Interferon-α/β enhances temozolomide activity against MGMT-positive glioma stem-like cells

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Abstract. Glioma is one of the most common primary tumors of the central nervous system in adults. Glioblastoma (GBM) is the most lethal type of glioma, whose 5-year survival is 9.8% at best. Glioma stem-like cells (GSCs) play an important role in recurrence and treatment resistance. MGMT is a DNA repair protein that removes DNA adducts and therefore attenuates treatment efficiency. It has been reported that interferon-α/β (IFN-α/β) downregulates the level of MGMT and sensitizes glioma cells to temozolomide. In the present study, we assessed whether IFN-α/β is able to sensitize GSCs to temozolomide by modulating MGMT expression. Upon the treatment of IFN-α/β, the efficacy of temozolomide against MGMT-positive GSCs was markedly enhanced by combination treatment with IFN-α/β when compared with the temozolomide single agent group, and MGMT expression was markedly decreased at the same time. Further mechanistic study showed that IFN-α/β suppressed the NF-κB activity, which further mediated the sensitization of MGMT-positive GSCs to temozolomide. Our data therefore demonstrated that the application of IFN-α/β is a promising agent with which to enhance temozolomide efficiency and reduce drug resistance, and our findings shed light on improving clinical outcomes and prolonging the survival of patients with malignant gliomas.

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Introduction

Glioma is one of the most common primary tumors of the central nervous system in adults, accounting for ~31% of all brain tumors (1). Although multimodal treatments including surgery, chemotherapy and radiotherapy have shown some efficacy, the outcome of patients with malignant glioma remains poor. Recently, it was proven by a randomized phase III clinical study that glioblastoma (GBM) patient survival was improved when combining alkylating agent temozolomide (TMZ) with radiotherapy. Overall patient survival was 27.2% at 2 years and 9.8% at 5 years in the combination group vs. 10.9% at 2 years and 1.9% at 5 years in the radiotherapy alone group (2,3). However, the recurrence rate of malignant glioma patients remained high even when they were sensitive to TMZ (4).

A high level of MGMT and the existence of glioma stem-like cells (GSCs) are considered as the two main causes of chemoresistance in malignant glioma patients. MGMT is a DNA repair protein that is unique in its ability to remove DNA adducts and to self-inactivate (5). Therefore, the protein level of MGMT was found to be inversely related to the chemosensitivity of gliomas to alkylating agents (6-9). GSCs are known as tumor-initiating cells due to their stem cell-like properties and the pivotal role in tumor development (10-12). The expression levels of multiple drug resistance enzymes in GSCs were also found to contribute to chemoresistance (13,14).

IFN-α and IFN-β, cytokines that elicit pleiotropic biological effects, have been widely used either alone or in combination with others in the treatment of malignant glioma (15-19). Both IFN-α and IFN-β have been reported to downregulate expression of MGMT and re-sensitize resistant glioma cells to TMZ *in vitro* and *in vivo*. However, how MGMT expression is regulated following IFN-α and IFN-β treatment, and whether IFN-α and IFN-β help against GSCs have not yet been addressed. In the present study, the effects of IFN-α and IFN-β on the efficacy of TMZ therapy, as well as the possible mechanism of this enhancement were investigated.

Table I. Primer sequences for PCR.

Gene name	Direction	Sequence (5'-3')	Product size (bp)
MGMT	Forward	GTTATGAATGTAGGAGCCCTTATG	239
	Reverse	TGACAACGGGAATGAAGTAATG	
NFκB	Forward	TGTGGGTTTCCTGTGCTAATG	208
	Reverse	GAGACCAGCCTTTCTCCGTA	
GAPDH	Forward	CGCTCTCTGCTCCTCCTGTTC	108
	Reverse	ATCCGTTGACTCCGACCTTCAC	

Materials and methods

Cell lines and GSC culture. Human glioma cell lines U251, SKMG-4 and U87 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen, New York, NY, USA) in a humidified atmosphere containing 5% CO_2 at 37˚C. GSCs were enriched from U251, SKMG-4 and U87 cell cultures using serum-free DMEM/F12 (Invitrogen) supplemented with 2% B27 (Gibco, Grand Island, NY, USA), 20 ng/ml EGF and 10 ng/ml bFGF (PeproTech, Rocky Hill, NJ, USA) according to the procedure established by Singh *et al* (11).

