Specificity protein 1-modulated superoxide dismutase 2 enhances temozolomide resistance in glioblastoma, which is independent of O6-methylguanine-DNA methyltransferase

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ABSTRACT

Acquisition of temozolomide (TMZ) resistance is a major factor leading to the failure of glioblastoma (GBM) treatment. The exact mechanism by which GBM evades TMZ toxicity is not always related to the expression of the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT), and so remains unclear. In this study, TMZ-resistant variants derived from MGMT-negative GBM clinical samples and cell lines were studied, revealing there to be increased specificity protein 1 (Sp1) expression associated with reduced reactive oxygen species (ROS) accumulation following TMZ treatment. Analysis of gene expression databases along with cell studies identified the ROS scavenger superoxide dismutase 2 (SOD2) as being disease-related. SOD2 expression was also increased, and it was found to be co-expressed with Sp1 in TMZ-resistant cells. Investigation of the SOD2 promoter revealed Sp1 as a critical transcriptional activator that enhances SOD2 gene expression. Co-treatment with an Sp1 inhibitor restored the inhibitory effects of TMZ, and decreased SOD2 levels in TMZ-resistant cells. This treatment strategy restored susceptibility to TMZ in xenograft animals, leading to prolonged survival in an orthotopic model. Thus, our results suggest that Sp1 modulates ROS scavengers as a novel mechanism to increase cancer malignancy and resistance to chemotherapy. Inhibition of this pathway may represent a potential therapeutic target for restoring treatment susceptibility in GBM.

1. Introduction

Glioblastoma (GBM) usually has a poor prognosis, and is never considered curable even following treatment with the most widely used first-line chemotherapeutic agent temozolomide (TMZ). In one phase III clinical trial, approximately 90% of the patients suffered from disease recurrence within two years of treatment, regardless of the initial response [1]. TMZ is known to cause lethal DNA damage (O6-methyl
dation-mediated DNA base mismatching) and subsequent reactive oxygen species (ROS) production [2]. Resistance to TMZ treatment is well known to occur because of the presence of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) [3]. It is important to note that regardless of MGMT status, the acquisition of TMZ resistance inevitably occurs, suggesting that non-MGMT resistance
mechanisms are operative in GBM. Compared with innate resistance, the development of acquired resistance is considered more complex, with multiple factors being involved, such as a mitochondrial adaptive response to TMZ genotoxic stress [4], and the stress-activated kinase p38, against TMZ-induced cell death [5]. It is therefore of interest to elucidate how cellular stress responses serve to protect GBMs from the effects of TMZ.

Specificity protein 1 (Sp1) is a nuclear transcription factor that is ubiquitously expressed in mammalian cells, and regulates multiple genes and cellular functions. It has been demonstrated previously that Sp1 is upregulated in most cancer cell types, such as breast, gastric, cervical, and GBM [6–8]. We have previously shown that Sp1 is a stress-sensitive transcription factor that increases the expression of genes that can protect against stress-induced cellular damage [9,10]. Chemotherapy is commonly performed after surgery in order to target cancer cells, but whether TMZ-induced environmental stress affects Sp1 levels and its functions, remains unclear.

Strict regulation of ROS levels is essential for cells to maintain viability and to avoid oxidative damage from stress overload [11]. Multiple antioxidant enzymes including superoxide dismutases (SODs), catalase, and glutathione peroxidases (GPx) are important, and have individual roles in the steps required to convert superoxide into water and oxygen [12]. Under certain circumstances, tumors have been shown to upregulate antioxidant enzyme expression to promote survival and resistance to certain anticancer agents [12]. For example, SOD2-overexpressing human ovarian and prostate cancer cells are prone to survive radiation toxicity, demonstrating increased resistance to the radiation treatment compared with control cells [13,14]. Notably, TMZ is known to induce ROS production in glioma cells, thus leading to the activation of cell death signaling pathways [2]. The question remains, however, whether antioxidant enzymes promote the development of TMZ resistance in GBM, and especially in MGMT-deficient cells.

