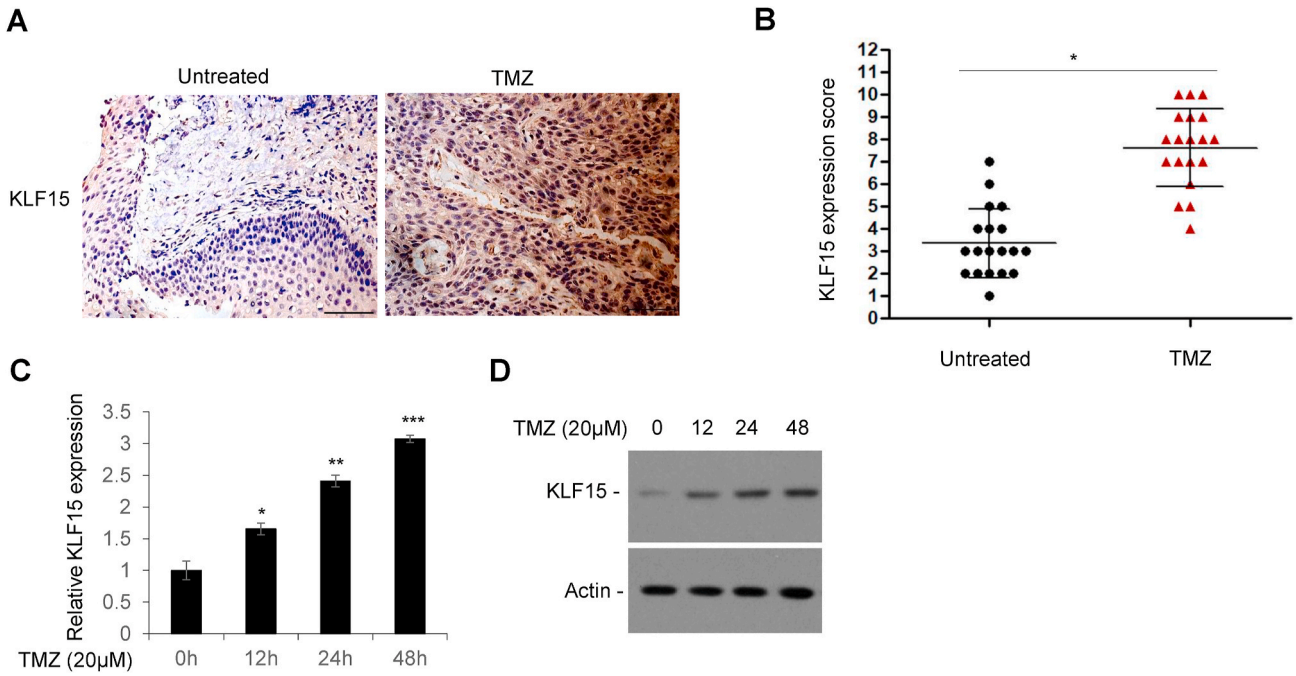

Fig. 1. KLF15 was upregulated in glioma tissues and glioma cell lines upon TMZ treatment. (A–B) Examples of immunohistochemical images of tumor tissues in treated gliomas with or without TMZ treatment, followed by staining with anti-KLF15 antibodies (scale bar, 100 μm). The levels of KLF15 expressions were classified as different grades (1–12) according to the staining signals in each group. (C) U87G cells were treated with TMZ (20 μM) for the indicated times and KLF15 mRNA levels were detected using a qPCR assay. Data represent the mean ± s.d., $^{*}P < 0.05$, $^{**}P < 0.01$, Student's t-test. (D) U87G cells were treated with TMZ (20 μM) for the indicated times and KLF15 protein levels were detected using western blotting.

