O^6-*Methylguanine-DNA Methyltransferase* Regulation by p53 in Astrocytic Cells

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Abstract

Methylation of the *O*^{6}*-methylguanine-DNA methyltransferase* (*MGMT*) gene promoter (i.e., gene silencing) occurs in 40% to 50% of patients with glioblastoma and predicts benefit from temozolomide chemotherapy; when unmethylated, MGMT repairs DNA damage induced by temozolomide, contributing to chemoresistance. In this study, we tested the hypothesis that *MGMT* is regulated by p53 in astrocytic cells, the precursors of which may give rise to glioblastoma. p53 is of interest because, in addition to often being mutated in glioblastoma, inactivation sensitizes some astrocytoma cell lines to temozolomide. *MGMT* expression was examined in neonatal murine astrocytes and SF767 human astrocytic glioma cells following *p53* inactivation by knockout (murine only) or RNAi methods. *MGMT* mRNA and protein were detected in murine wild-type *p53* astrocytes. However, in knockout murine astrocytes and wild-type cells in which p53 was inhibited by RNAi, *MGMT* expression was reduced by >90%. This effect of p53 on *MGMT* expression was unrelated to *MGMT* promoter methylation—in both wild-type and *p53*-null astrocytes, the *MGMT* promoter was unmethylated. In wild-type astrocytes, the p53 protein localized to a regulatory region of the *MGMT* promoter. In SF767 human astrocytic glioma cells, transient knockdown of *p53* led to the down-regulation of *MGMT* gene expression. In murine astrocytes and SF767 cells, p53 regulates *MGMT* expression without affecting promoter methylation; in astrocytes, this effect may be due to direct binding of p53 to the *MGMT* promoter. These results imply that the best use of temozolomide requires a thorough understanding of *MGMT* regulation. [Cancer Res 2007;67(2):580–4]

Introduction

Progress in the treatment of glioblastoma, an aggressive cancer of the brain, has been slow, with only a handful of patients surviving 2 years. A significant insight and therapeutic advance in the management of glioblastoma occurred recently, however, when Stupp et al., observed that the addition of temozolomide chemotherapy to radiotherapy in newly diagnosed cases significantly prolonged tumor control and patient survival (1). Subsequently, by analyzing glioblastoma tissues from study patients, Hegi et al., found that the survival advantage conferred by temozolomide chemotherapy was associated with methylation of the promoter region of the DNA repair gene *O*^{6}*-methylguanine DNA methyltransferase* (*MGMT*; ref. 2). MGMT repairs DNA damaged by temozolomide, thereby contributing to drug resistance. As such, *MGMT* methylation status, an indicator of gene silencing, emerges as a potentially important molecular test to determine which patients with glioblastoma will benefit from, and should receive, temozolomide.

From Hegi et al., an important clinical question arises: will a diagnostic test of *MGMT* promoter methylation be sufficient to identify all patients who will benefit from treatment with temozolomide? To answer this question with certainty, new knowledge will be needed: why is a ubiquitous DNA repair protein like *MGMT* silenced in some glioblastomas? Which molecules regulate MGMT promoter methylation and expression in glioblastomas? Is methylation of the promoter the primary mechanism of *MGMT* down-regulation in glioblastomas? In Hegi et al., *MGMT* promoter methylation was detected in 45% of glioblastomas (2). In this regard, the tumor suppressor p53, a transcriptional regulator mutated in 30% to 50% of glioblastomas (3), may be of interest. Inactivation of *p53* has been reported to enhance the sensitivity of some human astrocytoma cell lines to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide (4), drugs which alkylate *O*^{6}-guanine, the site of repair by MGMT. Here, we provide evidence that p53 regulates *MGMT* expression in murine astrocytes, and present data suggesting that p53 may also contribute to the regulation of *MGMT* gene expression in the human astrocytic glioma cell line, SF767.