In order to determine the major factors involved in acquired TMZ drug resistance, we hypothesized that exposure of GBM to TMZ would activate the antioxidant defensive system in malignant cell populations that are prone to survive TMZ-induced cytotoxicity. By using MGMT-negative cell lines, we identified Sp1 as a key factor. Sp1 expression was induced in TMZ-resistant cells. Further exploration of this process identified SOD2 as a downstream and critical target of Sp1 action. Inhibition of Sp1 restored the TMZ effect in both TMZ-resistant cells and xenografts.

Fig. 1. MGMT does not contribute to TMZ resistance in MGMT-negative GBM cells. (A) MGMT expression in MCF7 breast cancer cells, two GBM cell lines (U87MG, A172), and three patient-derived GBM cell lines (P#3, P#5, P#11). (B to D) U87MG cells were treated with either 50 µM or 100 µM TMZ for different time intervals as indicated. After treatment, (B) the number of viable cells was measured using the trypan blue dye exclusion method, (C) cell viability was assessed using the MTT colorimetric assay, and (D) representative images of cell morphology were acquired by microscopy. (E) Expression of MGMT in five GBM cell lines and TMZ-resistant cells (TMZ-R), formed after treatment with 50 µM or 100 µM TMZ for 1 month. HONE-1 cells and MCF7 cells were used as representative MGMT-positive cell lines. (t-test: *p < 0.05; **p < 0.01).
2. Materials and methods

2.1. Cells culture

Three patient-derived GBM lines, P#3, P#5, and P#11, were obtained according to the Taipei Medical University IRB protocol (201006011). These cell lines as well as human U87MG (ATCC) and A172 (ATCC) GBM cells, human MCF7 breast cancer cells (ATCC), and human Hone-1 nasopharyngeal carcinoma cells [15] were cultured in DMEM medium (Invitrogen) containing 10% FBS, 100 μg/mL streptomycin sulfate, and 100 U/mL penicillin-G sodium at 37 °C and 5% CO2. TMZ-resistant cells were maintained in the same culture medium containing 50 μM or 100 μM TMZ as indicated.

2.2. MTT assay

Cells were plated onto 24-well culture plates at an initial density of 1 × 10^5 cells/well. After one day of incubation, cells were treated with different doses of drugs as indicated for various time intervals. Subsequently, fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (final concentration of 0.5 mg/mL, Sigma Aldrich) was added to each well, and cells were then incubated for 1 h at 37 °C. Finally, the MTT medium was removed and the resultant formazan crystals were dissolved in 200 μL of DMSO. The absorbance readings of the DMSO extracts were measured at 570 nm by using an iMark Microplate Absorbance Reader (Bio-Rad).

2.3. Databases

To investigate the transcriptional levels of Sp1, SODs, catalase, and GPxs, human brain tumor gene expression profiles and the associated clinical pathological parameters were obtained from publicly available cancer microarray databases ONCOMINE (www.oncomine.org) [16]. In ONCOMINE, gene expression data from similar studies using the same methodology were used to compare gene expression for a tumor type with its normal counterpart. All data were log2-transformed and median-centered per array, and the standard deviation was normalized to one per array data [16]. We considered a gene to be overexpressed in a brain tumor when its mean expression level in the tumor samples was higher than the mean expression value in the normal tissue counterpart (Threshold: p-value < 0.05, fold change > 1.5).

2.4. Western blotting analysis

Protein samples were separated by SDS-PAGE, and transferred onto a PVDF membrane (Bio-Rad Laboratories). Subsequently, the PVDF membrane was blocked for 1 h by using 5% nonfat milk in TBST buffer and incubated with primary antibodies, including anti-Sp1 (1:1000, Millipore), anti-SOD2 (1:1000, GeneTex), anti-MGMT (1:1000, Santa Cruz Biotechnology), anti-phospho-Rad50 (Ser635) (1:1000, Cell Signaling), and anti-tubulin (1:3000, Proteintech) antibodies, for 2 h. After incubation, the membranes were washed with TBST buffer and then incubated with the appropriate secondary antibodies (1:3000, Santa Cruz Biotechnology) for 1 h. Finally, the membranes were washed again and the peroxidase was detected using chemiluminescence with Amersham Hyperfilm ECL (GE Healthcare).

