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ORIGINAL ARTICLE

A combination of IFN-β and temozolomide in human glioma xenograft models: implication of p53-mediated MGMT downregulation

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Abstract
Purpose Methylation of the O^6-methyguanine-DNA methyltransferase (MGMT) gene promoter in gliomas has been reported to be a useful predictor of the responsiveness to temozolomide (TMZ). In our previous experiments, we observed that IFN-β sensitized TMZ-resistant glioma cells with the unmethylated MGMT promoter and that the mechanism of action was possibly due to attenuation of MGMT expression via induction of TP53. In this study, (1) we explored the synergistic effect of IFN-β and TMZ in the animal model, and (2) clarified the role of IFN-β induced TP53 in the human MGMT promoter.
Methods (1) Nude mice with either subcutaneous T98 (TMZ-resistant) or U251SP (TMZ-sensitive) tumor were treated with IFN-β/TMZ for 5 consecutive days. (2) The MGMT promoter activity was assayed by a luciferase reporter system in Saos2 (p53-null) cells transduced with a p53-adenoviral vector, and T98 glioma cells treated with IFN-β.
Results (1) A combination of IFN-β/TMZ had significant synergistic antitumor activity on the growth of both T98 and U251SP tumors. (2) MGMT promoter activity was suppressed by either adenovirally transduced p53 or IFN-β.
Conclusions It would be appealing to consider a prospective clinical trial in which genetic markers are used for personalized drug selection, eliciting other forms of treatment or inhibition of MGMT for those with MGMT expression. In this context, IFN-β inactivates MGMT via p53 gene induction and enhances the therapeutic efficacy to TMZ.

Keywords Glioma · Temozolomide · IFN-beta · MGMT · Methylation · P53

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Introduction

High-grade (WHO grades III and IV) gliomas consisting of anaplastic astrocytoma (AA), anaplastic oligodendroglioma (AO), anaplastic oligoastrocytoma (AOA), and glioblastoma multiforme (GBM) often resist treatment; GBM, the most common glioma in adults, kills patients within a median time span of a year after diagnosis, even when aggressive surgical resection, chemotherapy, and radiotherapy are carried out. This dismal outcome has not been improved substantially over the last three decades. Temozolomide (TMZ) is a well-tolerated orally bioactive alkylating agent used in glioma patients. It has been adopted as the first-line treatment in patients with high-grade gliomas with the expectation that it might improve poor prognosis [2, 4, 11, 25–27]. TMZ induces the O^6 methyl-guanine (MG) lesion. Subsequently, during DNA synthesis, thymine is mispaired to the O^6 MG by DNA polymerase, where aberrant mismatch repair (MMR) processing of O^6-MG:T mispair generates DNA single-strand gaps and/or double-strand breaks, which leads to cell killing [5, 20]. A cellular DNA repair protein—the O^6-methylguanine-DNA methyltransferase (MGMT) protein—repairs the alkylation at the O^6 position of guanine [15, 21]. A number of studies have suggested that *MGMT* deficiency of brain tumors is closely related to the sensitivity to alkylating agents [1, 13, 16]. Furthermore, since MGMT protein loss may be a result

of promoter hypermethylation, methylation of the MGMT promoter in gliomas has been reported to be a useful predictor of their responsiveness to alkylating agents [4, 12]. Thus, MGMT promoter methylation may enable the selection of patients that are most likely to benefit from TMZ treatment.

For patients with gliomas in which the promoter is not methylated, alternative treatments involving a different mechanism of action or methods that inhibit *MGMT* should be developed. One such inhibitor is interferon-β (IFN-β), and it is being investigated for this purpose [18]. IFN-β, one of the type I IFNs, in its role as a drug sensitizer, has been widely used in combination with other antitumor agents such as nitrosoureas. In our previous experiments, we found that IFN-β sensitized TMZ-resistant glioma cells containing an unmethylated MGMT promoter [18]. With regard to the mechanism of action, the sensitizing effect of IFN-β was possibly due to the attenuation of MGMT expression via induction of TP53, which binds to the MGMT promoter, as determined by the ChIP assay. In this study, we further explore the synergistic effect of IFN-β and TMZ on nude mice with subcutaneously transplanted human gliomas. Here, we report that a combination of IFN-β and TMZ decreased tumor growth to a very significant extent, and complete regression was observed in 20% of the treated animals.

