IFN-β Down-Regulates the Expression of DNA Repair Gene *MGMT* and Sensitizes Resistant Glioma Cells to Temozolomide

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Abstract

Alkylating agents, such as temozolomide, are among the most effective cytotoxic agents used for malignant gliomas, but responses remain very poor. The DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) plays an important role in cellular resistance to alkylating agents. IFN-β can act as a drug sensitizer, enhancing toxicity against a variety of neoplasias, and is widely used in combination with other antitumor agents such as nitrosoureas. Here, we show that IFN-β sensitizes glioma cells that harbor the unmethylated MGMT promoter and are resistant to temozolomide. By means of oligonucleotide microarray and RNA interference, we reveal that the sensitizing effect of IFN-β was possibly due to attenuation of MGMT expression via induction of the protein p53. Our study suggests that clinical efficacy of temozolomide might be improved by combination with IFN-β using appropriate doses and schedules of administration. (Cancer Res 2005; 65(17): 7573-9)

Introduction

Gliomas are the most common primary tumors of the central nervous system; they account for 30% of adult primary brain tumors. The prognosis for patients with the advanced glioma, glioblastoma multiforme, is very poor; the mean survival period is 8 to 10 months (1). Alkylating agents are among the most effective cytotoxic agents used for treating malignant gliomas, including glioblastoma multiforme, but responses remain very poor. The most frequent site of alkylation in DNA is the O^6 position of guanine, which forms cross-links between adjacent strands of DNA, leading to cell death. A cellular DNA-repair protein, namely O^6-methylguanine-DNA methyltransferase (MGMT) protein, reverses alkylation at the O^6 position of guanine, thereby inhibiting the lethal cross-linking and bringing about resistance to alkylating agents (2, 3). A number of studies have suggested that MGMT deficiency is closely related to the sensitivity of brain tumors to alkylating agents (4–6). Furthermore, because MGMT protein loss may be a result of promoter hypermethylation, it was reported that methylation of the MGMT promoter in gliomas is a useful predictor of the responsiveness to alkylating agents (7). Temozolomide is a novel alkylating agent that has been currently approved for use in treatment of anaplastic astrocytoma in Europe and the United States. A phase II clinical trial has been organized in Japan. This drug is of significance because it can be administered orally; it readily crosses the blood-brain barrier and has minimal side effects (8). Before considering the treatment of malignant gliomas with temozolomide, a major obstacle may be the resistance pathway that occurs due to the actions of MGMT. Hence, efficient attenuation of the function of MGMT, which is expressed in ~70% of gliomas (9), either by direct interaction with protein or by indirect means such as the transcriptional control, is required. It could be advantageous if MGMT depletion can be accomplished by a drug that also has antitumor activity, and, therefore, synergistic effects with the alkylating agent may occur. Type I IFNs, including IFN-α and IFN-β, a family of cytokines that elicit 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, type I IFNs, particularly IFN-β, can act as a drug sensitizer enhancing toxicity against a variety of neoplasias when given in combination with nitrosoureas (10). Therefore, it is of interest to examine whether IFN-β can enhance chemosensitivity of malignant gliomas against temozolomide, the new alkylkating agent; to evaluate the mechanism; and to provide an experimental basis for the rational clinical use of such combinations.

Materials and Methods

Cell lines and reagents. Human glioma cell lines (T98, AO2, SKMG1, U251nu/nu, U251SP, and U251MG) derived from the Memorial Sloan-Kettering Cancer Institute (New York, NY) were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air in Eagle's medium (Nissui, Tokyo, Japan). Medium was supplemented with 10% fetal bovine serum (FBS), 5 mmol/L L-glutamine, 2 mmol/L nonessential amino acids, and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). Human IFN-β and temozolomide were kindly supplied by Toray, Co., Ltd. (Kamakura, Japan), and the Schering-Plough Research Institute (Kenilworth, NJ), respectively. Temozolomide readily discomposes in aqueous solution into DMSO.

RNA interference experiments. The target sequence in p53 and the control nonsilencing sequence are CGGCAUGAACCGGAGGCCCAU and AATTCTCCGAACGTGTCACGT, respectively. These synthetic sense and antisense oligonucleotides were obtained from Qiagen (Hilden, Germany). For the annealing of small interfering RNA (siRNA) oligonucleotides, sense and antisense oligonucleotides were incubated in siRNA Suspension Buffer (Qiagen) for 1 minute at 90°C, followed by 60 minutes at 37°C. siRNA oligonucleotides were mixed with Oligofectamine reagent (Invitrogen, Carlsbad, CA) in Opti-MEM (Life Technologies, Gaithersburg, MD). Cultured cells were washed with medium without serum and added to the siRNA-Oligofectamine mixture, of which the final concentration was 200 nmol/L. Medium with 10% FBS was added 4 hours later.