Materials and Methods

Cell culture. Cultures of wild-type and knockout *p53* astrocytes were prepared from the cerebrums of newborn C57bl/6 mice (The Jackson Laboratory, Bar Harbor, ME) with known *p53* genotypes using a method adapted from McCarthy and de Vellis (5). Briefly, the cerebral hemispheres were isolated from unanesthetized neonates, dissected free of meninges and blood vessels, and a single cell suspension prepared by enzymatic treatment (0.025% trypsin; Invitrogen, Carlsbad, CA) and mechanical dissociation. Astrocyte cultures were grown in DMEM containing 10% heat-inactivated fetal bovine serum and 50 units/mL penicillin and streptomycin. The status of the *p53* gene was assessed by PCR using genomic DNA isolated from mouse tails, following the protocol provided by the supplier. Astrocytes with identical genotypes were pooled from littermates (two to six mice) for each independent experiment. SF767 cells, generously provided by Dr. Mitchell Berger (Brain Tumor Research Center, University of California, San Francisco, San Francisco, CA), were grown in MEM (Sigma, St. Louis, MO) supplemented with 10% FCS.

Fluorescent microscopy. Astrocytes were grown on coverslips for immunostaining. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and treated with blocking solution (2% goat serum, 0.4% Triton X-100 in PBS) for 10 min. The coverslips were rinsed (0.2% Triton X-100, 0.2% bovine serum albumin in PBS) and exposed to anti–glial fibrillary acidic protein (GFAP) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in rinsing solution for 2 h. Subsequently, the coverslips were re-rinsed in PBS, exposed to FITC-conjugated goat anti-mouse

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secondary antibody (Santa Cruz Biotechnology) for 1 h, and visualized with a fluorescent microscope (Carl Zeiss Canada, Toronto, ON).

Reverse transcription-PCR. After washing twice with ice-cold PBS, 1 mL of Trizol (Life Technologies, Carlsbad, CA) reagent was added to each culture plate. Samples were removed by scraping and stored at −70°C. RNA extraction was done following the Trizol protocol as described by the manufacturer. PowerScript Reverse Transcriptase (ClonTech, Mountain View, CA) was used for reverse transcription as instructed by the manufacturer. PCR was done using Qiagen Hotstart Taq (Qiagen, Valencia, CA) also as instructed. Bands were quantitated using Quantity One software (Bio-Rad, Richmond, CA), readily available on the Internet. The primers used for PCR and MS-PCR were as follows:

Murine *p53* forward 5′-CCTCACTGCATGGACGATCTGTTG-3′,
Murine *p53* reverse 5′-GTGGATGGTGGTATACTCAGAGCC-3′,
Murine *MGMT* forward 5′-GGTGTTATGGAAGCTGCTGA-3′,
Murine *MGMT* reverse 5′-CGACTCGAAGGATGACTTGA-3′,
Murine *β-2-microglobulin* forward 5′-ATGGGAAGCCGAACATACTG-3′,
Murine *β-2-microglobulin* reverse 5′-GAAAGACCAGTCCTTGCTGA-3′,
Murine *MGMT* unmethylated forward 5′-TTTGGTAGTTTTTAGAGTTATGTTTTGTGT-3′,
Murine *MGMT* unmethylated reverse 5′-CCACAAACACATACACAAAATAAAAACAAA-3′,
Murine *MGMT* methylated forward 5′-GGTAGTTTTTAGAGTTACGTTTCGCGT-3′,
Murine *MGMT* methylated reverse 5′-CAAACGCGTACACGAAATAAAAACGAAA-3′,
Human *p53* forward 5′-ACATCTGGCCTTGAAACCAC-3′,
Human *p53* reverse 5′-CGAGACCCAGTCTCAAAGAA-3′,
Human *MGMT* forward 5′-GTCGTTCACCAGACAGGTGTTA-3′,
Human *MGMT* reverse 5′-ACAGGATTGCCTCTCATTGCTC-3′,
Human *MGMT* unmethylated forward 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′,
Human *MGMT* unmethylated reverse 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′,
Human *MGMT* methylated forward 5′-TTTCGACGTTCGTAGGTTTTCGC-3′,
Human *MGMT* methylated reverse 5′-GCACTCTTCCGAAAACGAAACG-3′,
Human *β-2-microglobulin* forward 5′-GCTATCCAGCGTACTCCAAA-3′,
Human *β-2-microglobulin* reverse 5′-GATGGATGAAACCCAGACAC-3′.