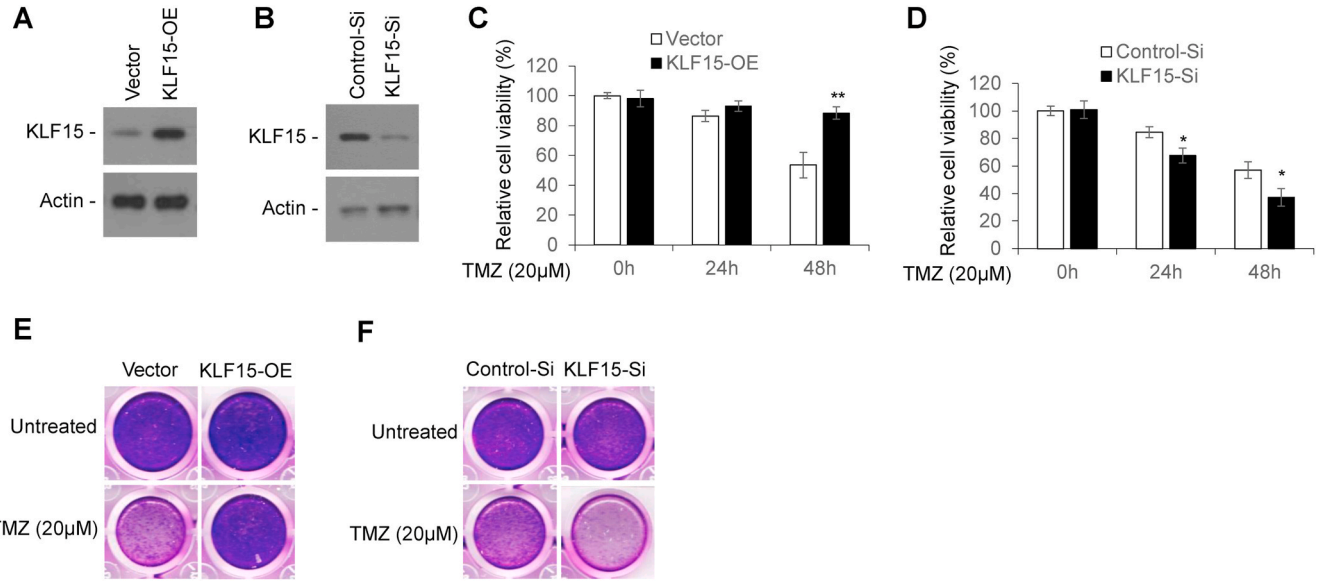

Fig. 2. KLF15 knockdown sensitized cells to TMZ treatment. (A) U87G cells with stable overexpression of KLF15 (KLF15-OE) or a vector control (Vector) were established. Western blots show KLF15 expression. (B) U87 cells with stable knockdown of KLF15 (KLF15-Si) or a vector control (Control-Si) were established. Western blots show KLF15 expression. (C) Vector and KLF15-OE U87 cells were treated with TMZ (20 μM) for 24 h and 48 h respectively, and cell death were analyzed by MTT. (D) Control-Si and KLF15-Si U87 cells were treated with TMZ (20 μM) for 24 h and 48 h, respectively, and cell death were analyzed using an MTT assay. (E) A clonogenic survival assay was conducted in Vector and KLF15-OE U87G cells treated with TMZ. (F) A clonogenic survival assay was conducted in Control-Si and KLF15-Si U87 cells treated with TMZ. Data represent the mean ± s.d., $^{*}P < 0.05$, $^{**}P < 0.01$, Student's t-test.

3.3. KLF15 upregulated MGMT

Given that KLF15 is a transcription factor and the resistance to TMZ is mainly contributed by the base excision repair (BER) pathway mediated by MGMT, we then determined whether KLF15 affected MGMT expression. Notably, KLF15 overexpression showed a strong enhancement effect on MGMT-promoter luciferase activity (Fig. 3A). Furthermore, MGMT mRNA level and protein level were dramatically upregulated in KLF15 transient expression cells (Fig. 3B and C). Similarly, MGMT mRNA level and protein level also increased in KLF15-OE stable cells (Fig. 3D and E). Conversely, MGMT mRNA and protein levels were decreased in KLF15-knockdown stable cells but not in the KLF15-