The mechanism of MGMT inactivation caused by IFN-β-induced p53 upregulation in glioma cells is not completely understood. The possibility that MGMT may be regulated by p53 has received much attention. Briefly, studies in murine cells have indicated that MGMT can be induced by ionizing radiation in a wild-type *p53* gene-dependent manner [6, 22], and p53 may upregulate the basal expression of MGMT [19]. On the contrary, the findings in human cells have been inconsistent with p53 being a negative regulator of MGMT expression [7, 9]. A clear explanation for the specific role of IFN-β inducible p53 in *MGMT* gene expression in human glioma cells is critical. In this context, we have examined the role of TP53 in the human MGMT promoter in a luciferase reporter plasmid. We have demonstrated that MGMT promoter activity in a reporter gene system was suppressed by adenoviral vector-mediated wild-type p53 expression in Saos-2, a p53-null cell line, or by IFN-β mediated p53 overexpression in a glioma cell line, i.e., T98.

Materials and methods

Cell lines and reagents

We used two human glioma cell lines (T98 and U251SP), which were determined to be TMZ-resistant and TMZ-sensitive, respectively, in a previous study [18]. These cell lines were obtained from the American Tissue Culture Collection. Human IFN-β (Toray Co., Ltd., Kamakura, Japan) and TMZ (the Schering-Plough Research Institute, Kenilworth, NJ, USA) were resolved in phosphate-buffered saline (PBS) and dimethyl sulfoxide, respectively.

Animal experiments

Balb/c nude mice (female and 5–6 weeks old) bearing T98 or U251SP tumors were randomized to separate them into six (T98) or four (U251SP) groups (five to six animals per group) and treated when the subcutaneous tumors had reached a volume between 200 and 400 mm^3. IFN-β(2×10^5 IU/animal) was administered i.p. 6 h before an i.p. injection of TMZ (50 or 100 mg/kg). Control mice, or mice receiving IFN-β or TMZ alone, also received the corresponding vehicle. Treatments were repeated at 24-h intervals for a total of five doses. Tumor length (a) and width (b) were measured in situ with digital calipers at 7-day intervals, and tumor volumes were calculated according to the following formula: V (mm^3) = $a \times b^2/2$. The statistical significance between treated and control tumors was evaluated by a one-tailed Mann–Whitney test.

MGMT expression in tumors after treatment with IFN-β

Mice bearing T98 tumors of volume 200–400 mm^3 were randomized; divided into groups of three; and treated as described with PBS, TMZ (50 mg/kg) alone, and a combination of TMZ (50 mg/kg) and IFN-β (2×10^5 IU/animal). Treatments were repeated at 24-h intervals for a total of five doses. Seven days after the initial treatment, total RNA was immediately isolated from the removed tumor samples by using a standard Trizol preparation protocol (Invitrogen, Carlsbad, CA, USA). To investigate MGMT mRNA expression, semiquantitative RT-PCR was performed using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen), as previously described [18]. β-Actin-specific PCR products from the same RNA samples were amplified and used as internal controls.

Construction of a reporter plasmid

The 955-bp DNA fragment containing the human MGMT promoter was amplified from T98 genomic DNA by PCR amplification using the forward primer with the *Mlu*I site, 5′-cg*acgcgt*atcctgctccctctgaaggctc-3′ and the reverse primer with the *Bgl*II site, 5′-ga*agatctg*gacctgagaaaagcaagagag-3′.

The fragment was then subcloned into the pGL3-luciferase enhancer vector (Promega, Madison, WI, USA) through the *Mlu*I and *Bgl*II sites to generate the luciferase expression plasmid under the human MGMT promoter, i.e., p-952/+3 MGMT LUC. The corresponding construct was amplified with the primer sets RV primer3 (5′-ctagcaaaataggctgtccc-3′) and GL primer2 (5′-ctttatgtttttggcgtcttcc-3′) and sequenced using the BigDye Terminator Sequencing Kit (Applied Biosystems) in order to confirm the vector construct.