Cell proliferation analysis. GSCs, namely U251G (MGMT-positive), SKMG-4G (MGMT-positive), and U87G (MGMT-negative), were plated at 5,000 cells/well in 96-well plates and incubated for 24 h. These GSCs were then divided into 6 groups: i) the control group, in which the inhibition of the growth rate of untreated cells was regarded as 0%; ii) TMZ (500 μM)-treated for 72 h; iii) IFN-α (100 U/ml)-treated for 72 h; iv) IFN-β (100 U/ml)-treated for 72 h; v) IFN-α (100 U/ml)-treated for 24 h and then co-incubated with TMZ (500 μM) for 48 h; vi) IFN-β (100 U/ml)-treated for 24 h and then co-incubated with TMZ (500 μM) for 48 h. Four hours prior to harvest, 10 μl/well of the reagent in the Cell Counting Kit-8 (CCK-8; Dojindo, Japan) was added, and the cells were incubated for 4 h at 37˚C. The absorbance was measured by Labsystems MK3 ELIASA (Thermo Fisher, Waltham, MA, USA) at a wavelength of 490 nm. The percentage of cell survival (survival rate) was calculated by dividing the absorbance value of the treated sample by the absorbance value of the untreated control for every group.

Subcutaneous xenograft tumor growth inhibition experiments. BALB/c nude mice (male, 5-6 weeks of age) were purchased from the SLAC Laboratory Animal Company (Shanghai, China). The mice were housed in laminar flow cabinets under specific pathogen-free conditions in the animal facility at our institute. All animal studies were performed according to the institutional ethical guidelines for experimental animal care and were approved by the Medical Ethics Committee of Sun Yat-sen University Cancer Center. U251G and SKMG-4G cells were harvested by Accutase (Gibco) and injected ($1x10^7$ cells/mouse, suspended in 100 μl PBS and 100 μl Matrigel) subcutaneously into the right flank of the mice. When the subcutaneous tumors had

reached a volume of ~50 mm^3, the animals were randomly divided into groups: treated with saline, TMZ only (TMZ 50 mg/kg), IFN-α only (IFN-α $2x10^5$ IU), IFN-β only (IFN-β $2x10^5$ IU), TMZ+IFN-α (TMZ 50 mg/kg + IFN-α $2x10^5$ IU) or TMZ + IFN-β (TMZ 50 mg/kg + IFN-β $2x10^5$ IU). IFN-α or IFN-β was administered by subcutaneous injection 6 h before an intraperitoneal injection of TMZ. Treatments were repeated at 24-h intervals for a total of 5 doses. Subcutaneous tumors were measured once a week, and the tumor growth inhibition rates were analyzed at week 5. Tumor volume was calculated according to the following formula: Volume = length x width^2/2. All animal experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University and in accordance with the national guidelines for the care and maintenance of laboratory animals.