2.5. Immunohistochemistry (IHC)

All immunohistochemical staining was performed on 10-μm-thick sections of paraformaldehyde-fixed, paraffin-embedded xenograft tumor tissues as well as on a human Glioma Tissue Microarray (US Biomax). The IHC method has been described previously [8]. The slides were incubated with a primary antibody at a dilution of 1:200 after

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### Table 1

| Author          | Normal brain | GBM | Fold | t-test | P-value |
|-----------------|--------------|-----|------|--------|---------|
| Sun et al. [40] | 23           | 81  | 1.69 | 9.240  | 9.84E−12|
| Bredel et al. [41] | 4    | 27  | 2.053| 4.136  | 0.007   |
| Murat et al. [42] | 4    | 80  | 1.299| 6.006  | 3.37E−4 |
| Liang et al. [43] | 2    | 30  | 1.376| 2.056  | 0.073   |
antigen retrieval, and then developed using the EnVision+ staining kit (DAKO). The antibodies used were Sp1 (Millipore) and SOD2 (Cell Signaling).

2.6. Soft agar colony formation assay

Cells (2 × 10⁴) were suspended in 2 mL of a 37 °C, 0.4% SeaKem LE Agarose (Lonza) solution in DMEM containing 10% FBS and the same culture medium supplements as described above. This suspension formed the upper layer, which was poured onto a lower layer formed of 0.8% agarose in a composition otherwise identical to the upper layer, in one 6-cm dish. Subsequently, the cells were incubated at 37 °C in a 5% CO₂ atmosphere. A few drops of fresh medium were added twice a week. After fourteen days, the number of viable cells was measured using the trypan blue dye exclusion method. (D) One day after treatment with TMZ and/or MA, the activation of Rad50 in A172 cells was measured by Western blotting. (E) A172 cells were transfected with pEGFP or pEGFP-Sp1. One day after protein expression (upper panel), cells were treated with DMSO (D) or 100 µM TMZ (T) for the different time intervals as indicated, and cell viability was analyzed using the MTT assay. (*p < 0.05; ***,##p < 0.01; ***,###p < 0.001).

2.7. Measurement of cellular ROS

Intracellular ROS levels were measured using dihydroethidium (DHE) [17]. Cells were collected and incubated with 10 µM DHE at 37 °C for 30 min. After incubation, cells were washed and DHE fluorescence was detected by flow cytometry (BD FACSCalibur), and analyzed using BD CellQuest software.

2.8. Real-time PCR

Complementary DNA was converted from total mRNA by reverse-transcription PCR [18]. The level of SOD2 cDNA were determined using 2 × SYBR real time master mix (Applied Biosystems) and primers specific for SOD2 (F: 5'-GCGCTAAGTGAACACCTGAA; R: 5'-CTGTAAACATCTCCTGGGCCA), or the control gene GAPDH (F: 5'-GAAGGTGAAGGTCGGAGTC; R: 5'-GAAGATGGTGATGGGATTC). SYBR green fluorescence was then monitored using an ABI 7000 Sequence Detection System (Applied Biosystems).

2.9. DNA affinity precipitation assay (DAPA)

The DAPA method has been described previously [9,19]. A 5′-biotin end-labeled double-stranded probe, derived from the human SOD2 promoter (sequence -103 to -75: 5′-
CCGGGCGGGGGGGGGGGGGGGGGGGGGG containing two Sp1 binding elements), was synthesized by Mission Biotech, Inc.

2.10. Experimental animals

Male NOD-SCID mice (5–6 weeks old, BioLASCO, Taiwan) were maintained at the animal facility of the National Health Research Institutes (Taiwan). For subcutaneous inoculation, 2 × 10^6 TMZ-resistant cells were injected into the right flank. Tumor volume was measured twice a week based on the following formula provided by the National Cancer Institute: tumor volume = length × width^2 × 3.14/6. When the volume reached 200 mm^3, animals were randomly assigned at the start of treatment. For the orthotropic model, a skull burr hole was first created in the right frontal brain area. An ultra-fine needle was then inserted to a depth of 3 mm using stereotatic guiding and 5 × 10^5 TMZ-resistant cells were injected. Treatment was initiated 10 days later. The scheduled treatment was interrupted when body weight loss became greater than 10% and was reinitiated after weight recovery. The endpoint was death or profound tumor impact leading to sacrifice.

With regard to the treatment, mithromycin A (MA) or vehicle (DMSO) was dosed intraperitoneally, and TMZ was dosed by oral gavage. In the combined treatment group, TMZ was administered 3–4 h after MA injection. The survival curve was plotted using SPSS (IBM).