Assay for transiently expressed reporter gene

In the preliminary experiment, Saos2 (p53-null) osteosarcoma cells were plated in a 35-mm dish at a density of 2×10^5 cells/well. At 24 h, they were infected with the recombinant wild-type p53 or lacZ adenoviral vector under the control of the cytomegalovirus promoter (a gift from Dr. Hamada, Hokkaido, Japan) at multiplicity of infection (MOI) = 1. The MOI was necessary to infect 100% of the cells, and TP53 was successfully transduced in the cells, as determined by western blotting analysis. Cell lysis and western blotting were carried out as described previously [18]. Antibodies against the following proteins were purchased: p53 (DO-1: Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (AC-15: Sigma–Aldrich, St. Louis, MO, USA). In this study, Saos2 and T98 glioma cells were plated in a 35-mm dish at a density of 2×10^5 cells/well, respectively. At 24 h, the Saos2 cells were infected with the p53 adenoviral vector at MOI = 1, and the T98 cells were treated with 1,000 IU/ml of IFN-β. After an additional 24 h, Saos2 and T98 cells were transfected with the p-952/+3 MGMT LUC plasmid (1 μg) along with the β-galactosidase expression plasmid (0.5 μg) as the internal control; transfection was carried out by using FuGENE6 (Roche, Indianapolis, IN, USA) and Lipofectamine (Invitrogen), respectively. The cells were harvested 48 h after plasmid cotransfection. Luciferase and β-galactosidase activity were measured using kits from Promega and the protocols recommended by the manufacturer. Luciferase activity was normalized based on the β-galactosidase activity to correct for variations in tranfection efficiency. The assay was performed in triplicates.

Results

A combination of TMZ and IFN-β reduced the growth of the human glioma xenografts

As shown in Fig. 1, IFN-β alone did not decrease the growth of the T98 tumor significantly (Fig. 1, filled diamond). TMZ (100 mg/kg) alone suppressed T98 tumor growth (filled square, $P \leq 0.05$) but increased the associated body weight loss (Fig. 2); it produced toxicity-related deaths in two of seven animals. In contrast, a combination of IFN-β and 50 mg/kg TMZ decreased T98 tumor growth to a very significant extent (triangle, $P \leq 0.05$), and complete regression was observed in one of five animals (20%). A combination of IFN-β and 100 mg/kg TMZ reached more statistical significance (filled triangle, $P \leq 0.01$), however increased the body-weight loss. Semiquantitative RT-PCR demonstrated that MGMT expression decreased substantially in the T98 tumor 7 days after the combined treatment with TMZ and IFN-β (Fig. 3).

Similarly, for the U251SP (TMZ-sensitive) tumor, while a TMZ dose of 50 mg/kg alone reduced tumor growth, IFN-β preadministration marginally enhanced tumor growth delay ($P \leq 0.05$) (Fig. 4).

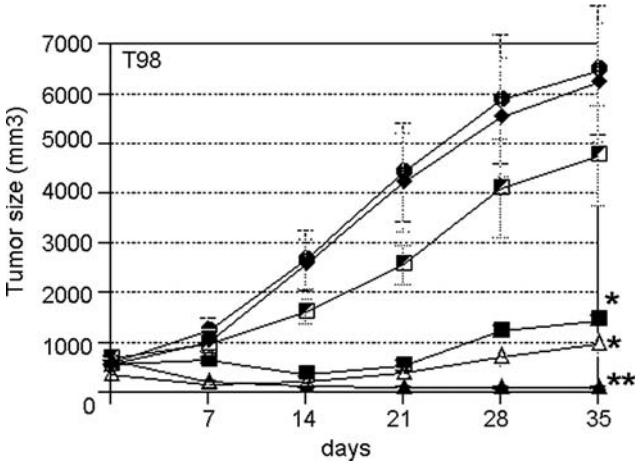

Fig. 1 Growth inhibition of T98 xenografts treated with IFN-$\beta \pm$ TMZ. BALB/c nude mice (female, 5–6 weeks old) bearing T98 were separated into six treatment groups; *filled circle* vehicle; *filled diamond* IFN-β (2×10^5 IU); *half filled square* TMZ (50 mg/kg); *filled square* TMZ (100 mg/kg); *triangle* IFN-β (2×10^5 IU) +TMZ (50 mg/kg); *filled triangle* IFN-β (2×10^5 IU) +TMZ (100 mg/kg). IFN-β was administered i.p. 6 h before an i.p. injection of TMZ. Control mice, or mice receiving IFN-β or TMZ alone, also received the corresponding vehicle. Treatments were repeated at 24-h intervals for a total of five doses. Tumor length (**a**) and width (**b**) were measured in situ with digital calipers at 7-day intervals, and tumor volumes were calculated according to the following formula: V (mm^3) = $a \times b^2/2$. Points represent mean values ± SE. The statistical significance between treated and control tumors was evaluated by a one-tailed Mann–Whitney test. *$P < 0.05$ compared with vehicle; **$P < 0.01$ compared with vehicle