Determination of cell growth. To compare chemosensitivity of glioma cell lines to temozolomide, the agent (final concentration of 0-1,000 μmol/L) was added to the culture medium at 24 hours after aliquots of 2×10^4 cells/well were placed in triplicate wells. Incubation was continued for 72 hours, and the number of viable cells was counted by trypan blue exclusion method. The number was expressed as a percentage of untreated control. The statistical significance of difference was determined by ANOVA using

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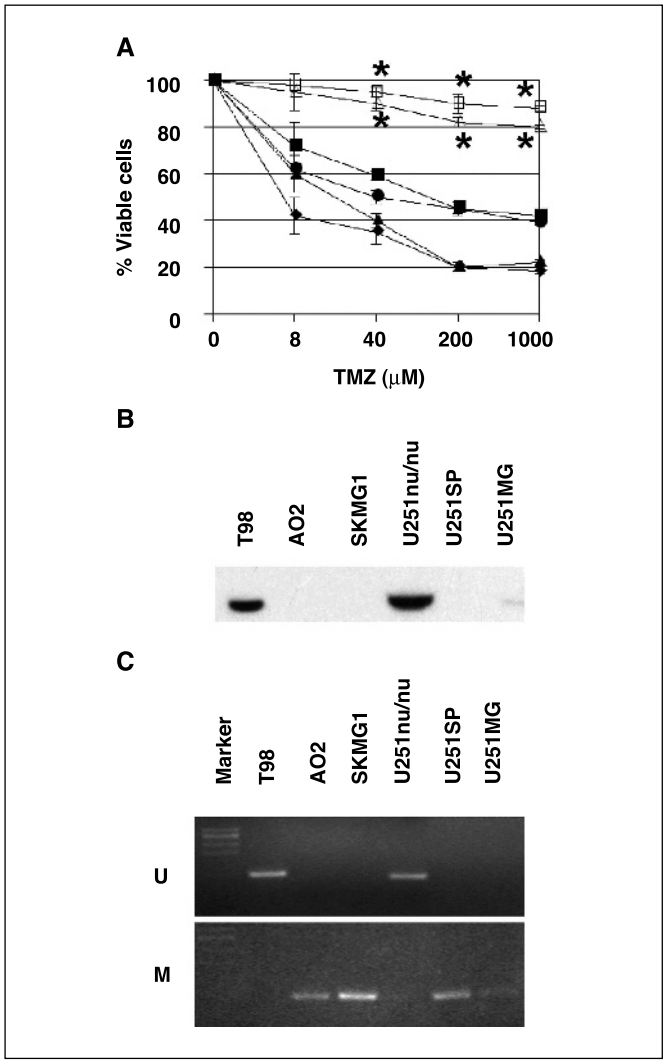


Figure 1. Comparisons among human glioma cell lines. *A,* the antitumor effects of temozolomide against six human glioma cell lines: T98 (□), AO2 (▲), SKMG1 (●), U251nu/nu (△), U251SP (■), and U251MG (♦). At 72 hours after temozolomide (final concentration of 0-1,000 μmol/L) was added to the culture medium, the number of viable cells was counted. The number was expressed as a percentage of untreated control. *$P < 0.05$ versus AO2, SKMG1, U251SP, and U251MG at each dose. *B,* Western blot analysis of MGMT in human glioma cell lines. The cell lysate was subjected to Western blotting with anti-MGMT antibody. *C,* methylation-specific PCR analysis of human glioma cell lines. *U* and *M,* reactions for unmethylated and methylated sequences, respectively.

Bonferroni's correction for the multiple comparisons used. For sensitizing assay of IFN-β to temozolomide, IFN-β (100 IU/mL) and temozolomide (100 μmol/L) were added at 24 and 48 hours after cell inoculation, respectively. The number of viable cells was counted at 96 hours. To examine the effect of siRNA for p53 on the growth of T98 cells treated with IFN-β and temozolomide, either siRNA for p53 or nonsilencing siRNA was transfected as described above at 24 hours after aliquots of 4×10^4 cells/well were placed in triplicate wells. IFN-β (100 IU/mL) and temozolomide (100 μmol/L) were added at 28 and 48 hours, respectively. The number of viable cells was counted at 96 hours.

Western blot analysis. Cell lysis and immunoblotting were carried out as described (11). Antibodies against the following proteins were purchased: p53 (DO-1: Santa Cruz Biotechnology, Santa Cruz, CA), p21 (EA10: Oncogene Research Products, San Diego CA), MGMT (MT3.1: Neomarkers, Fremont, CA), and β-actin (AC-15: Sigma-Aldrich, St. Louis, MO). Band intensities were quantified by densitometric scanning using the NIH IMAGE program.