Western blotting. Packed cells were resuspended in an equal volume of radioimmunoprecipitation assay lysis buffer (50 mmol/L Tris, 10% SDS, 150 mmol/L NaCl, 12 mmol/L sodium deoxycholate, 50 mmol/L NaF, 1% Triton X-100, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 1 μg/mL aprotinin, and 1 μg/mL pepstatin). The suspension was placed on ice for 15 min, vortexed for 10 s and spun-down for 10 min at 12,000 rpm at 4°C. The supernatant was removed and the protein concentration determined using the Bio-Rad Protein Assay kit (specifications from Bio-Rad). Lysates were supplemented with 500 mmol/L of DTT and protein sample buffer (Invitrogen) and heated to 70°C for 10 min. Samples were loaded and run on precast 4% to 12% gradient SDS-PAGE gels (Nupage, Invitrogen), and transferred to nitrocellulose membranes in a semidry gel transfer module (Nupage, Invitrogen). Hybridization to antibodies was done, and signals visualized using an enhanced chemiluminescence kit (Amersham, Piscataway, NJ). The antibody to MGMT (FL-207), the secondary goat anti-rabbit (G1305), and anti-actin (C2) were all obtained from Santa Cruz Biotechnology.

Gene knockdown. Predesigned RNAi specific for murine and human *p53* were obtained from Invitrogen. Control oligos and the transfection reagent, HiPerFect, were purchased from Qiagen. Manufacturer's instructions were followed to transiently inhibit *p53* in murine astrocytes and SF767 cells. Transfections were carried out immediately after plating cells, and the expression of *p53* and *MGMT* were analyzed 2 days later. Transfection efficiency was monitored using FITC-labeled RNA duplex (Qiagen). Reverse transcription-PCR (RT-PCR) was done to examine *MGMT* and *β-2M* expression.

***MGMT* promoter analysis.** The *MGMT* promoter sequence (NCBI accession no. S82865) was obtained from the National Center for Biotechnology Information and searched for the p53 consensus site 5′-PuPuPuC(A/T)-(T/A)GPyPyPy-3′ using DNA analysis software.[1]

Bisulfite modification. A methylation-specific PCR procedure similar to Herman et al., was used (6). Briefly, DNA was isolated using the DNeasy kit (Qiagen). Two micrograms of DNA in 20 μL of water was denatured in NaOH (0.2 mol/L final concentration) for 15 min at 37°C. Following denaturation, 30 μL of 10 mmol/L hydroquinone (Sigma) and 520 μL of 3 mol/L sodium bisulfite (Sigma) were added and incubated overnight at 55°C in the dark. Modified DNA was then purified using the Wizard DNA Purification kit (Promega, Madison, WI) and eluted in 20 μL of water. Modification was completed by treatment with NaOH (final concentration, 0.3 mol/L) at 37°C for 15 min, followed by ethanol precipitation. DNA was resuspended in water and PCR was done as described above.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (CHIP) assay was done as instructed by the manufacturer (Upstate, Charlottesville, VA). In brief, protein/DNA complexes were crosslinked in astrocytes with formaldehyde, followed by sonication to shear DNA. Immunoprecipitation was done using the p53 antibody FL393 (Santa Cruz Biotechnology), or normal mouse IgG as a control (Upstate). Protein/DNA complexes were separated and purified. Analysis of p53-bound sequences ensued by PCR to determine if the *MGMT* promoter had precipitated with p53. As a control for nonspecific p53-DNA interactions, PCR primers were designed for a sequence of the *MGMT* gene 5,000 bp downstream of the *MGMT* promoter. The primers used for the PCR component of the assay were:

MGMT promoter forward 5′-GCTCAGGCACCTAAAACTTG-3′,
MGMT promoter reverse 5′-GAGGTCCTAGAGAAAGGAAGGA-3′,
Control forward 5′-AAGTCCAGTTTCCAGGACCA-3′,
Control reverse 5′-GCCTTTGAGAAGTGATTGGA-3′.