RNA preparation and RT-PCR. Total RNA from U251G and SKMG-4G cells (cell cultures grouped as previously described) was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using 2 μg of total RNA from each sample in a total volume of 20 μl using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, USA). GAPDH was used as a loading control. The sequences of the forward and reverse primers for each gene are listed in Table I. Each PCR mixture (20 μl) contained 1 μl cDNA, 10 μl Green GoTaq DNA polymerase (Promega, USA) and 0.5 μl of each forward and reverse primers (10 μM). The PCR program was carried out as follows: 94˚C for 2 min for hot start; 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, for 35 cycles; 72˚C for 2 min. The PCR products were separated on 2% agarose gel, visualized by ethidium bromide staining and photographed with Gel Doc XR (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry. Subcutaneous xenograft tumors were established as previously described. When the subcutaneous tumors had reached a volume ~50 mm^3, the animals were randomly divided into 4 groups (6 animals per group) and treated as described: with saline water, TMZ only (TMZ 50 mg/kg), TMZ+IFN-α (TMZ 50 mg/kg + IFN-α $2x10^5$ IU), or TMZ+IFN-β (TMZ 50 mg/kg + IFN-β $2x10^5$ IU). The treatments were repeated at 24-h intervals for a total of 5 doses. Seven days after treatment, tumor samples were harvested and flash frozen in liquid nitrogen. Half of the tumor samples were used for histological analysis, while the other half were
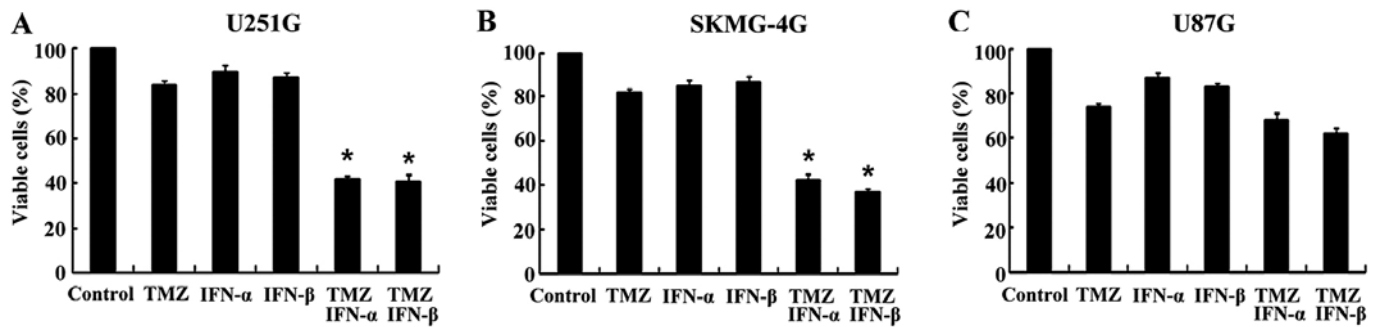
Figure 1. IFN-α/β sensitizes MGMT-positive GSCs to TMZ *in vitro*. Following combined treatment of TMZ with IFN-α or IFN-β, the cell viability was markedly decreased in the (A) U251G and (B) SKMG-4G cells (*p<0.05). (C) The cell viability was not significantly decreased in the U87G cells, following combined treatment of TMZ with IFN-α or IFN-β. The detailed treatment conditions are as follows: control group, TMZ (500 μM) for 72 h, IFN-α (100 U/ml) for 72 h, IFN-β (100 U/ml) for 72 h, IFN-α (100 U/ml) for 24 h and then co-incubated with TMZ (500 μM) for 48 h, IFN-β (100 U/ml) for 24 h and then co-incubated with TMZ (500 μM) for 48 h. One-way ANOVA was applied for statistical analysis.
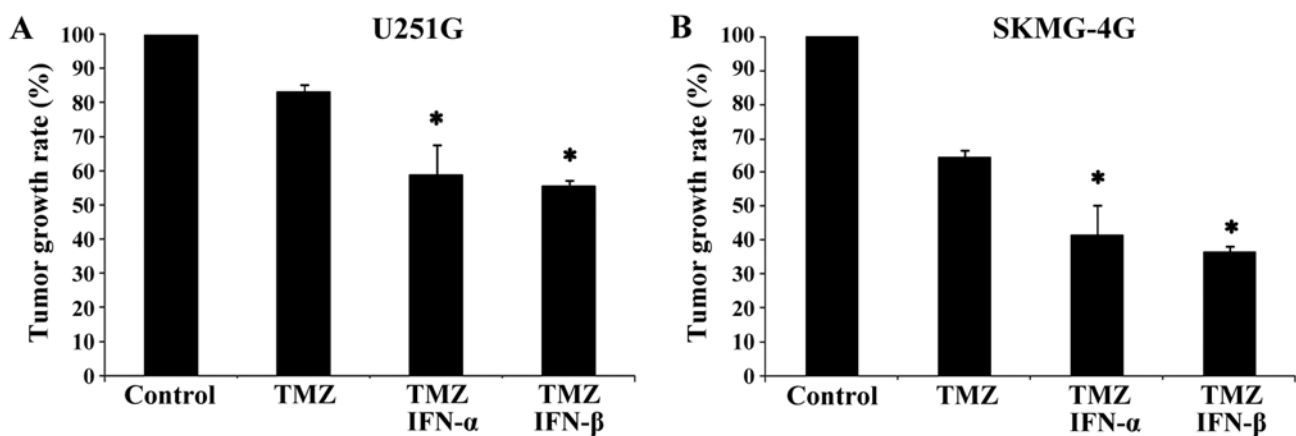

Figure 2. IFN-α/β sensitizes MGMT-positive GSC xenografts to TMZ *in vivo*. The tumor growth rate was markedly reduced following combined treatment with TMZ and IFN-α/β in the (A) U251G and (B) SKMG-4G xenografts (*p<0.05). The treatment conditions were as follows: control group, TMZ only, TMZ+IFN-α and TMZ+IFN-β. The differences between the TMZ only and combination groups were analyzed by one-way ANOVA test.

used for western blot analysis. For histological analysis, the tumor samples subjected to immunohistochemical analysis were fixed in 4% paraformaldehyde and stained with anti-human-MGMT (Invitrogen) and anti-human-NF-κB (BioVision, USA) antibodies. MGMT and NF-κB protein quantification was performed by counting the number of stained cells in 10 random fields at x400 magnification for each tumor sample.

Western blotting. Tumor cells or tissues were washed with ice-cold PBS and lysed on ice by RIPA buffer (Beyotime, China). The protein concentration was measured using the BCA assay kit (Beyotime) according to the manufacturer's protocol. Equal amounts (60 μg) of total protein from each sample were boiled at 95°C for 5 min, separated on 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). The membranes were then incubated in 5% milk for 1.5 h. The following primary antibodies were probed by overnight incubation at 4°C: anti-MGMT (Invitrogen), anti-β-actin (Beyotime) and anti-NF-κB (BioVision). The next day, the membranes were washed and incubated with the secondary antibody for 1 h. Proteins were visualized using the ECL system (Millipore), and the bands were exposed to BioMax MR film (Kodak, Japan).