Genomic DNA extraction and methylation-specific PCR. Genomic DNA was extracted using the QIAmp DNA Mini kit (Qiagen) following the manufacturer's instructions. DNA methylation patterns in the promoter region of the *MGMT* gene (Genbank accession no. X_61657) were determined by methylation-specific PCR as previously described (12). Primers for either methylated or unmethylated alleles were 5′-TTTCGACGTT-CGTAGGTTTTCGC-3′ (sense) and 5′-GCACTCTTCCGAAAACGAAACG-3′ (antisense) or 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (sense) and

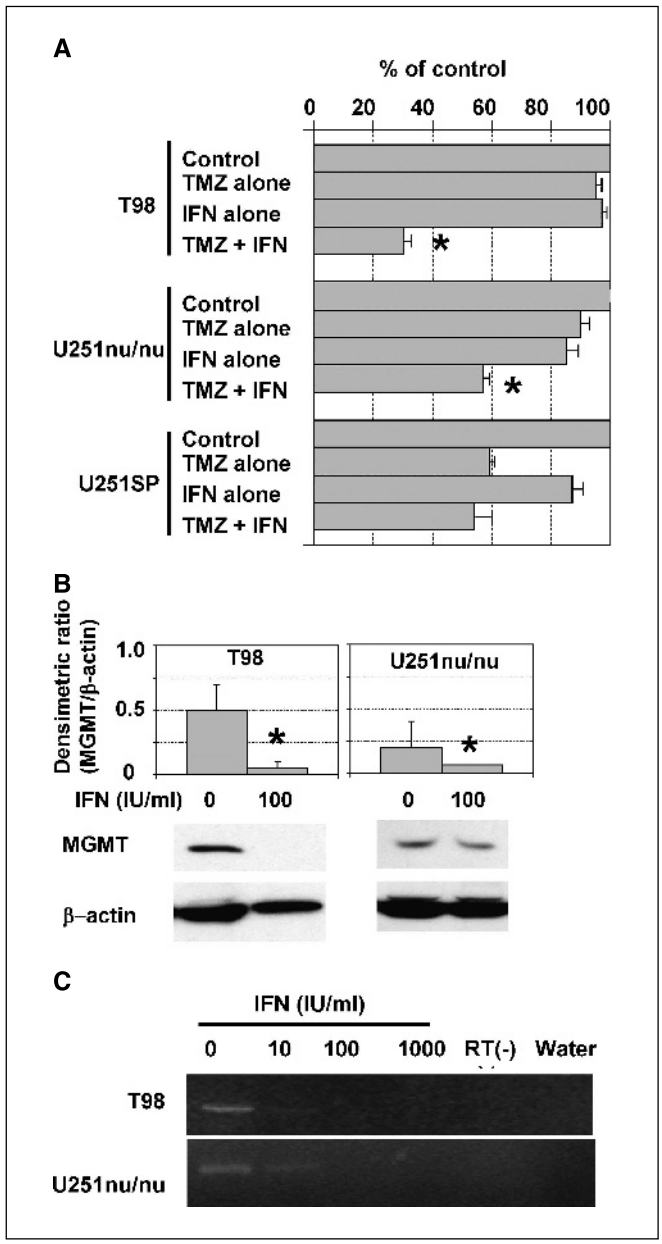


Figure 2. IFN-β sensitizes T98 and U251nu/nu glioma cells to temozolomide and down-regulated MGMT expression. *A,* IFN-β sensitizes the resistant T98 and U251nu/nu cell lines, but not the sensitive U251SP cell line, to temozolomide. The cells were incubated in a culture medium containing IFN-β (100 IU/mL) for 24 hours before the addition of temozolomide (final concentration of 100 μmol/L). Seventy-two hours after, the number of viable cells was counted and expressed as a percentage of untreated control (*$P < 0.05$). *B,* Western blot analysis of MGMT in T98 and U251nu/nu cells treated with IFN-β. Western blotting was done as in Fig. 1*B*. The histogram shows the amount of MGMT relative to that of β-actin. *Columns,* mean from three independent experiments; *bars,* SD (*$P < 0.05$). *C,* RT-PCR analysis for MGMT mRNA. No reverse-transcription control (*RT*−) is also shown.

Table 1.