Results

Astrocytes were isolated from the cerebral cortices of 3-day-old wild-type and knockout *p53* C57bl/6 pups and cultured for 96 h as described. Virtually 100% of these brain-derived cells expressed GFAP, affirming their astrocytic nature (Fig. 1). From these astrocyte-enriched cultures, RNA was harvested and RT-PCR done to assess *MGMT* gene expression. We observed that *MGMT* gene expression was substantially lower in knockout *p53* astrocytes than in wild-type *p53* astrocytes (Fig. 2*A*). To confirm an interaction at the protein level, extracts were prepared from knockout and wild-type *p53* astrocytes and the levels of MGMT protein were examined. As seen in Fig. 2*B*, MGMT protein was detected in extracts from wild-type *p53* astrocytes but was not readily discernible from knockout *p53* astrocytes, correlating with the RT-PCR data. To ascertain whether the *p53*-null state was a major contributor to the reduction in *MGMT* expression, we quantitated the differences in *MGMT* gene expression in knockout compared with wild-type astrocytes, and noted that an absence of *p53* expression was associated with a >90% reduction in *MGMT* gene expression (data not shown). To confirm that down-regulation of *MGMT* expression in the absence of *p53* was not simply an idiosyncrasy of genetically modified mice, *p53* expression was transiently inhibited in wild-type astrocytes by RNAi. We observed that successful inhibition of *p53* using a mixture of three different RNAi duplexes specific for *p53* (Fig. 3, RNAi) substantially reduced *MGMT* gene expression. The specificity of this effect on *MGMT*

[1] http://rsat.ulb.ac.be/rsat/.

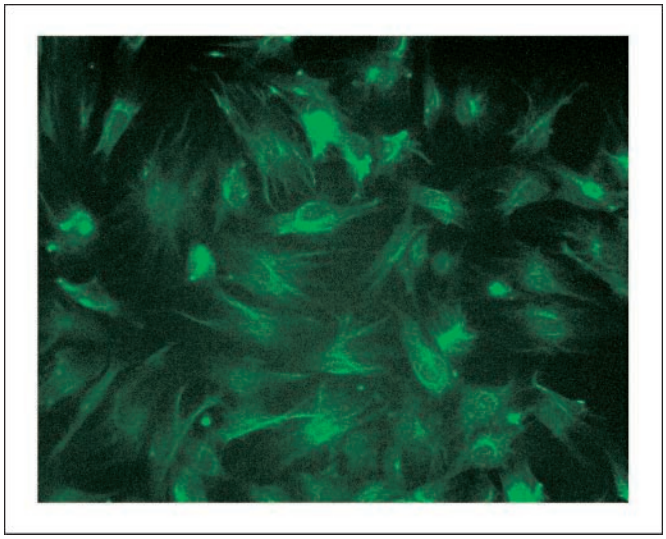

Figure 1. Murine astrocytes harvested from the cerebral hemispheres and stained for GFAP.

expression was confirmed using a random duplex (CNTRL), as well as an unrelated RNAi duplex (data not shown). Taken together, these findings further support the conclusion that p53 positively regulates *MGMT* gene expression in murine astrocytes.

In human glioblastomas, the expression of *MGMT* seems to be regulated by promoter methylation, an epigenetic modification that prevents transcription factor binding and gene expression (7). Accordingly, we tested the supplementary hypothesis that p53 regulates *MGMT* expression in murine astrocytes by controlling the methylation status of the *MGMT* gene promoter. As shown in Fig. 4*A*, we found no relationship between *p53* genotype and *MGMT* promoter methylation; the *MGMT* promoter was unmethylated in both wild-type and knockout *p53* astrocytes. This finding indicated that p53 regulated *MGMT* gene expression in murine

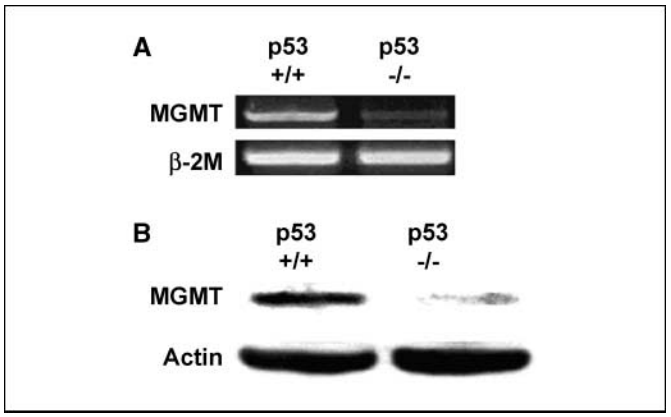

Figure 2. *A, MGMT* gene expression in wild-type and knockout *p53* murine astrocytes. RNA was isolated from wild-type and knockout astrocytes and RT-PCR was done to assess *MGMT* gene expression. *MGMT* expression was significantly higher in wild-type than in knockout *p53* astrocytes. Murine *β2-microglobulin* was used to control for the PCR reaction. *B,* MGMT protein expression in wild-type and knockout *p53* murine astrocytes. Protein extracts were prepared from wild-type and knockout astrocytes and expression of MGMT was analyzed by Western blot. Actin was used as a loading control. MGMT protein levels were higher in wild-type than in knockout *p53* astrocytes.