Statistical analysis. Each experiment was repeated at least three times and data are presented as mean ± SD. The difference was analyzed using one-way ANOVA test. The correlations were determined by bivariate correlation procedures (Spearman correlation coefficient). All statistical analyses were performed using SPSS 16.0. The differences were considered as significant at p<0.05.

Results

IFN-α/β enhances the sensitivity of MGMT-positive GSCs to TMZ. Previous studies have shown that both IFN-α and IFN-β enhance TMZ activity against MGMT-positive glioma cells (20,21). However, their effects on GSCs remain unknown. Therefore, whether IFN-α or IFN-β enhances the effect of TMZ on MGMT-positive GSCs was initially assessed. Following treatment with 100 IU/ml IFN-α or 100 IU/ml IFN-β, the viable cell rates of the U251G cells were 42.05±1.54 and 40.86±2.83% compared to 83.87±2.33% for the TMZ only group (p<0.05, Fig. 1A). In the SKMG-4G cells, the viable cell rates of the combination groups were 42.26±2.91 and 37.38±1.55%, respectively, compared to 81.97±1.35% for the TMZ only group (p<0.05, Fig. 1B). However, in the MGMT-negative GSCs derived from U87G cells, the viable cell rates
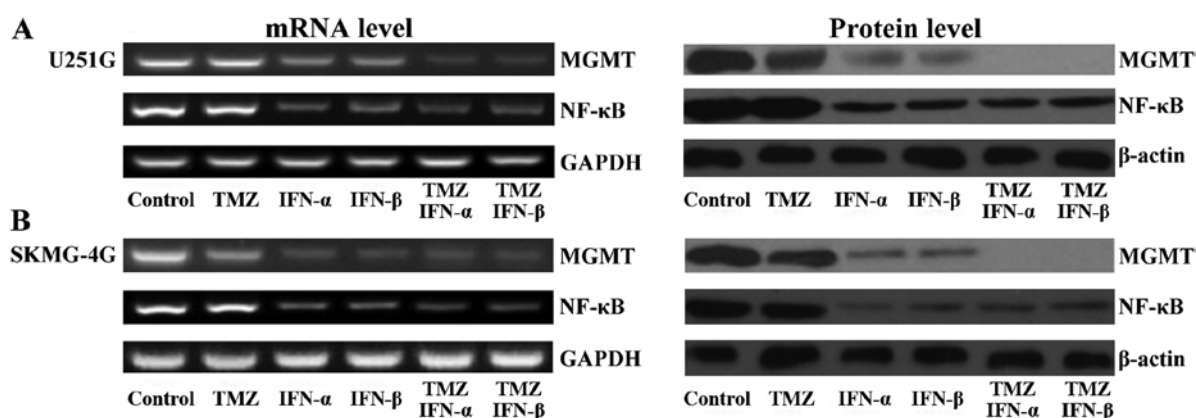
Figure 3. NF-κB and MGMT are decreased in the MGMT-positive GSCs following combination treatment *in vitro*. (A) The mRNA (left panel) and protein (right panel) levels of MGMT and NF-κB in the U251G cells. (B) The mRNA (left panel) and protein (right panel) levels of MGMT and NF-κB in the SKMG-4G cells. The treatment conditions were as follows: control group, TMZ (500 μM) for 72 h, IFN-α (100 U/ml) for 72 h, IFN-β (100 U/ml) for 72 h, IFN-α (100 U/ml) for 24 h and then co-incubated with TMZ (500 μM) for 48 h, IFN-β (100 U/ml) for 24 h and then co-incubated with TMZ (500 μM) for 48 h.
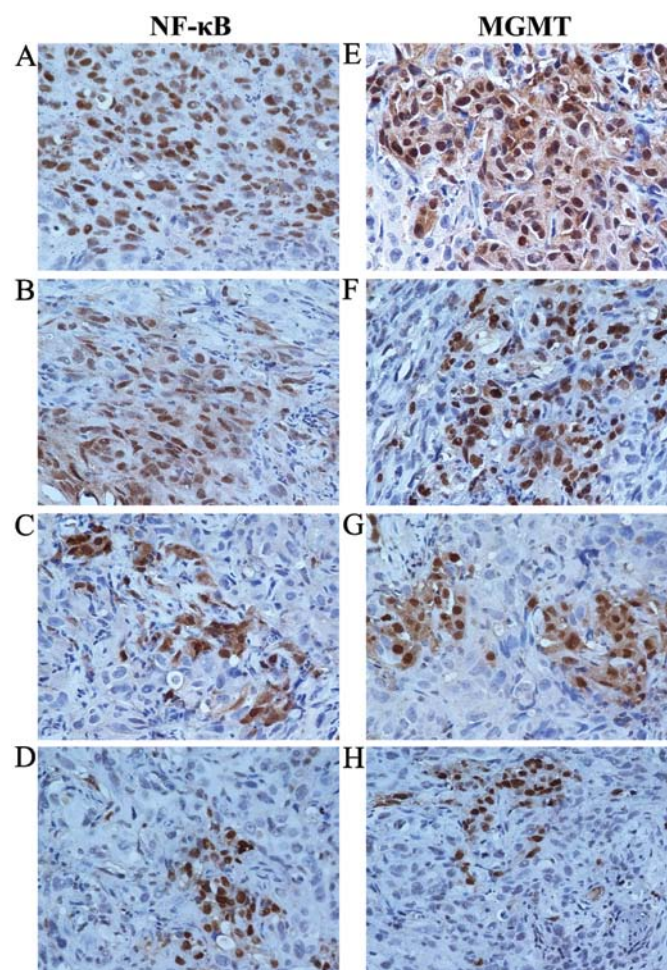