2.11. Statistics

Similar results were obtained from more than three independent experiments, and are expressed as the mean ± standard deviation. Comparisons among multiple groups were performed using a one-way ANOVA with appropriate post hoc tests, whereas comparisons between two groups were performed using unpaired, two-tailed Student's t-test with a significance level set at p < 0.05.

3. Results

3.1. TMZ resistance in MGMT-deficient GBM cells is not associated with de novo expression of MGMT

To investigate the MGMT-independent mechanisms of chemoresistance in GBM, we treated MGMT-negative GBM cells, including U87MG, A172, P#3, P#5, and P#11 cells (Fig. 1A), with TMZ. Although the drug significantly reduced cellular proliferation and survival immediately after treatment (U87MG cells, Fig. 1B–D; other cell lines, data not shown), cell viability recovered after approximately one week (U87MG and P#11) to one month (A172, P#3 and P#5) of TMZ treatment. The resistance to TMZ was not related to MGMT expression, which remained undetectable (Fig. 1E). This finding confirmed that the survival of the several GBM cells treated with TMZ was MGMT-independent.

3.2. Upregulation of Sp1 enables GBM cells to escape TMZ-mediated toxicity

TMZ is also known to induce ROS generation, leading to cell death [2,20]. Our previous studies reported that Sp1 in brain acts as a pleiotropic oxidative stress response protein [9,10], providing protection to cells from peroxide damage. Using the ONCOMINE database (Fig. 2A and Table 1) and immunostaining of a tissue array (Fig. 2B), we found that Sp1 expression was increased in GBM compared with normal controls. Subsequent analysis of Sp1 expression in TMZ resistance revealed that Sp1 protein levels in TMZ-resistant cells were significantly higher than their parental tumor lines, including two established GBM lines, and three patient-derived GBM lines (Fig. 2C).

We next hypothesized that reducing Sp1 functionality may sensitize these cells to the anti-neoplastic agent. The Sp1 inhibitor mithromycin A (MA), was thus used in combination with TMZ in U87MG and P#5 GBM cells, which resulted in a significant inhibition of cell viability (Fig. 3A) and proliferation (Fig. 3B) compared to either drug alone. Additionally, we examined the viability of parental and TMZ-resistant
A172 cells following TMZ exposure, and showed that the IC50 value for TMZ-inhibited growth in resistant cells (approximately 380 μM) was higher than in wild-type cells (approximately 60 μM, Fig. 3C). How-ever, co-treatment with MA strongly sensitized resistant cells to TMZ (Fig. 3C) and increased TMZ-induced genotoxic stress, as shown by increased radiation-sensitive mutant 50 (Rad50) phosphorylation in response to DNA damage (Fig. 3D). Furthermore, we enhanced Sp1 expression by transient transfection, and showed that Sp1 over-expression reduced the inhibitory effect of TMZ on the growth of A172 cells (Fig. 3E). These findings collectively suggest that Sp1 protects GBM cells against TMZ-mediated cytotoxicity.

3.3. TMZ-resistant cells exhibit low levels of intracellular ROS, but Sp1 inhibition abolishes this

Since Sp1 is an oxidative stress-inducible, anti-death transcription factor [21], upregulated Sp1 may regulate the antioxidant system altering the production and/or removal of ROS in cells. Therefore, we examined intracellular ROS levels in TMZ-resistant cells using the superoxide indicator DHE. As shown in Fig. 4A background intracellular ROS levels in TMZ-resistant cells were significantly lower compared with parental cells. To further investigate whether Sp1 plays a role in the reduction of ROS levels, we treated TMZ-resistant cells with the Sp1 inhibitor MA and found that Sp1 inhibition induced a significant accumulation of intracellular ROS levels in resistant cells (Fig. 4B). These data suggest that increased Sp1 activity confers low levels of intracellular ROS, and that the antioxidant defensive system may be driven by Sp1.