IFN-β and TP53 expression inhibit MGMT promoter function

Saos-2 cells were used in this study because they are p53-null and therefore contain no endogenous p53 protein, wild type, or mutant, which might influence the transfection

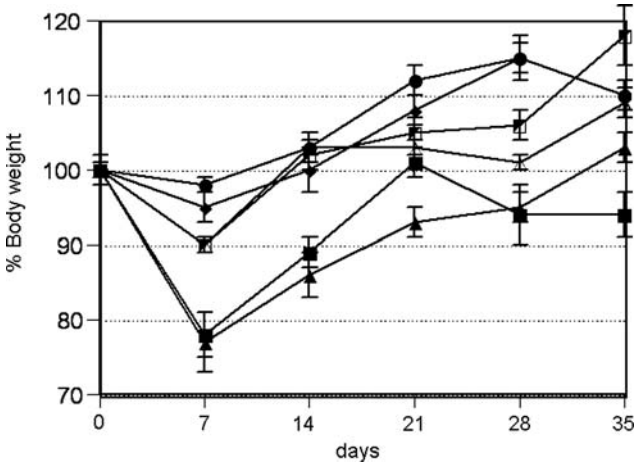

Fig. 2 Body weight of mice receiving IFN-$\beta \pm$ TMZ. The mean body weight of six treatment groups; *filled circle* vehicle; *filled diamond* IFN-β (2×105 IU); *half filled square* TMZ (50 mg/kg); *filled square* TMZ (100 mg/kg); *triangle* IFN-β (2×10^5 IU) +TMZ (50 mg/kg); *filled triangle* IFN-β (2×10^5 IU) +TMZ (100 mg/kg), was measured and expressed as a percentage of the day 0 untreated weight. The mice receiving TMZ (100 mg/kg) (*filled square* and *filled triangle*) had the associated body weight loss; toxicity-related deaths were found in four of 14 animals. Points represent mean values $\pm$ SE

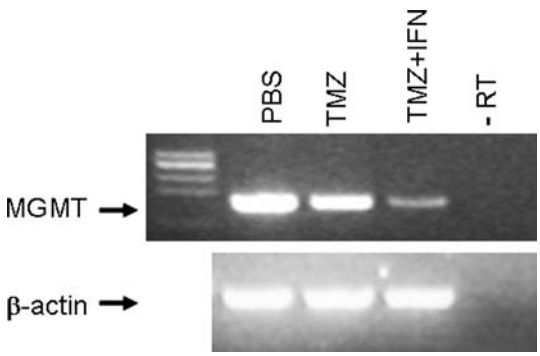

Fig. 3 Semi-quantitative RT-PCR for MGMT expression in T98 tumor. Mice bearing T98 tumors were treated with either PBS, TMZ (50 mg/kg) alone, or a combination of TMZ (50 mg/kg) and IFN-β (2×10^5 IU/animal). Treatments were repeated at 24-h intervals for a total of five doses. To investigate MGMT mRNA expression, 7 days after the initial treatment, semiquantitative RT-PCR was performed. β-Actin-specific PCR products from the same RNA samples were amplified and used as internal controls

result. To demonstrate that overexpression of wild-type p53 could affect MGMT promoter function, Saos-2 cells were transduced with a wild-type p53 adenovirus at MOI = 1. Overexpression of p53 was obtained 24 h after adenovirus-mediated transduction of p53 gene into Saos-2 cells (Fig. 5a, lower panel). Then, cells were transiently transfected with p-952/+3 MGMT LUC together with a control plasmid pSVβ-gal. Luciferase activity was normalized based on β-galactosidase activity to correct different transfection efficiencies. In the presence of wild-type p53, the