Figure 4. Expression levels of NF-κB and MGMT in GSC xenograft are reduced following IFN-α/β treatment *in vivo*. The expression levels of NF-κB (A-D) and MGMT (E-H) were reduced in the combination group when compared with levels in the TMZ group, which were lower than those in the control group in the U251G xenografts. (A and E) Control group, (B and F) TMZ only group, (C and G) TMZ+IFN-α group, (D and H) TMZ+IFN-β group. Magnification, x400.

vs. 73.84±1.65%) (p>0.05, Fig. 1C). These results revealed that the co-treatment of IFN-α/β significantly enhanced the sensitivity of MGMT-positive GSCs to TMZ, while no marked elevation was found in the MGMT-negative GSCs.

IFN-α/β enhances the antitumor efficacy of TMZ in GSC xenografts. Subcutaneous xenografts were established via implantation of MGMT-positive U251G and SKMG-4G GSCs in BALB/c nude mice. After treatment for 5 weeks, the tumor growth rates of the U251G xenografts in the combination groups (TMZ+IFN-α and TMZ+IFN-β) were reduced when compared with the growth rate in the TMZ only group (58.9±8.66 and 55.5±1.90 vs. 83.3±1.96%, p<0.05, Fig. 2A). Similarly, the growth rates of SKMG-4G xenografts in the TMZ+IFN-α and TMZ+IFN-β groups were much lower than the rate in the TMZ only group (41.6±4.34 and 36.6±1.08 vs. 64.8±2.28%, p<0.05, Fig. 2B). These data demonstrated that IFN-α/β enhanced the antitumor efficiency of TMZ *in vivo*.

NF-κB and MGMT are downregulated by the combination treatment. As a previous study showed that NF-κB regulates the expression of MGMT in the process of DNA repair (22), we detected the levels of NF-κB and MGMT by RT-PCR and western blot assays following the treatments. Both the mRNA and protein levels of MGMT were reduced following treatment with IFN-α/β and further decreased with the combination treatment (Fig. 3A). NF-κB was reduced following IFN-α/β treatment but remained at the same level in the co-treatment cell lysates (Fig. 3A). The effect of TMZ on the level of MGMT was not marked. In SKMG-4G cells, we obtained consistent data that IFN-α/β treatment led to a reduction in both MGMT and NF-κB; the combined treatment further decreased MGMT but not NF-κB (Fig. 3B). Our data indicated that IFN-α or IFN-β sensitized MGMT-positive GSCs to TMZ, which may be through the donwregulation of NF-κB leading to a reduction in MGMT.