3.4. SOD2 is highly expressed in TMZ-resistant GBM

Investigation of the expression of individual ROS scavenging factors in GBM was first conducted by examining the ONCOMINE [16] database of clinical samples, in which the gene expression of the major cellular antioxidants including SOD 1–3, catalase, and GPx 1–8 was analyzed. In thirteen microarray datasets, SOD2 was increased to the
vels were signiﬁcantly overexpressed in these TMZ resistance models. As shown in Fig. 7A, the human SOD2 promoter contains several Sp1 binding sites [22], indicating that Sp1 likely regulates SOD2 expression in resistant cells. To investigate the association between Sp1 and SOD2, we selected random single-cell clones that were simultaneously derived from TMZ-resistant U87MG cells. The data showed that 9 of 11 clones had increased expression of SOD2 with concurrently upregulated Sp1 (Fig. 7B), demonstrating a similar expression pattern of Sp1 and SOD2 in the majority of these resistant clones. Next, we studied U87MG xenografts and their recurrent tumors after continuous treatment with TMZ, and found increased expression of Sp1 and SOD2 in resistant tissues (Fig. 7C). Notably, areas in TMZ-resistant tumors with high Sp1 expression also exhibited increased SOD2 expression to a similar extent (upper panel, indicated by the dotted line). Subsequently, real-time PCR was performed to investigate the mRNA levels of SOD2, demonstrating that SOD2 mRNA levels were elevated in TMZ-resistant cells (Fig. 7D). Next, we used an Sp1 inhibitor to evaluate the role of Sp1 in TMZ-mediated SOD2 expression. As shown in Fig. 7E, Sp1 inhibition with MA reduced SOD2 protein expression in resistant cells. Finally, we performed a DAPA assay using a biotin-labeled Sp1 probe corresponding to the SOD2 promoter in U87MG cells. The results indicated that Sp1 bound to the SOD2 promoter, and its DNA binding was increased in resistant variants (#9 and #10, Fig. 7F). In summary, these results indicate that Sp1 plays a critical role in the upregulation of SOD2 in resistant GBM.

3.6. Inhibition of Sp1 reinstates the TMZ treatment effect in xenograft GBM models

To evaluate the in vivo effect of Sp1 inhibition, a TMZ-resistant cell xenograft model was generated. Oral TMZ was given with or without intraperitoneal administration of MA (Fig. 8A). The results showed that tumor growth was significantly slower with the combined treatment compared to the other treatment groups. Notably, TMZ alone exerted only a marginal effect in resistant cells, whereas MA alone did not show any anti-tumor effect. Examination of tumor tissues following the combined treatment with TMZ/MA revealed decreased SOD2 expression (Fig. 8B), suggesting a similar mechanism to that found in the in vitro experiments.

We next used a TMZ-resistant inoculated brain orthotopic model to conduct a survival study. As shown in Fig. 8C, treatment with TMZ exhibited a similar survival curve compared to that with vehicle only.

Fig. 6. SOD2 inhibition by diethyldithiocarbamate signiﬁcantly reduces the TMZ resistance of GBM cells. (A) A colony formation assay was performed on P#5 GBM cells treated with TMZ (T) and/or SODi (Si) at the indicated concentrations. (B) One day after treatment with TMZ and/or SODi, the activation of Rad50 in U87MG cells was measured by Western blotting. (C) U87MG TMZ-resistant cells were treated with 50 µM TMZ alone or co-treated with SODi at different doses as indicated for two and three days. Cell proliferation was then assessed using a colorimetric MTT assay. (D) The wild-type (Wt, dotted line) and resistant (TMZ-R, solid lines) A172 cells were exposed to various concentrations of TMZ and/or SODi as indicated for two days, and cell viability was measured by the trypan blue exclusion method. (*p < 0.05; **p < 0.01; ***p < 0.001).
An increased understanding of the mechanism for TMZ resistance in GBM could point to potential strategies to overcome this treatment dilemma. Importantly, our study provides the first evidence that MGMT-independent TMZ resistance relies upon the defensive mechanism of Sp1-modulated ROS scavenging to mitigate TMZ-induced stress. By studying TMZ-resistant cell lines, enhanced Sp1 expression was shown to accompany enhanced SOD2 expression. Furthermore, we demonstrated that Sp1 plays a critical role in SOD2 expression through promoter binding, which could be downregulated by co-incubation with the Sp1 inhibitor MA. Cellular viability in TMZ-resistant GBM could also be decreased by Sp1 inhibition, suggesting a potential strategy to overcome the acquired TMZ resistance. These findings were further confirmed using in vivo xenograft studies. Overall, our findings indicate that the Sp1-SOD2 pathway protects GBM against TMZ toxicity, and that inhibition of this pathway could mitigate the cellular resistance to this anti-neoplastic agent.