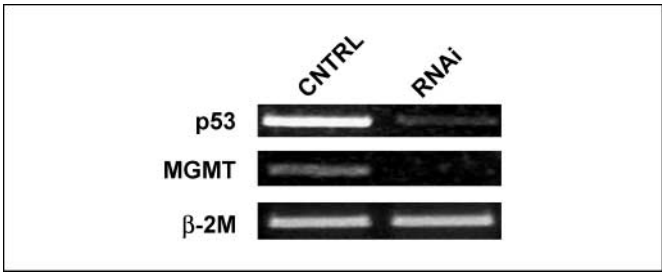

Figure 3. RT-PCR to examine the effect of *p53* knockdown on *MGMT* expression in wild-type *p53* astrocytes. Cells were treated with a control oligo (*CNTRL*) or RNAi specific for *p53* (*RNAi*). *p53* knockdown was successful and accompanied by a significant reduction in *MGMT* gene expression. Murine *β2-microglobulin* was used to control for the PCR reaction.

astrocytes in a manner unrelated to promoter methylation and led us to explore a direct mechanism of interaction. We searched the murine *MGMT* promoter for the p53 consensus site 5′-PuPuPuC(A/T)-(T/A)GPyPyPy-3′. This search yielded a previously unidentified perfect p53 binding site, consisting of two half sites separated by 11 bases in the first intron of the murine *MGMT* gene (Fig. 4*B*). Aware that Iwakuma et al., had reported a regulatory region for an E2F-like enhancer element for *MGMT* expression in this region (8), we used the CHIP assay to explore the possibility that p53 interacted with this DNA segment. As seen in Fig. 4*C*, the p53 protein was detected at this *MGMT* regulatory region.

To determine if regulation of *MGMT* expression by p53 occurred in human cells, we searched the literature for a glioma cell line expressing both functional wild-type *p53* and *MGMT*. We found

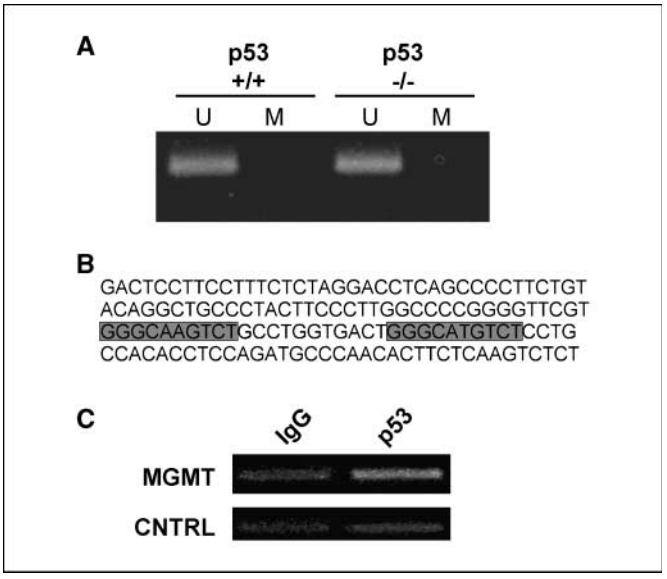

Figure 4. *A, MGMT* promoter methylation analysis in wild-type and knockout *p53* murine astrocytes. DNA was extracted from wild-type and knockout *p53* astrocytes. After bisulfite modification, methylation-specific PCR for the *MGMT* promoter was done. In both wild-type and knockout *p53* astrocytes, the *MGMT* promoter is unmethylated. Unmethylated *MGMT* promoter (*U*), methylated *MGMT* promoter (*M*). *B, MGMT* promoter examined for a p53 consensus site. Two previously unidentified p53 half sites are highlighted. *C,* CHIP assay to examine the interaction of p53 with the *MGMT* promoter. IgG controlled for the immunoprecipitation component of the assay and a region of DNA ~5,000 bp downstream of the *MGMT* promoter was controlled for nonspecific p53-DNA interactions (*CNTRL*). p53 was detected at the first intron of the *MGMT* gene promoter.