IFN-α/β decreases NF-κB and MGMT protein levels in the GSC xenografts. We collected U251G xenografts and performed immunohistochemical staining following standard procedures. The expression levels of NF-κB and MGMT in
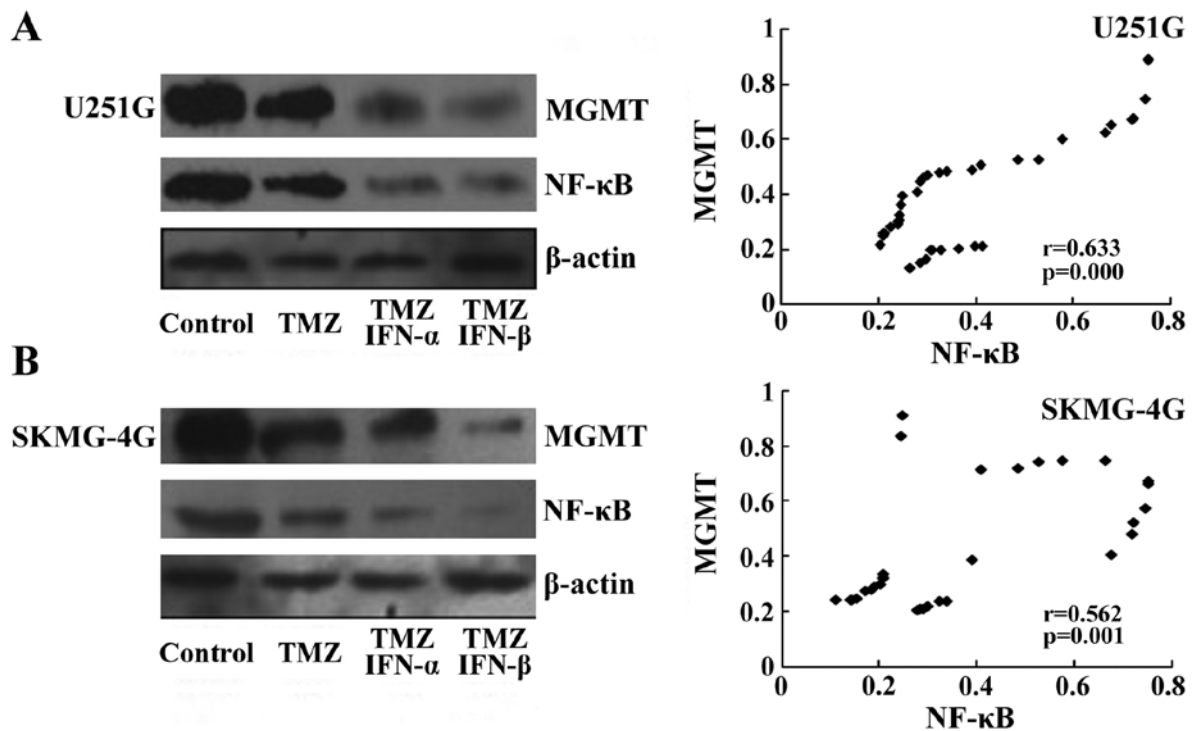
were not markedly reduced in the combination groups when compared with the TMZ only group (67.94±3.57 and 62.0±2.68

Figure 5. Expression levels of NF-κB and MGMT are correlated in the GSC xenografts (A) The expression of MGMT and NF-κB in U251G xenografts (left panels), and the correlation between their expression levels (right panels). (B) The expression of MGMT and NF-κB in SKMG-4G xenografts (left panels), and the correlation between their expression levels (right panels). Each dot indicates the level of NF-κB and MGMT for each xenograft.

the control group were considered as normal (Fig. 4A and E). In the TMZ-treated group, the expression levels of NF-κB and MGMT were slightly reduced when compared with these levels in the control group (Fig. 4B and F), which was further decreased in the xenografts co-treated with TMZ and IFN-α or IFN-β (Fig. 4C and G, D and H). Our data demonstrated that IFN-α or IFN-β decreased the level of NF-κB. Consistent with previous reports, MGMT was reduced accompanied by decreased NF-κB, which may be the potential mechanism for the enhanced sensitivity of MGMT-positive GSCs to TMZ by IFN-α/β.

MGMT levels are positively correlated with NF-κB in the GSC xenografts. To confirm the results from IHC analysis, we collected xenografts derived from U251G and SKMG-4G cells, and determined the levels of NF-κB and MGMT by western blot assay. Levels of NF-κB and MGMT were slightly decreased following TMZ treatment compared to levels in the untreated control group, while NF-κB and MGMT levels were markedly reduced in the groups treated with TMZ and IFN-α/β (Fig. 5A and B, left panels). The protein levels of NF-κB and MGMT in each xenograft were detected by western blotting, and then subjected to Spearman correlation analysis. We found that a lower level of MGMT was detected with a decrease in NF-κB. Statistical analysis also confirmed that the MGMT level was positively associated with NF-κB in both the U251G (r=0.633, p<0.05, Fig. 5A, right panel) and SKMG-4G xenografts (r=0.562, p<0.05, Fig. 5B, right panel). Therefore, our data confirmed that the MGMT level was correlated with NF-κB upon the combination treatment of TMZ and IFN-α/β.

Discussion

The unique characteristics of GSCs, including self-renewal, unlimited proliferation and differentiation abilities, provide them with the ability to promote tumor initiation, growth and recurrence. They also have altered signaling pathways that are involved in the regulation of cell survival and proliferation. GSCs have also been demonstrated to correlate with chemo-resistance and poor survival since they express high levels of multidrug resistance proteins and DNA repair enzymes (23). A previous study carried out by us showed that *MGMT* was unmethylated, and the protein level was increased in U251G and SKMG-4G cells when compared with their parental U251 and SKMG-4 cells. *In vitro* experiments revealed that U251G and SKMG-4G cells are more resistant to TMZ than their parental cells with low level of MGMT expression (24).