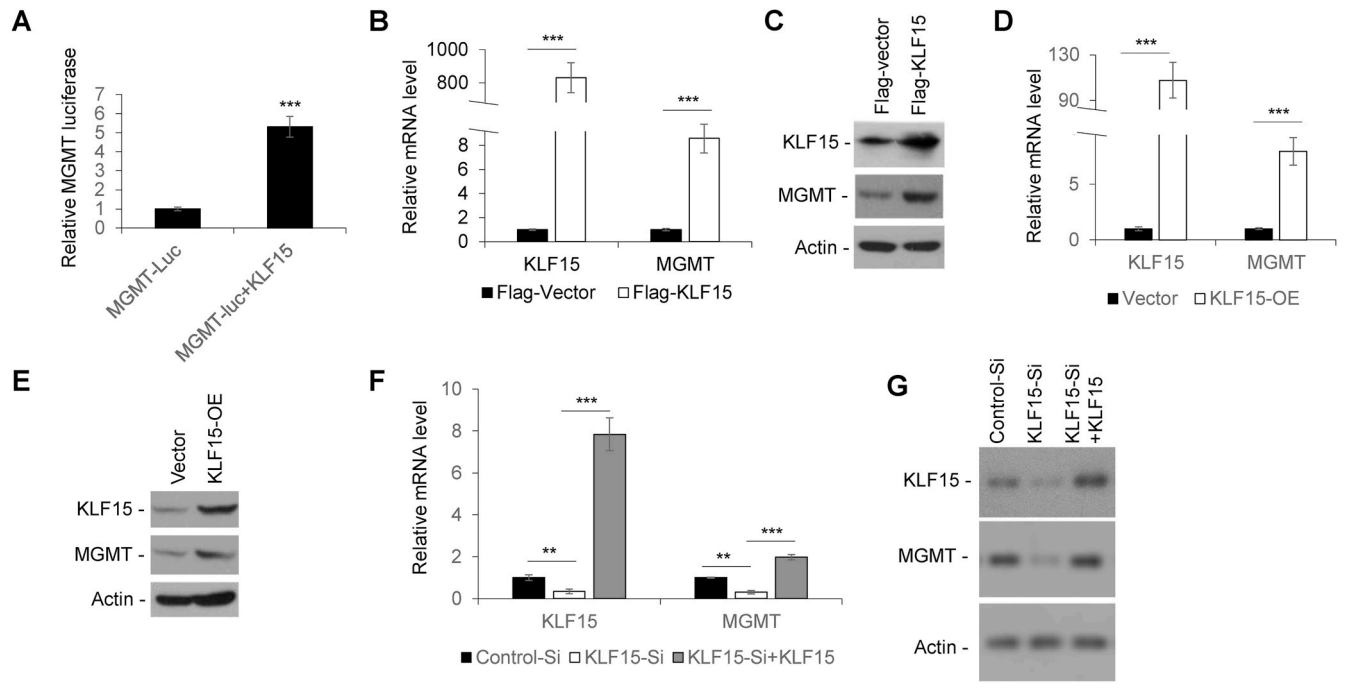

Fig. 3. KLF15 upregulated MGMT. (A) U87G cells were transfected with the MGMT-promoter luciferase reporter plasmid with or without the KLF15 plasmid for 24 h, followed by the determination of the relative luciferase activity levels. (B) Relative KLF15 and MGMT mRNA expression levels in U87G cells transfected with KLF15 plasmid were analyzed using qRT-PCR. (C) Western blots show KLF15 and MGMT levels in U87 cells transfected with KLF15 plasmid using anti-KLF15 and anti-MGMT antibodies. (D) Relative KLF15 and MGMT mRNA expression levels in KLF15-OE U87 cells were analyzed by qRT-PCR. (E) Western blots show KLF15 and MGMT levels in Vector and KLF15-OE U87 cells using anti-KLF15 and anti-MGMT antibodies. (F) Relative KLF15 and MGMT mRNA expression levels in KLF15-Si and KLF15-resued U87 cells were analyzed by qRT-PCR. (G) Western blots show KLF15 and MGMT levels in KLF15-Si and rescued of KLF15 U87 cells using anti-KLF15 and anti-MGMT antibodies. Data represent the mean ± s.d., $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P \leq 0.001$, Student's *t*-test.

rescued cells (Fig. 3F and G). Together, these results indicated that KLF15 functioned as a transcriptional activator of MGMT.