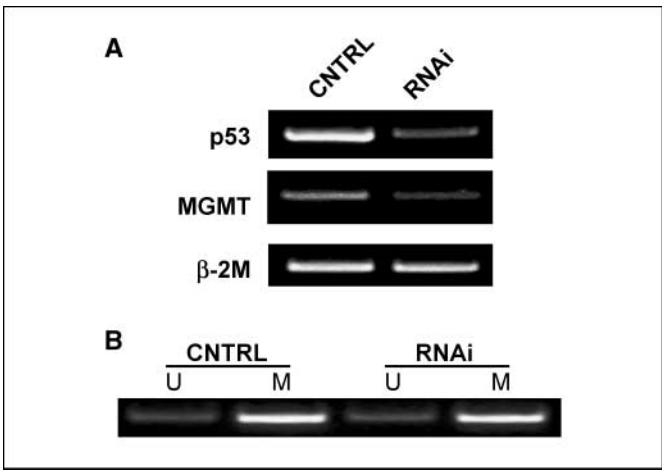

Figure 5. *A,* RNAi knockdown of *p53* in SF767 human astrocytic glioma cells. Cells were treated with RNAi specific for *p53* and RT-PCR was done to examine *MGMT* gene expression. Cells in which *p53* expression was inhibited (*RNAi*) expressed lower levels of *MGMT* than cells treated with a control duplex (*CNTRL*). *B, MGMT* promoter methylation analysis of SF767 cells. After *p53* knockdown, bisulfite modification and methylation-specific PCR were done. The methylation status of the *MGMT* promoter in SF767 cells was unaffected by knockdown of *p53*. Unmethylated *MGMT* promoter (*U*), methylated *MGMT* promoter (*M*).

only one human astrocytic glioma cell line, designated SF767, that simultaneously expresses both genes (9–14). Using SF767 cells, we transiently inhibited *p53* expression by applying a mixture of two RNAi duplexes. As shown in Fig. 5*A*, inhibition of *p53* by RNAi was accompanied by down-regulation of *MGMT* gene expression; the average decrease in *MGMT* expression in three independent experiments was 43% (P = 0.0199). SF767 cultures seemed to contain a mixed population of glioma cells; in some, the *MGMT* promoter was methylated, whereas in others, the promoter was unmethylated (Fig. 5*B*). Furthermore, down-regulation of *MGMT* expression accompanying *p53* inhibition by RNAi was not associated with a discernible change in the pattern of *MGMT* promoter methylation (Fig. 5*B*). Although additional experiments are required to fully ascertain the functional significance of *MGMT* down-regulation, early results suggest that *p53* inhibition also sensitizes SF767 cells to temozolomide (data not shown). Moreover, we have identified several potential p53 binding sites in the human *MGMT* gene promoter.

Discussion

Many effective cancer chemotherapeutics alkylate DNA. Although a variety of DNA adducts are generated by such drugs, O^6-methylguanine (O^6MeG) is a commonly acquired and clinically important lesion. The addition of a methyl group to O^6-guanine leads to interstrand cross-linking, faulty DNA replication, and cancer cell death. O^6MeG lesions induced by chemotherapeutics can be repaired, however. Repair of O^6MeG is mediated by the suicide protein MGMT (15). MGMT restores the structural integrity of DNA by irreversibly transferring methyl adducts to an internal cysteine residue; thereafter, MGMT is degraded by the proteosome. Thus, in the context of cancer treatment, DNA repair by MGMT emerges as a potentially significant determinant of the effectiveness of certain chemotherapies. Indeed, the role of MGMT in resistance to chemotherapy is increasingly apparent. For example, among patients with high-grade astrocytomas (i.e., glioblastoma), the duration of tumor control and patient survival after BCNU or temozolomide are highly associated with evidence of MGMT activity: both MGMT immunonegativity and *MGMT* promoter methylation predict good tumor control and long survival, whereas active expression of *MGMT* is associated with early tumor progression and short survival time (2). Yet to be explained, however, is the dichotomous expression of *MGMT* in histologically indistinguishable glioblastomas. Seeking to clarify this issue, we studied the influence of p53 function on *MGMT* expression in astrocytes.

