

## STAT3 Inhibition Overcomes Temozolomide Resistance in Glioblastoma by Downregulating MGMT Expression

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### Abstract

Glioblastoma multiforme (GBM) is one of the most aggressive human tumors with a poor prognosis. Current standard treatment includes chemotherapy with the DNA-alkylating agent temozolomide concomitant with surgical resection and/or irradiation. However, a number of cases are resistant to temozolomide-induced DNA damage due to elevated expression of the DNA repair enzyme O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT). Here, we show that upregulation of both MGMT and STAT3 was accompanied with acquisition of temozolomide resistance in the GBM cell line U87. Inactivation of STAT3 by inhibitor or short hairpin RNA (shRNA) downregulated MGMT expression in GBM cell lines. MGMT upregulation was not observed by the treatment of interleukin (IL)-6 which is a strong activator of STAT3. Contrarily, forced expressed MGMT could be downregulated by STAT3 inhibitor which was partially rescued by the proteasome inhibitor, MG132, suggesting the STAT3-mediated posttranscriptional regulation of the protein levels of MGMT. Immunohistochemical analysis of 44 malignant glioma specimens showed significant positive correlation between expression levels of MGMT and phosphorylated STAT3 (p-STAT3;  $P < 0.001$ ,  $r = 0.58$ ). Importantly, the levels of both MGMT and p-STAT3 were increased in the recurrence compared with the primary lesion in paired identical tumors of 12 cases. Finally, we showed that STAT3 inhibitor or STAT3 knockdown potentiated temozolomide efficacy in temozolomide-resistant GBM cell lines. Therefore, STAT3 inhibitor might be one of the candidate reagents for combination therapy with temozolomide for patients with temozolomide-resistant GBM. *Mol Cancer Ther*; 11(6); 1289–99. ©2012 AACR.

### Introduction

Glioma is the most common primary tumors of the central nervous system, accounting approximately for 30%, and classified into 4 clinical grades as I to IV. The most aggressive and lethal tumors is glioblastoma multiforme (GBM; ref. 1). The median survival of patients with GBM is less than 1 year, mainly because conventional postsurgical chemotherapeutic agents and irradiation exhibit limited effects (2).

Temozolomide is an alkylating agent applied to malignant glioma including GBM (3). Temozolomide induces DNA methylation of guanine at O<sup>6</sup> position and O<sup>6</sup>-methylguanine incorrectly pairs with thymine and triggers

the mismatch repair (MMR) system leading to double-strand break of the genome that result in the arrest of cell cycle and induction of apoptosis (4, 5). O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) removes methylation from O<sup>6</sup> position of guanine and induces temozolomide resistance (6). Thus, the patients with silenced of MGMT expression through methylation of the MGMT promoter were reported to have an improved 2-year survival with temozolomide treatment together with irradiation (7). Despite pseudosubstrates of MGMT such as O<sup>6</sup>-benzylguanine were expected to suppress resistance by depleting MGMT (8–10), clinical trials did not show significant restoration of temozolomide sensitivity in patients with temozolomide-resistant GBM (11). Therefore, other therapeutic agents which suppress MGMT expression and sensitize the efficacy of temozolomide are highly desired (12, 13).

It is well known that receptor tyrosine kinases including the EGF receptor (EGFR), platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$ ), and the VEGF receptor (VEGFR), contribute to the growth of GBM through the activation of Ras/ERK- or phosphoinositide-3 kinase (PI3K)/AKT-dependent signaling pathways (14–16). These diverse signals converge at specific transcription factors, including STAT3 (17). STAT3 was initially identified as a signal mediator for IFN stimulation with various gene targets

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controlling cell growth, and subsequently, STAT3 has been shown to play an important role for cellular transformation (18). In fact, aberrant activation of STAT3 has been identified in GBM as well as in a number of other human cancers (19–21).

To date, profiling analysis has proposed molecular-based classification of GBM as proneuronal, proliferative, and mesenchymal type. Primary tumor tends to be a proneuronal type, however along with the recurrence, GBM becomes mesenchymal type with invasion and angiogenesis (22, 23). Recently, STAT3, C/EBP, bHLH-B2, RUNX1, FOSL2, and ZNF238 have been shown to collectively control more than 74% of the mesenchymal gene expression signature gene (24). Furthermore, expression of STAT3 correlates with mesenchymal differentiation and predicts poor clinical outcome in human glioma (24). While STAT3 is considered to be a key molecule regulating mesenchymal phenotype, MGMT is not classified into any specific subtype according to the previous microarray data comparing each subtype (22). Furthermore, methylation status of MGMT was not associated with subtype (25). Several studies have shown that STAT3 inhibitors suppressed STAT3 activation and expression of its downstream target genes including cyclin D1 and VEGF (26–29). However, the relation between STAT3 and temozolomide resistance has not yet been assessed.