A. Increase in gene expression in T98 cells by IFN-β

Symbol	Genes	Genbank accession no.	Mean
RBP2	*Retinoblastoma-binding protein 2*	S66431.1	2.012
PKB	*Protein kinase B*	X61037.1	2.024
FGF-9	*Fibroblast growth factor 9*	D14838.1	2.035
Grb14	*Grb14*	L76687.1	2.035
VCAM-1	*Vascular cell adhesion molecule 1 (VCAM-1)*	X53051.1	2.035
IL18	*Interleukin 18 (IFN-γ–inducing factor)*	NM_001562.2	2.047
HOX-11	*Homeobox protein (HOX-11)*	M75952.1	2.055
bcl-w	*Gi|1572492|gb|U59747.1|HSU59747 Human Bcl-w (bcl-w) mRNA, complete cds*	U59747.1	2.074
IGFBP-5	*Insulin-like growth factor binding protein 5*	M62782.1	2.081
Clk2	*Clk2*	L29216.1	2.098
NIK	*Serine/threonine protein kinase*	Y10256.1	2.106
p68K	*p68 kinase*	M35663.1	2.112
IGF-II	*Human insulin–like growth factor II*	M29645.1	2.121
GALNAC4S-6ST	*B-cell RAG-associated protein*	NM_014863.1	2.128
FGF-10	*FGF-10*	AB002097.1	2.129
FRA-2	*Human fra-2*	X16706.1	2.134
FASL	*Fas ligand*	D38122.1	2.146
PDGF	*Platelet-derived growth factor A-chain*	A09204.1	2.158
E1A-F	*E1A-F*	D12765.1	2.163
c-myc	*c-myc*	D89667.1	2.167
hTRIP	*hTRIP (hTRIP)*	U77845.1	2.185
CALM2	*Calmodulin 2 (phosphorylase kinase, δ)*	NM_001743.3	2.207
PUMP-1	*PUMP-1 gene encoding PUMP*	Z11887.1	2.207
Bcl2	*Bcl2, p53-binding protein Bbp/53BP2 (BBP/53BP2)*	U58334.1	2.207
MKK6	*MAP kinase kinase 6*	U39657.1	2.218
JNK3A1	*NK3 α1 protein kinase*	U34820.1	2.242
pS2	*pS2 mRNA induced by estrogen from human breast cancer cell line MCF-7*	X00474.1	2.273
ICAM-2	*ICAM-2, cell adhesion ligand for LFA-1*	X15606.1	2.276
CREM	*Cyclic AMP-responsive element modulator (CREM)*	S68271.1	2.289
Humig	*Humig*	X72755.1	2.289
NFAT1	*Transcription factor NFAT1 isoform C (NFAT1)*	U43342.1	2.289
SFPQ	*Splicing factor proline/glutamine–rich (polypyrimidine tract binding protein associated; SFPQ)*	NM_005066.1	2.302
N-CoR	*Nuclear receptor corepressor*	AF044209.1	2.333
IGFBP3	*Growth factor-binding protein-3 precursor (IGFBP3)*	M35878.1	2.359
IRF-1	*IFN regulatory factor 1*	X14454.1	2.4
IL-11R	*Interleukin-11 receptor*	Z38102.1	2.417
hsp70	*Heat shock protein 70 (hsp70)*	L12723.1	2.424
bcl-3	*B-cell lymphoma 3-encoded protein (bcl-3)*	M31732.1	2.441
FAS/Apo 1	*FAS/Apo 1 mRNA for FAS soluble protein (clone FAS Exo4Del)*	Z70519.1	2.471
HSP75	*Homo sapiens mitochondrial HSP75*	L15189.1	2.481
ICE-LAP6	*Cysteine protease ICE-LAP6*	U56390.1	2.485
G0S8	*Helix-loop-helix basic phosphoprotein*	L13463.1	2.487
HEK2	*HEK2 mRNA for protein tyrosine kinase receptor*	X75208.1	2.493
ERK2	*40 kDa protein kinase related to rat ERK2*	Z11695.1	2.503
KIAA0347	*KIAA0347*	AB002345.2	2.504
APAF1	*Apoptotic protease–activating factor (APAF1), transcript variant 2*	NM_001160.2	2.513
TRA1	*Tumor rejection antigen (gp96) 1*	NM_003299.1	2.574
TNF	*Tumor necrosis factor superfamily member LIGHT*	AF036581.1	2.582
FGF-5	*Fibroblast growth factor-5 (FGF-5)*	M37825.1	2.616
PPAR	*Peroxisome proliferator–activated receptor*	L02932.1	2.616
TIPM3	*Tissue inhibitor of metalloproteinases-3*	U14394.1	2.628
Mch3	*Mch3 isoform α(Mch3)*	U37448.1	2.698
PTPRZ	*Protein tyrosine phosphatase ζ-polypeptide (PTPRZ)*	M93426.1	2.698
RPGR	*Retinitis pigmentosa GTPase regulator (RPGR)*	NM_000328.1	2.741
ENO3	*Enolase 3, (β, muscle; ENO3), transcript variant 1*	NM_001976.2	2.754

(*Continued on the following page*)

Table 1. (Cont'd)

A. Increase in gene expression in T98 cells by IFN-β

Symbol	Genes	Genbank accession no.	Mean
NFKB1	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105; NFKB1)*	NM_003998.2	2.761
HRK	*Activator of apoptosis Hrk (HRK)*	U76376.1	2.774
IFITM3	*IFN-induced transmembrane protein 3 (1-8U; IFITM3)*	NM_021034.1	2.882
IL15RA	*Interleukin-15 receptor αchain precursor (IL15RA)*	U31628.1	2.918
AMPD2	*AMP deaminase 2 (isoform L; AMPD2)*	NM_004037.5	2.989
DNAJA1	*DnaJ (Hsp40) homologue, subfamily A, member 1*	NM_001539.1	3.184
HLA-C	*MHC class I HLA-C allele HLA-4*	M11886.1	3.265
IFN-γ	*IFN-γ*	X13274.1	3.27
p53	*p53 cellular tumor antigen*	M14694.1	3.301
HLA-B	*MHC, class I*	NM_005514.4	3.374
CHED	*cdc2-related protein kinase*	M80629.1	3.686
GADD45	*Gadd45*	S40706.1	3.694
BPAG1	*Bullous pemphigoid antigen 1, 230/240kDa (BPAG1), transcript variant 1e, mRNA*	NM_001723.3	3.793
ISGF-3	*Transcription factor ISGF-3*	M97936.1	3.938
TGFR	*Transforming growth factor-βtype III receptor*	L07594.1	4.341
MC4R	*Melanocortin 4 receptor*	NM_005912.1	10.821