MGMT is a DNA repair enzyme that specifically removes unfavorable methyl groups from the O^6 position of guanine, thereby restoring the nucleotide to its native form without causing any DNA damage. It is a crucial protein both for cancer prevention and treatment due to its ability to repair mutagenic lesions in DNA and limit the effectiveness of alkylating chemotherapies (25). Previous studies have shown that high MGMT expression is correlated with poor clinical outcome in glioma patients treated with chloroethylnitrosoureas and alkylating agents (26-28). In the clinical setting, the benefits of TMZ therapy are mainly observed in those patients with methylated *MGMT* promoter for whom TMZ-induced DNA damage is unable to be repaired. Patients with unmethylated *MGMT* promoter fail to benefit from TMZ chemotherapy. Even with methylated *MGMT*, gliomas are usually sensitive to

TMZ chemotherapy at the beginning and develop resistance shortly after.

Natsume *et al* reported that IFN-β treatment decreases MGMT levels in glioma cells via transcription inhibition. Moreover, pre-treatment with IFN-β markedly enhances chemosensitivity of glioma cells to TMZ (20,21). Another study showed that IFN-β provides a survival benefit for patients after radiation (29). Recently, a multi-center study demonstrated that both the presence of methylated *MGMT* and patient response to combination treatment (IFN-β and TMZ) were independent favorable prognostic indicators for GBM patients (30). It was reported that the MGMT level is related with NF-κB activity, since there are two putative NF-κB binding sites in the *MGMT* promoter region that regulates MGMT expression. Thus, NF-κB plays an important role in MGMT-mediated chemoresistance (31,32). A study by Lavon *et al* showed that cell lines with high constitutive NF-κB activity are less sensitive to nitrosourea treatment, and suppression of MGMT activity by small molecules completely abolished the chemoresistance regulated by NF-κB (22).

The present study aimed to explore whether GSCs also contribute to TMZ-resistance in malignant glioma patients with unmethylated *MGMT* and whether manipulating the MGMT level by combination therapy will benefit glioma patients. We demonstrated that the efficacy of TMZ on MGMT-positive GSCs was markedly enhanced by the combination treatment with IFN-α/β when compared with the TMZ single agent group, and MGMT expression was markedly decreased at the same time. We also provided evidence that IFN-α/β suppressed NF-κB activity, which further mediated the sensitization of MGMT-positive GSCs to TMZ by decreasing the MGMT protein level. Our data therefore suggest that inhibition of NF-κB may improve clinical outcomes and prolong the survival of patients with malignant gliomas.