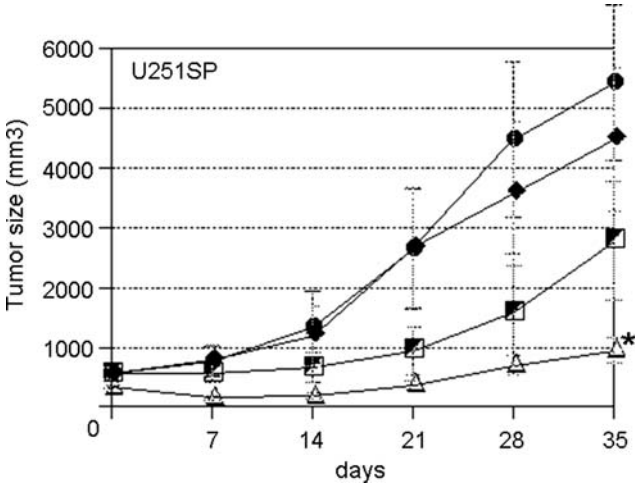

Fig. 4 Growth inhibition of U251SP xenografts treated with IFN-$\beta \pm$ TMZ. BALB/c nude mice (female, 5–6 weeks old) bearing U251SP were separated into four treatment groups; *filled circle* vehicle; *filled diamond* IFN-β (2×105 IU); *half filled square* TMZ (50 mg/kg); *triangle* IFN-β (2×10^5 IU) +TMZ (50 mg/kg). IFN-β was administered i.p. 6 h before an i.p. injection of TMZ. Control mice, or mice receiving IFN-β or TMZ alone, also received the corresponding vehicle. Treatments were repeated at 24-h intervals for a total of five doses. Points represent mean values $\pm$ SE. The statistical significance between treated and control tumors was evaluated by a one-tailed Mann–Whitney test. $^*P < 0.05$ compared with vehicle

MGMT promoter produced only 5% of the luciferase activity generated in the absence of p53 expression (Fig. 5a, upper panel).

IFN-β upregulated p53 expression in T98 glioma cells (Fig. 5b, lower panel). Previously, we have demonstrated that knockdown of p53 by siRNA increased MGMT expression in T98 cells, and the ChIP assay revealed that the MGMT promoter coprecipitated with p53 [18]. We analyzed whether the p53 induction caused by IFN-β curtailed the MGMT promoter function in T98 cells. T98 cells were treated with IFN-β and after 24 h, the cells were transfected with p-952/+3 MGMT LUC. Luciferase activity in T98 cells transfected with p-952/+3 MGMT LUC was dramatically decreased in the presence of IFN-β (Fig. 5b, upper panel).

Discussion

Previous studies have demonstrated that epigenetic silencing of the *MGMT* DNA repair gene by promoter methylation compromised DNA repair, and this was shown to be associated with longer survival in glioblastoma patients who received TMZ chemotherapy [4, 12]. The methylation status of the MGMT promoter may have prognostic value. Additionally, it may be a clinically relevant predictor of the beneficial effects of TMZ chemotherapy. Thus, MGMT

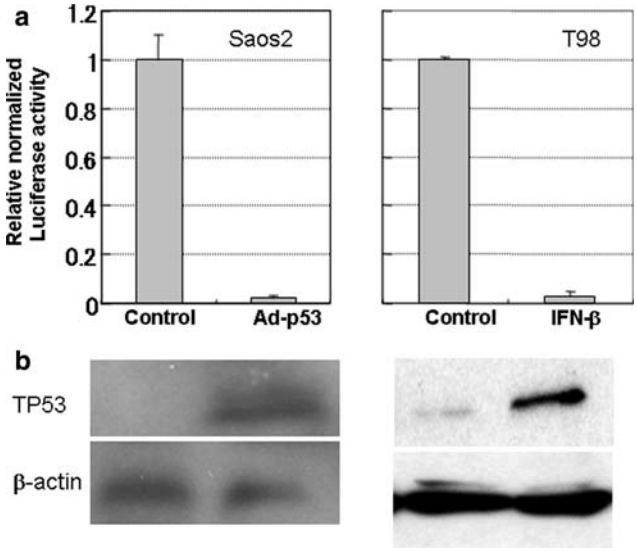

Fig. 5 Relative MGMT promoter luciferase activity. Saos2 (**a**) and T98 glioma (**b**) cells were plated in a 35-mm dish at a density of 2×10^5 cells/well, respectively. At 24 h, the Saos2 cells were infected with the p53 adenoviral vector at MOI = 1, and the T98 cells were treated with 1,000 IU/ml of IFN-β. Overexpression of TP53 was confirmed by immunoblotting, and β-actin was also immunoblotted as a loading control (*lower panels*). After an additional 24 h, Saos2 and T98 cells were transfected with the MGMT promoter-luciferase construct (1 μg) along with the β-galactosidase expression plasmid (0.5 μg) as the internal control. The cells were harvested 48 h after plasmid co-transfection. Luciferase activity was normalized based on the β-galactosidase activity to correct for variations in tranfection efficiency. The assay was performed in triplicates

promoter methylation may enable the selection of patients that are most likely to benefit from TMZ treatment.