3.4. KLF15 depletion displayed drug sensitivity via MGMT

To confirm that KLF15 regulated drug resistance via MGMT, we first detected MGMT levels after TMZ treatment. The results showed that MGMT levels increased following TMZ treatment (Fig. 4A). While MGMT levels remained unchanged under TMZ treatment in KLF15-knockdown stable cell lines (Fig. 4B). In addition, we established KLF15-knockdown and rescued of MGMT stable cell lines and found that MGMT-rescue reversed cell death induced by TMZ treatment in KLF15 knockdown cells (Fig. 4C). We also determined whether KLF15 knockdown enhanced the therapeutic response of xenograft tumors in mice upon TMZ treatment. KLF15-normal and -knockdown U87G cells were subcutaneously injected into nude mice above the left and right legs, resulting in significant inhibition of tumor growth in the KLF15-knockdown groups receiving TMZ but not PBS treatment (Fig. 4D). These results suggested that knockdown of KLF15 sensitized glioma tumors to TMZ treatment via the downregulation of MGMT.

4. Discussion

These findings revealed a previously unknown function of KLF15 in the regulation of chemoresistance in gliomas. TMZ treatment resulted in an increase of KLF15. Moreover, KLF15 functioned as a transcriptional activator, to promote MGMT expression. Depletion of KLF15 resulted in an increase in cell death upon TMZ treatment via MGMT. Furthermore, KLF15 deficiency significantly improved the anti-tumor activity of TMZ in vivo. These findings provided the first evidence that KLF15 is directly involved in the regulation of glioma chemoresistance, suggesting that KLF15 has the potential to enhance the tumor-killing efficacy of TMZ.

This study showed that KLF15 expression is important in the control of tumor chemotherapy. Indeed, KLF15 expression always changed under different stress circumstances. The mRNA and protein levels of KLF15 were significantly increased in the neurons of mice undergoing neuropathic pain [24]. Exposure of cultured hepatocytes to metformin reduced the abundance of KLF15 through acceleration of its degradation and downregulation of its mRNA [25]. High expression of KLF15 predicated worse prognosis in lung adenocarcinoma [26]. These results implied that control of mRNA and protein levels of KLF15 is crucial to disease prevention. However, the mechanism of KLF15 upregulation is unknown. It is therefore important to identify the upstream regulators of KLF15 under various stresses.

DNA damage repair is one of the leading causes for chemoresistance in tumors. Targeting the DNA damage response (DDR) is proving to be a beneficial strategy to sensitize tumor cells to treatment. Most of the methylation sites induced by TMZ are N7-meG and N3-meA. Less frequently TMZ creates O6-meG adducts [6]. O6-meG is more lethal for the cell and is repaired via the methylguanine-DNA-methyltransferase (MGMT) pathway, mediated by the MGMT enzyme. MGMT expression has been used as a biomarker for glioma response to treatment with TMZ. In our study, we found that MGMT enzyme expression was regulated by KLF15, whereas, other DNA repair enzymes involved in tumor chemoresistance need to be further identified.

In summary, we identified the role of KLF15 in glioma tumor drug resistance. KLF15 acted as a transcriptional activator to regulate MGMT expression. In addition, knockdown of KLF15 significantly increased TMZ-induced tumor growth inhibition and cancer cell death in vivo and in vitro. Based on these results, we propose that KLF15 is a potential target to confer TMZ resistance in gliomas.