B. Decrease in gene expression in T98 cells by IFN-β

DR3	*Death receptor 3 (DR3)*	U72763.1	0.5
GST-pi-1	*Anionic glutathione-S-transferase (GST-pi-1)*	X15480.1	0.5
TSHR	*Thyroid stimulatory hormone receptor*	M32215.1	0.5
PRDX6	*Peroxiredoxin 6*	NM_004905.2	0.499
IGFBP6	*Insulin-like growth factor binding protein 6*	M62402.1	0.499
HRR-1	*Farnesol receptor*	U68233.1	0.498
IL8RBA	*Interleukin-8 receptor type A*	U11870.1	0.497
RPS2	*Ribosomal protein S2*	NM_002952.2	0.493
COX6C	*Cytochrome coxidase subunit Vic*	NM_004374.2	0.49
HINT1	*Histidine triad nucleotide–binding protein 1*	NM_005340.2	0.489
E2F-1	*pRB-binding protein*	M96577.1	0.483
SKP1A	*S-phase kinase–associated protein 1A (p19A; SKP1A), transcript variant 1*	NM_006930.2	0.483
HS1	*HS1 protein*	X57347.1	0.478
PPP2CA	*Protein phosphatase 2 (formerly 2A), catalytic subunit, αisoform*	NM_002715.1	0.478
G3PD	*Glyceraldehyde-3-phosphate dehydrogenase*	X01677.1	0.473
VitDR	*Vitamin D receptor*	J03258.1	0.472
SEC61B	*Sec61 βsubunit*	NM_006808.2	0.47
SHC	*SHC*	X68148.1	0.47
COX6A1	*Cytochrome coxidase subunit VIa polypeptide 1*	NM_004373.2	0.46
PIN1	*Peptidyl-prolyl isomerase and essential mitotic regulator*	U49070.1	0.457
IL5Rα	*Interleukin 5 receptor α*	M75914.1	0.443
RAB	*Cellular cofactor*	L42025.1	0.435
CD6	*T-cell glycoprotein CD6*	X60992.1	0.434
NDUFB3	*NADH dehydrogenase (ubiquinone) 1 βsubcomplex, 3, 12 kDa*	NM_002491.1	0.433
TXN	*Thioredoxin*	NM_003329.1	0.431
COX4I1	*Cytochrome coxidase subunit IV isoform 1*	NM_001861.2	0.426
SMRT	*Silencing mediator of retinoid and thyroid hormone action*	U37146.1	0.423
ERK3	*ERK3*	X80692.1	0.422
TCRB	*T-cell receptor βchain*	L07294.1	0.421
LGALS1	*Lectin, galactoside-binding, soluble, 1 (galectin 1)*	NM_002305.2	0.417
SOD1	*Superoxide dismutase 1*	NM_000454.2	0.414
ROX	*ROX protein*	X96401.1	0.41
BAG1	*BCL2-associated athanogene*	NM_004323.2	0.409
DC50	*Hypothetical protein DC50*	NM_031210.3	0.407
FTH1	*Ferritin, heavy polypeptide 1*	NM_002032.1	0.398
XRCC5	*X-ray repair complementing defective repair in Chinese hamster cells 5*	NM_021141.2	0.387

(*Continued on the following page*)

Table 1. (Cont'd)

B. Decrease in gene expression in T98 cells by IFN-β

Symbol	Genes	Genbank accession no.	Mean
GSTO1	*Glutathione S-transferase ω1*	NM_004832.1	0.384
TPM2	*Tropomyosin 2 (β)*	NM_003289.1	0.377
CD27L	*CD27 ligand*	L08096.1	0.374
PRDX1	*Peroxiredoxin 1*	NM_002574.2	0.372
MAD-3	*MAD-3 mRNA encoding IkB-like activity*	M69043.1	0.369
CRAF1	*CD40 receptor associated factor 1*	U21092.1	0.365
KIP2	*Cdk-inhibitor p57KIP2*	U22398.1	0.362
RPLP1	*Ribosomal protein, large, P1*	NM_001003.2	0.356
PIG3	*Pig3*	AF010309.1	0.341
GAS	*Growth-arrest–specific protein*	L13720.1	0.33
TMSB10	*Thymosin, β10 (TMSB10)*	NM_021103.2	0.322
ME491	*Melanoma-associated antigen ME491*	X07982.1	0.313
ANXA2	*Annexin A2*	NM_004039.1	0.29
CollagenaseV	*Collagenase type IV*	J03210.1	0.29
S100A2	*S100 calcium–binding protein A2*	NM_005978.2	0.281
calpain	*Calcium-activated neutral protease large subunit*	X04366.1	0.262
FTL	*Ferritin, light polypeptide*	NM_000146.2	0.239

NOTE: p53-related genes are in bold. The mean was based on three replicate arrays analyzed statistically as described in the text.