Molecular studies have shown that specific tumor characteristics such as the presence of a silenced *MGMT* gene may allow the treatment of individual patients to be tailored. The role of MGMT in the resistance to alkylating chemotherapy has been shown; therefore, in several studies, an MGMT-depleting agent was added in order to increase efficacy. For this purpose, both drugs with intrinsic cytotoxic activity as well as those that deplete MGMT were used. O^6-Benzylguanine (O^6-BG) was developed based on its restricted mechanism of action [3]. O^6-BG reacts with MGMT by covalent transfer of the benzyl group to the active site cysteine and thereby causes irreversible inactivation of the enzyme. The first results of this strategy showed that adding O^6-BG to carmustine did not induce regression in carmustine-resistant gliomas, although a similar trial in TMZ-pretreated patients showed some evidence that addition of O^6-BG to TMZ was beneficial. At therapeutic levels, O^6-BG alone is not toxic, and one might expect that it could be advantageous if MGMT depletion can be accomplished by a drug that also has antitumor activity; therefore, synergistic effects with the alkylating agent may occur. Type I IFNs, including IFN-α and IFN-β, a family of cytokines that elicits pleiotropic biological effects, are widely used either alone or in combination with other antitumor agents such as nitrosoureas in the treatment of malignant gliomas. Among the multiple functions of type I IFNs against human neoplasias, IFN-β, in particular, can act as a drug sensitizer that enhances the toxicity against a variety of neoplasias when administered in combination with nitrosoureas [10]. Consistent with the results of in vitro studies [18], the present study indicates that administration of IFN-β 6 h prior to TMZ for 5 consecutive days has a significant synergistic antitumor activity on the growth of both T98 (TMZ-resistant) and U251SP (TMZ-sensitive) tumors in a xenograft model. Intensive TMZ treatment (100 mg/kg) alone suppressed the ever-resistant T98 tumor growth but increased the associated body weight loss and produced toxicity-related deaths in two of the seven animals. In contrast, a combination of IFN-β and a 50 mg/kg dose of TMZ decreased T98 tumor growth to a very significant extent, and complete regression was observed in one of five animals (20%). Additionally, while TMZ (50 mg/kg) alone suppressed TMZ-sensitive U251SP tumor growth, the two drugs in combination enhanced antitumor activity significantly. This study suggests that prior administration of IFN-β can lead to an increase in the therapeutic index of TMZ. RT-PCR analysis revealed a decrease in MGMT expression in a tumor xenograft treated with an IFN-β/TMZ combination; this observation was consistent with the results of a previous study [18].

With regard to the mechanism of action, we have speculated that IFN-β suppresses MGMT transcription via TP53 induction. Previous studies using murine cells [6, 19, 22] and human cells [7, 9] in which different experimental approaches were used have yielded confusing results on the possibility that MGMT may be regulated by p53. Our study sought to clarify the direct and specific effects of p53 on *MGMT* gene expression in human glioma cells. Our observations clearly show that p53 downregulates the transcription of the *MGMT* gene, and may have clinical relevance. Our studies agree with the initial observations of Harris et al. and Srivenugopal et al. who showed suppression of *MGMT* gene expression in IMR human fibroblasts after infection with an adenoviral p53 construct [9] and in p53-null H1299 human lung cancer cells engineered to express p53 in a tetracycline-regulated system [24]. It appears that modest to high level induction of p53 expression by either infection with an adenoviral construct, regulated gene expression, or treatment with a drug such as IFN-β (this study) is capable of inhibiting MGMT expression. The MGMT gene promoter lacks the TATA and CAAT boxes and has 10 Sp1 transcription factor binding sites [8]. There is growing evidence that TATA-less promoters are subject to p53 repression [14], and the *MGMT* gene may belong to this category. Alternatively, a variety of mechanisms

involving protein–protein interactions between p53 and the TATA-binding protein [23], p300/CBP transcriptional coactivator/histone deacetylation machinery [17], and Sp1 transcription factor have been proposed to explain p53-mediated transcriptional repression. It is possible that p53 could operate through one or more of these mechanisms to curtail the transcription of the *MGMT* gene. Since MGMT expression occurs in approximately 70% of gliomas, the present study highlights the important role of p53 in *MGMT* gene expression.

In summary, it would be attractive to inhibit MGMT in cases with MGMT expression. In this context, IFN-β inactivates MGMT via *p53* gene induction and enhances the therapeutic efficacy to TMZ. Clinical examination of an IFN-β/TMZ combination may therefore be warranted.

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