Author contributions

X.Q. and X.L. performed most of the experiments. X. W and Z. S interpreted data and wrote the manuscript. Y. Z. supervised the study

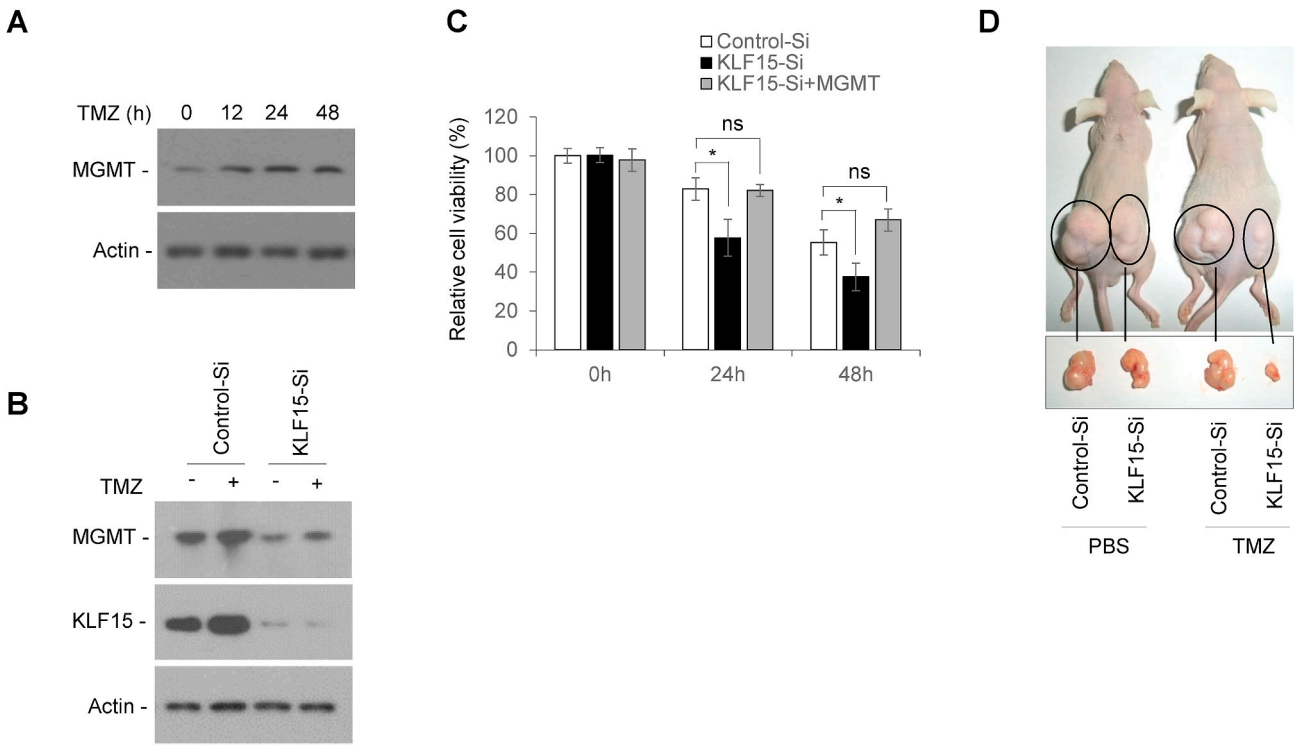

Fig. 4. KLF15 depletion displayed drug sensitivity via MGMT. (A) U87G cells were treated with TMZ (20 μM) for the indicated times and MGMT protein levels were detected using western blotting. (B) Stable Control-Si and KLF15-Si U87 cell lines were treated with TMZ (20 μM) for 24h and MGMT protein levels were detected using western blotting. (C) Stable Control-Si and KLF15-Si U87 cell lines were infected with lentivirus expression of MGMT and cultured for 48 h, and then treated with TMZ for the indicated times. Cell death was measured using MTT assay. (D) Mice with xenograft tumors originating from stable U87G cells with Control-Si or KLF15-Si were treated with TMZ (10 mg/kg) by intraperitoneal injections. Images show tumors after 30 days of treatment of PBS or TMZ. Data represent the mean ± s.d., $*P < 0.05$, $**P < 0.01$, Student's *t*-test.

and reviewed the manuscript.

Declaration of competing interest

None of the authors have competing financial interests related to this work.

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