5′-AACTCCACACACTCTTCCAAAAACAAAACA-3′ (antisense). All PCRs were done with positive controls for methylated alleles and no DNA control. Human placental DNA was treated *in vitro* with excess SssI methyltransferase (New England Biolabs, Beverly, MA), generating DNA completely methylated at CpG sites, served as the positive control for methylated MGMT, and then the PCR conditions were determined. Each PCR product was loaded onto a 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

Reverse transcriptase-PCR. To investigate MGMT mRNA expression, reverse transcription-PCR (RT-PCR) was done using the Superscript first-strand synthesis system for RT-PCR (Invitrogen) as previously described (13). β-actin–specific PCR products from the same RNA samples were amplified and served as internal controls.

Microarray analysis. T98 cells were incubated with 100 IU/mL of IFN-β for 48 hours, and RNA was isolated. Standard Trizol preparation protocol (Invitrogen) and reagents were used for total RNA isolation. RNA amplification and labeling were done using the Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX). Briefly, after reverse transcription reactions (2 μg total RNA/sample) were preformed, double-stranded cDNA was transcribed *in vitro* into amino allyl cRNA. The purified and concentrated cRNA (5 μg) was coupled with either Cy3 or Cy5 dyes (Amersham Biosciences). The dye-labeled aRNA was purified from uncoupled dye using Micro Bio-Spin P-30 Tris chromatography columns (Bio-Rad, Hercules, CA) and MicroconYM-30 centrifugal filter devices (Millipore, Billerica, MA). The cRNA was fragmented in fragmentation buffer [40 mmol/L Tris-acetate (pH 8.1), 100 mmol/L potassium acetate, 30 mmol/L magnesium acetate] at 94°C for 15 minutes and purified with Microcon YM-10 (Millipore). Microarrays were preblocked with 1% bovine serum albumin solution. Fragmented cRNA was added to microarrays in hybridization solution and hybridized at 42°C for 16 hours. After this, arrays were washed, scanned at 10 μm pixel size, gridded, and analyzed (GenePix 4000B; Axon Instruments, Union City, CA). Background was subtracted, and the median sum and median ration were calculated. Flagged spots and spots with sum intensity (CH1, CH2) <100 absorbance units were excluded. Data were normalized by trimmed mean at 10% to account for differences in the amounts of RNA labeled or labeling efficiencies.

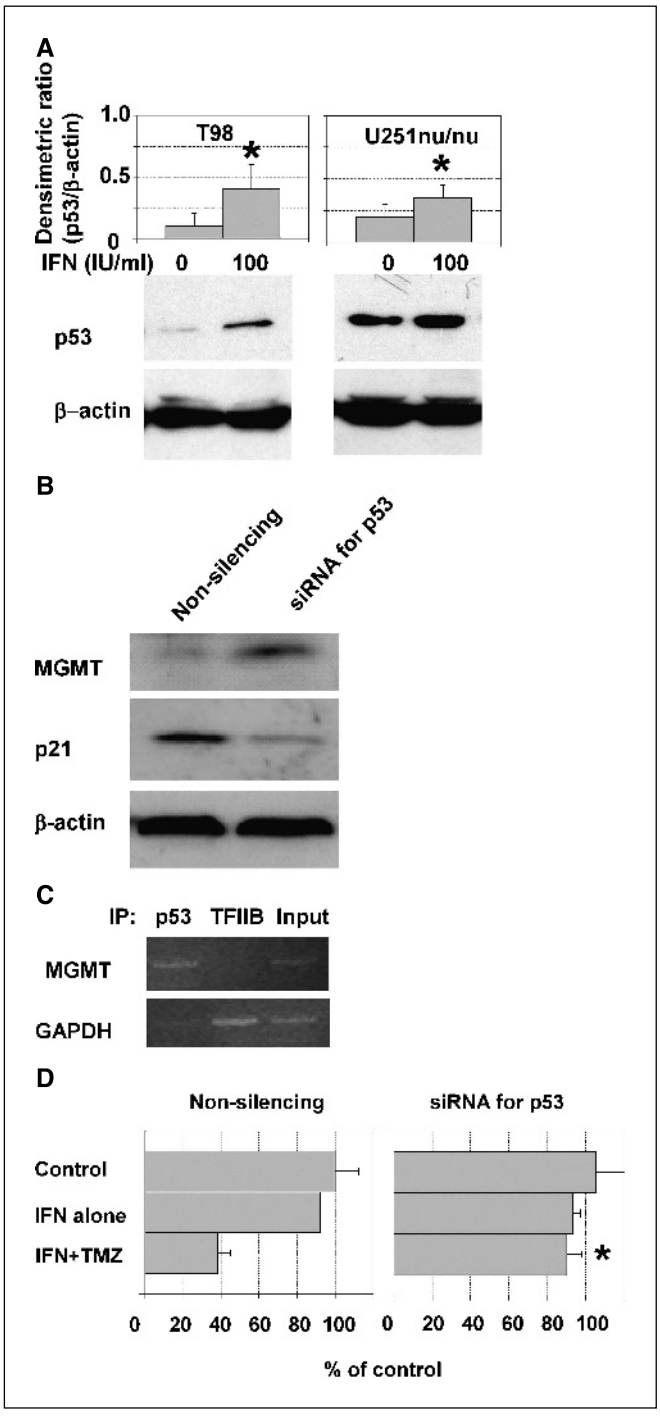
Chromatin immunoprecipitation assay. The chromatin immunoprecipitation assay was done according to the protocol of the manufacturer (Upstate Biotechnology, Lake Placid, NY) with a slight modification. The specific antibodies used for immunoprecipitations were the anti-p53 antibody and a control antibody. After protein-DNA cross-links in the immunoprecipitates were reversed, the purified DNA was analyzed by PCR (35 cycles; 45 seconds at 95°C, 45 seconds at 55°C, 60 seconds at 72°C) with primers that detect the MGMT promoter sequence [5′-GCTCCAGGGAA-GAGTGTCCTCTGC-TCCCT-3′ (sense) and 5′-GGCCTGTGGTGGGCGAT-GCCGTCCAG-3′ (antisense)] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (provided with the kit). The PCR products were visualized on an ethidium bromide gel.