Acknowledgements

References

1. CBTRUS: CBTRUS Statistical report: Primary brain and central nervoussystem tumors diagnosed in the United States in 2004-2007. Source: Central Brain Tumor Registry of the United States CBTRUS, Hindsdale, IL, USA, 2011. www.cbtrus.org.
2. Stupp R, Mason WP, van den Bent MJ, *et al*; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987-996, 2005.
3. Stupp R, Hegi ME, Mason WP, *et al*; European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group: Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 10: 459-466, 2009.
4. Fukushima T, Takeshima H and Kataoka H: Anti-glioma therapy with temozolomide and status of the DNA-repair gene *MGMT*. Anticancer Res 29: 4845-4854, 2009.
5. Sharma S, Salehi F, Scheithauer BW, Rotondo F, Syro LV and Kovacs K: Role of MGMT in tumor development, progression, diagnosis, treatment and prognosis. Anticancer Res 29: 3759-3768, 2009.
6. Fruehauf JP, Brem H, Brem S, Sloan A, Barger G, Huang W and Parker R: In vitro drug response and molecular markers associated with drug resistance in malignant gliomas. Clin Cancer Res 12: 4523-4532, 2006.
7. Hermisson M, Klumpp A, Wick W, *et al*: O^6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. J Neurochem 96: 766-776, 2006.
8. Esteller M, Garcia-Foncillas J, Andion E, *et al*: Inactivation of the DNA-repair gene *MGMT* and the clinical response of gliomas to alkylating agents. N Engl J Med 343: 1350-1354, 2000.
9. Hegi ME, Diserens AC, Gorlia T, *et al*: *MGMT* gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352: 997-1003, 2005.
10. Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD and Steindler DA: Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. Glia 39: 193-206, 2002.
11. Singh SK, Hawkins C, Clarke ID, *et al*: Identification of human brain tumour initiating cells. Nature 432: 396-401, 2004.
12. Kondo T, Setoguchi T and Taga T: Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci USA 101: 781-786, 2004.
13. Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G, Pilozzi E, Larocca LM, Peschle C and De Maria R: Chemotherapy resistance of glioblastoma stem cells. Cell Death Differ 13: 1238-1241, 2006.
14. Salmaggi A, Boiardi A, Gelati M, *et al*: Glioblastoma-derived tumorospheres identify a population of tumor stem-like cells with angiogenic potential and enhanced multidrug resistance phenotype. Glia 54: 850-860, 2006.
15. Jonasch E and Haluska FG: Interferon in oncological practice: Review of interferon biology, clinical applications, and toxicities. Oncologist 6: 34-55, 2001.
16. Wakabayashi T, Natsume A, Hashizume Y, Fujii M, Mizuno M and Yoshida J: A phase I clinical trial of interferon-beta gene therapy for high-grade glioma: Novel findings from gene expression profiling and autopsy. J Gene Med 10: 329-339, 2008.
17. Groves MD, Puduvalli VK, Gilbert MR, *et al*: Two phase II trials of temozolomide with interferon-alpha2b (pegylated and non-pegylated) in patients with recurrent glioblastoma multiforme. Br J Cancer 101: 615-620, 2009.
18. Ohno M, Natsume A, Fujii M, Ito M and Wakabayashi T: Interferon-beta, MCNU, and conventional radiotherapy for pediatric patients with brainstem glioma. Pediatr Blood Cancer 53: 37-41, 2009.
19. Warren K, Bent R, Wolters PL, *et al*: A phase 2 study of pegylated interferon α-2b (PEG-Intron®) in children with diffuse intrinsic pontine glioma. Cancer 118: 3607-3613, 2012.
20. Natsume A, Ishii D, Wakabayashi T, *et al*: IFN-beta downregulates the expression of DNA repair gene *MGMT* and sensitizes resistant glioma cells to temozolomide. Cancer Res 65: 7573-7579, 2005.
21. Natsume A, Wakabayashi T, Ishii D, *et al*: A combination of IFN-beta and temozolomide in human glioma xenograft models: Implication of p53-mediated MGMT downregulation. Cancer Chemother Pharmacol 61: 653-659, 2008.
22. Lavon I, Fuchs D, Zrihan D, *et al*: Novel mechanism whereby nuclear factor kappaB mediates DNA damage repair through regulation of O^6-methylguanine-DNA-methyltransferase. Cancer Res 67: 8952-8959, 2007.
23. Liu G, Yuan X, Zeng Z, *et al*: Analysis of gene expression and chemoresistance of CD133$^+$ cancer stem cells in glioblastoma. Mol Cancer 5: 67, 2006.
24. Qiu ZK, Shen D, Chen YS, *et al*: Enhanced MGMT expression contributes to temozolomide resistance in glioma stem-like cells. Chin J Cancer 33: 115-122, 2014.
25. Tubbs JL, Pegg AE and Tainer JA: DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by O^6-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy. DNA Repair 6: 1100-1115, 2007.

26. Chen ZP, Yarosh D, Garcia Y, *et al*: Relationship between O^6-methylguanine-DNA methyltransferase levels and clinical response induced by chloroethylnitrosourea therapy in glioma patients. Can J Neurol Sci 26: 104-109, 1999.
27. Paz MF, Yaya-Tur R, Rojas-Marcos I, *et al*: CpG island hypermethylation of the DNA repair enzyme methyltransferase predicts response to temozolomide in primary gliomas. Clin Cancer Res 10: 4933-4938, 2004.
28. Hegi ME, Liu L, Herman JG, *et al*: Correlation of O^6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. J Clin Oncol 26: 4189-4199, 2008.
29. Colman H1, Berkey BA, Maor MH, *et al*; Radiation Therapy Oncology Group: Phase II Radiation Therapy Oncology Group trial of conventional radiation therapy followed by treatment with recombinant interferon-beta for supratentorial glioblastoma: Results of RTOG 9710. Int J Radiat Oncol Biol Phys 66: 818-824, 2006.
30. Motomura K, Natsume A, Kishida Y, *et al*: Benefits of interferon-β and temozolomide combination therapy for newly diagnosed primary glioblastoma with the unmethylated MGMT promoter: A multicenter study. Cancer 117: 1721-1730, 2011.
31. Saccani S, Marazzi I, Beg AA and Natoli G: Degradation of promoter-bound p65/RelA is essential for the prompt termination of the nuclear factor kappaB response. J Exp Med 200: 107-113, 2004.
32. Sethi G, Ahn KS, Sung B and Aggarwal BB: Pinitol targets nuclear factor-kappaB activation pathway leading to inhibition of gene products associated with proliferation, apoptosis, invasion, and angiogenesis. Mol Cancer Ther 7: 1604-1614, 2008.