In terms of clinical treatment of GBM, the presence of MGMT factor has been shown to contribute to the innate tumor resistance to TMZ [1]. The major treatment limitation, however, emerges from acquired resistance rather than innate resistance. Unlike findings in rodent models, to date, studies examining whether anti-cancer therapies are capable of inducing MGMT in humans have been inconclusive [23]. Our study, using MGMT-negative models, showed a lack of MGMT expression even after acquisition of TMZ resistance, thus supporting a multifactorial resistance mechanism. In response to DNA damage-mediated death signals, certain tumor cells may increase their intracellular defensive mechanisms in order to survive [24]. This theory in turn led to our interest in ROS, which has been shown to be crucial to cell death caused by various DNA-damaging cytotoxic agents [25]. With regard to TMZ,
the drug itself and its analog, TMZ-perillyl alcohol conjugate, have been shown to upregulate ROS production in GBM cells and non-small cell lung cancer cells, respectively [2,26]. However, ROS is paradoxically critical both in the promotion of cancer progression and the induction of a detrimental cytotoxic response [27]. It is therefore unsurprising that certain cells possess an enhanced inherited antioxidant ability to tightly regulate ROS levels, thus maintaining viability and avoiding oxidative stress from the anticancer therapy. For example, overexpression of ROS scavengers, including CAT and NAC, has been reported to reverse TMZ analog-induced cell death in lung cancer [26]. In this study, we identified Sp1-upregulated SOD2 expression as a critical mechanism for protecting GBM cells against TMZ-induced cytotoxicity.

Sp1 has been recognized as critical in regulating multiple genes and influencing cellular functions in many cancer cell types such as breast, gastric, cervical, and pancreatic cancers [7,28]. In glioma, the expression of Sp1 is associated with disease grade and clinical outcome [29]. Although previous studies have shown that Sp1 inhibition by MA enhances the chemotherapeutic effect of TMZ [30] and reduces cancer stemness [31,32] in GBM, it remains unclear whether environmental stress induced by chemotherapy affects Sp1 and how Sp1 affects genes protective against the therapeutic stress. In this study, we showed that Sp1 was overexpressed in TMZ-resistant GBM and that it modulated SOD2 expression, which enabled cells to survive TMZ by eliminating hypoxia [35]. Multiple environmental stress-related transcription factors including Nrf2 and HIF-1 have been shown to be responsible for the induction of detoxifying enzyme gene expression [36]. Additionally, FoxO3a, a transcription factor produced in response to oxidative stress, has been shown to block hypoxia-mediated ROS generation [37]. These factors are, however, not related to increased SOD2 gene expression [37–39]. Although Sp1 is a stress-induced transcription factor [9,21] and our study indicated that Sp1 indeed plays an essential role in the transcriptional regulation of SOD2 gene, the inhibition of Sp1 by MA, which led to a decrease in SOD2, only diminished tumor growth and prolonged survival, but did not eliminate the tumor in these animal models. An important limitation of this study that needs to be recognized is that we only focused on the Sp1/SOD2 pathway and disregarded other transcription factors and ROS scavenging proteins, whose expression levels were also found to be increased in the clinical database, as shown in Fig. 5A. Thus, the role of microenvironment factor-mediated cellular protection resulting in chemotherapy resistance in GBM still needs to be further examined.

In conclusion, our study describes a mechanism by which TMZ resistance may be acquired, and suggests a treatment strategy to eliminate resistance by modulating oxidative pathways. This mechanism can be summarized as follows: firstly, Sp1 is upregulated in MGMT-deficient cells. Secondly, SOD2 accumulation regulated by Sp1 protects the GBM, resulting in TMZ tolerance. Finally, Sp1 and SOD2 antagonists allow TMZ susceptibility to be restored. Inhibition of this pathway may be of benefit in the clinical treatment with TMZ and represents a potential therapeutic strategy for the treatment of GBM.

Conflicts of interest

The authors declare no conflicts of interest in this work.
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