We reasoned that p53 might be a regulator of *MGMT* expression because *p53* inactivation has been reported to sensitize astrocytes and astrocytoma cells to BCNU and temozolomide (16, 17) and because *p53* mutations and *MGMT* promoter methylation each occur in ~40% of newly diagnosed glioblastomas. Concerned that glioma cell lines could harbor extraneous genetic abnormalities complicating the analysis, we first studied the association of p53 and *MGMT* in normal astrocytes cultured from the cerebral hemispheres of neonatal mice. In knockout *p53* astrocytes and in wild-type astrocytes in which *p53* has been transiently inhibited, *MGMT* expression is reduced. Quantitative analysis showed a >90% reduction in *MGMT* expression in knockout versus wild-type cells, suggesting that p53 is a major contributor to *MGMT* expression in murine astrocytes. These findings are consistent with the earlier observation by Nutt et al. (17) that MGMT activity is significantly decreased and sensitivity to BCNU is significantly increased in *p53*-null murine astrocytes compared with wild-type *p53* astrocytes.

We found no evidence that p53 regulates *MGMT* expression in murine astrocytes via an effect on *MGMT* promoter methylation, but discovered a p53 binding site in the murine *MGMT* promoter. We also observed a direct interaction between the p53 protein and the *MGMT* promoter. Although there may be promoter-independent mechanisms by which p53 regulates *MGMT* in murine astrocytes, our data suggest that p53 directly regulates *MGMT* expression. Indeed, p53 is known to interact with basal transcription machinery (18–20) to regulate the expression of many genes containing a p53 consensus site. Although only one consensus site was found in the murine *MGMT* promoter, *GADD45*, a p53 regulated gene, contains a single consensus site (21, 22), as do *transforming growth factor-α* (23) and *14-3-3σ* (24). Hence, a single consensus site is sufficient for p53-mediated transactivation. Furthermore, using less stringent search criteria (i.e., permitting base substitutions in the consensus sequence), we found multiple potential p53 binding sites in the *MGMT* promoter.

Determining whether p53 also plays a role in regulating the expression of *MGMT* in human tumors of astrocytic derivation is more challenging because there are few human experimental systems in which to examine the interaction and because correlative studies using human glioblastoma tissues are prone to a degree of uncertainty due to confounding variables. Nevertheless, there are data supporting a role for p53 regulation of *MGMT* expression in human gliomas. Russel et al., used an alkyltransferase activity assay to monitor the activity of MGMT in human tumor xenografts of anaplastic astrocytomas, glioblastomas, and medulloblastomas (13). They found that the average level of MGMT activity in tumors containing mutant p53 was 198 fmol/mg protein compared with 697 fmol/mg protein in samples containing wild-type p53. Anker et al., found that glioblastomas harboring mutant p53 expressed lower levels of MGMT than tumors containing wild-type p53 (25). Yuan et al., in an immunohistochemical analysis of 35 astrocytomas, noted that expression of mutant p53 correlated with

MGMT immunonegativity; the presence of mutant p53 and MGMT were mutually exclusive (26). Here, we have shown that disruption of *p53* in SF767 cells significantly decreases *MGMT* expression without affecting promoter methylation. Together, these observations support a relationship between p53 and *MGMT* expression in glioblastomas and encourage further study.

Whether p53 contributes to *MGMT* regulation in astrocyte precursors or neural stem cells, the likely cells of origin of human glioblastomas, or whether our data are relevant to the treatment of human glioblastomas, where inactivating mutations of p53 and silencing of *MGMT* are common, remain unanswered. Nevertheless, the demonstration that p53 modulates *MGMT* expression in murine astrocytes and the human astrocytic glioma cell line, SF767, raises the possibility that p53 function, or the p53 pathway, may be another determinant of MGMT activity. This observation potentially sheds light on long-surviving glioblastoma cases that tested negative for *MGMT* promoter methylation in the analysis done by Hegi et al. (2). Clearly, knowing all the mechanisms of *MGMT* regulation in gliomas will be critical to making the very best chemotherapy treatment decisions for patients with glioblastoma.

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