Results and Discussion

Chemosensitivity to temozolomide, MGMT expression, and MGMT promoter hypermethylation of human glioma cell lines. First, we compared chemosensitivity of six human glioma cell lines to temozolomide; T98 and U251nu/nu cell lines were significantly resistant, whereas the other four (AO2, SKMG1, U251SP, and U251MG) showed similar degrees of sensitivity in a dose-dependent manner (Fig. 1*A*). MGMT expression was not detected by Western blot in the sensitive cell lines (Fig. 1*B*), and the hypermethylation of MGMT promoter was as assessed by methylation-specific PCR (Fig. 1*C*), but this was not observed in the T98 and U251nu/nu cell lines. Thus, the results showed that hypermethylation of MGMT promoter could prevent expression of this gene and be associated with chemosensitivity of glioma cells to temozolomide, which was consistent with the results of reported studies (7). Because ~70% of gliomas express MGMT (9), it is essential to suppress the expression or function of MGMT in an efficient manner if treatment with the aid of temozolomide is to be considered.

IFN-β sensitizes resistant glioma cells to temozolomide and down-regulates MGMT expression. IFNs are a family of cytokines

that possess pleiotropic biological effects mediated by a number of responsive genes. IFNs were the first human proteins to be recognized as being effective in cancer therapy (14). Although identified and named for their action to interfere with viral replication, IFNs have immunomodulatory, cell differentiative, antiangiogenic, and antiproliferative effects (15, 16). In the previous studies, we showed that IFN-β has multiple functions relevant to antitumor activity: (*a*) cytostatic effect on glioma cells, (*b*) supportive action on the differentiation of CTLs and augmentation of their antitumor immune responses, and (*c*) behavior as a drug or radiation sensitizer enhancing toxicity against gliomas (10).



To examine whether IFN-β sensitizes T98 and U251nu/nu cell lines that are resistant to temozolomide, the cells were incubated in a culture medium containing IFN-β (100 IU/mL) for 24 hours before the addition of temozolomide (final concentration of 100 μmol/L). As shown in Fig. 2*A*, whereas IFN-β or temozolomide alone did not suppress cell growth of both T98 and U251nu/nu cells significantly, a combination of IFN-β and temozolomide markedly inhibits the growth. On the other hand, U251SP cell lines that are sensitive to temozolomide, but are resistant to IFN-β did not show the synergistic cytotoxic effect of temozolomide and IFN-β. Consequently, we hypothesized that IFN-β might enhance chemosensitivity due to reduction of MGMT expression and did Western blotting and RT-PCR for MGMT (Fig. 2*B* and *C*). It was shown that IFN-β decreased both MGMT protein and mRNA levels of both T98 and U251nu/nu cells at 48 hours after treatment. Thus, IFN-β decreases MGMT transcription directly or indirectly and sensitizes resistant glioma cells to temozolomide.

Microarray analysis of IFN-β–regulated genes. Because limited information regarding the transcriptional regulation of IFN-β is available, we introduced microarray technology that enables the simultaneous examination of expression of a large number of genes in an experimental condition. This microarray contains 1,300 functionally well-characterized genes involved in various important cellular processes, including the IFN-related pathway, apoptosis, cell cycle, transcription, and immunology. The microarray experiments were repeated thrice about the T98 cell lines that showed the most drastic change of MGMT expression after IFN-β treatment. Data analysis identified 71 significantly induced genes and 54 repressed genes in several different categories in T98 cells treated with IFN-β compared with the parental cells. The representative results of these comparisons are reported in Table 1. Some of the alterations in gene expression following IFN-β treatment confirm the involvement of pathways known to be active in this process, and this was anticipated from prior evidence. Consistent with a report that the *p53* gene is transcriptionally induced by IFN-β through ISGF3 activation (11), our microarray data showed that the *p53* gene and its downstream genes, including *bcl-2, FAS/Apo1*, and *Gadd45*, are up-regulated in glioma cells treated with IFN-β, suggesting that *p53* induction may be a key involved in antitumor action of IFN-β. Thus, we focused on IFN-β–mediated *p53* induction. By Western blot, we confirmed that *p53* expression level in both T98 cells treated with IFN-β was markedly higher than that in the

Figure 3. IFN-β down-regulates MGMT expression via induction of p53. *A,* up-regulation of the protein p53 in T98 and U251nu/nu cells by IFN-β. The cell lysate of cells treated with IFN-β (100 IU/mL) was subjected to Western blotting with anti-p53 and anti–β-actin antibodies. The histogram shows the amount of p53 relative to that of β-actin. *Columns,* mean values from three independent experiments; *bars,* SD (* $P < 0.05$). *B,* RNA interference experiments for p53. After p53 was knocked down by the siRNA specific for p53, Western blotting for MGMT, p21, and β-actin was done. The siRNA with nonsilencing sequence was used as a control. The specific knockdown was confirmed by the diminished expression level of p21, a well-known target gene of *p53*. *C,* chromatin immunoprecipitation assay. The protein p53 binding to the MGMT promoter element was examined. *Lane 1,* PCR amplification of MGMT and GAPDH sequences in immunoprecipitated chromatin fragments with anti-p53 antibody. *Lane 2,* result of PCR using immunoprecipitated samples with control antibody against TFIIB, the known transcriptional factor of GAPDH. *Lane 3,* PCR amplification of the total input DNA. *D,* cancellation of the sensitizing effect of IFN-β by p53 siRNA. T98 cells were treated with the siRNA for p53 or the nonsilencing siRNA before the treatments of IFN-β (100 IU/mL) and temozolomide (100 μmol/L). Seventy-two hours after, the number of viable cells was counted. *$P < 0.05$ versus the treatment group with nonsilencing siRNA, IFN-β, and temozolomide.

untreated cells and the similar phenomenon was observed in the U251nu/nu cells as well (Fig. 3*A*).

IFN-β down-regulates MGMT expression via protein p53. For >10 years, p53 has been the focus of intensive research. This has led to a plethora of information regarding p53, its biological roles, and its relevance to cancer (17). p53 is primarily a sequence-specific transcriptional activator. It binds to cognate p53-responsive elements within the genome and activates the transcription of genes residing in the vicinity of these binding sites. The proteins encoded by the p53 target genes, whose number is probably in the hundreds, contribute in multiple ways to the biological effects of p53. The biological outcomes of p53 activity include apoptosis, inhibition of cell cycle progression, senescence, differentiation, and accelerated DNA repair. We first confirmed that siRNA for p53 specifically knocked down the protein p53 function by examining the diminished expression level of *p21*, a well-known target gene of p53 (Fig. 3*B*). As shown in Fig. 3*B*, knockdown of p53 by siRNA increased MGMT expression in T98 cells. Therefore, p53 can down-regulate transcription of MGMT. To further investigate whether protein p53 directly interacts with MGMT promoter and down-regulates transcription of MGMT, we carried out chromatin immunoprecipitation assay. We observed that MGMT promoter, but not GAPDH promoter, coprecipitated with p53. The irrelevant antibody specific to TFIIB, which is a transcription factor of GAPDH, did not coprecipitate with MGMT promoter (Fig. 3*C*). These findings are in agreement with the previous reports showing that p53 reduces the basal activity of the MGMT promoter, and adenoviral vector–mediated overexpression of p53 reduces MGMT expression (18, 19), although some papers suggested that inactivated MGMT may be linked to cytotoxic effect of alkylating agents and cell signaling events, but was independent of p53 status (20). The reasons for these differences are unclear and may be cell type dependent. Moreover, in cell growth experiments, knocking down of p53 before IFN-β treatment nullified the synergistic inhibitory effect of IFN-β and temozolomide on T98 cell growth (Fig. 3*D*). Thus, acting together, IFN-β down-regulates MGMT transcription via induction of the p53 expression and sensitizes resistant glioma cells to temozolomide. In conclusion, this report shows that IFN-β is able to decrease MGMT levels in glioma cells via the inhibition of *MGMT* gene transcription. Moreover, pretreatment of glioma cells with IFN-β markedly enhances chemosensitivity to temozolomide. This suggests that clinical efficacy of temozolomide might be improved by combination with IFN-β using appropriate doses and schedules of administration.

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