In this study, to elucidate the regulatory mechanism of MGMT and identify suppressive reagent for MGMT expression in GBM for the increase of efficacy of temozolomide, we generated temozolomide-resistant cells and found the elevation of STAT3 expression in these cells. The roles for STAT3 in GBM were examined and the effect of STAT3 inhibitor on temozolomide-resistant GBM was evaluated.

## Materials and Methods

### Cells

The human GBM cell lines T98G (ATCC#CRL-1690), U138 (ATCC#HTB-16), and U87 (ATCC#HTB-14) were purchased from American Type Culture Collection. KMG4 cell line was kindly provided by Dr. Kazuo Tabuchi (Saga University, Saga, Japan; ref. 30). LN382, LN308, and LN235 cell lines were kindly provided by Dr. Erwin G. Van Meir (Emory University School of Medicine, Atlanta, GA; refs. 31, 32). All cell lines were cultured in Dulbecco's Modified Minimal Essential Medium (DMEM; Wako) supplemented with 10% FBS. Cell line authentication was not carried out by the authors within the last 6 months.

### Reagents

STAT3 inhibitor III WP1066 (Santa Cruz Biotechnology), STAT3 inhibitor V Stattic (Santa Cruz Biotechnology), STAT3 inhibitor VI NSC 74859 (Calbiochem), Janus-activated kinase (JAK) inhibitor I (Calbiochem), MET inhibitor PHA 665752 (Santa Cruz Biotechnology), and Src-family kinase inhibitor dasatinib (LC Laboratories) were used following the manufacturer's instruction.

### STAT3 phosphorylation by IL-6 stimulation

Glioma cells were cultured in DMEM supplemented with 10% FBS for 24 hours. The cells were treated with 10 ng/mL of interleukin (IL)-6 (Sigma) for 24 hours.

### Preparation of retrovirus and establishment of stable cell line

For retrovirus production, the pcx4 vector system was used (33, 34). The complete sequences of pcx4pur (puromycin) is available from the GenBank database (AB086386). Full-length cDNAs for human MGMT and IL-6 were subcloned into pcx4pur. Retroviruses were obtained by using 293T cells as packaging cells and were infected to GBM cell lines and selected with puromycin (2 µg/mL).

### Preparation of shRNA stably introduced cell lines by lentivirus system

For targeting STAT3, 3 sequences were designed by the BLOCK-It RNAi Designer (Invitrogen). The preparation of lentiviral vectors expressing human STAT3 short hairpin RNA (shRNA) was carried out using the BLOCK-It Lentiviral RNAi Expression System (catalog no. K4934-00; Invitrogen), following the manufacturer's instruction. Briefly, 3 pLenti6.4/shSTAT3 expression vectors containing the human STAT3 shRNA-expressing cassette were constructed. Replication-incompetent lentivirus was produced by cotransfection of the pLenti6.4/shSTAT3 expression vector and ViraPower Packaging Mix (Invitrogen) containing an optimized mixture of 3 packaging plasmids: pLP1, pLP2, and pLP/VSVG into 293FT cells with Lipofectamine 2000 (Invitrogen). Viral supernatant was harvested 48 hours after transfection, filtered through a 0.45-µm cellulose acetate filter, and frozen at –80°C. The lentivirus-only containing pLenti6.4/U6 mock vector was used as control. STAT3 shRNA lentiviral construct and control lentiviral constructs were introduced into glioblastoma cell lines, followed by selection with blasticidin S (5 µg/mL).

### Bromodeoxyuridine assays

BrdU Labeling ELISA Kit (Roche Molecular Biochemicals) was used to examine the effects of temozolomide on the proliferation of glioma cell lines. Bromodeoxyuridine (BrdUrd) is a thymidine nucleotide analogue that is incorporated during S-phase (instead of thymidine) only in the DNA of proliferating cells. Cells were plated in 96-well plates at a density of 2,500 cells per well and were incubated with DMEM supplemented with 10% FBS in the presence or absence of temozolomide for 5 days. The rate of BrdUrd incorporation was measured by ELISA technique, and the percentage of cells in S-phase was quantitated by colorimetric evaluation ( $\lambda = 450$  nm). The absorbance of cells treated with 10% FBS without temozolomide was assumed to be 100%, and temozolomide-induced decreases in S-phase were calculated as a percentage of the control FBS-treated cells. Each sample was

carried out in triplicate, and the entire experiment was repeated twice.

### MTT assay

MTT assay was used to determine cell viability following the manufacturer's protocols (Cayman Chemical Company). Cells were plated in 96-well plates at a density of 2,500 cells per well. The cells were cultured in 100  $\mu$ L of the medium with indicated concentration of temozolomide. After 5 days, 10  $\mu$ L of MTT solution (5 mg/mL) was added to each well, and the plates were incubated at 37°C for 4 hours. Then, the media was removed and 100  $\mu$ L of crystal dissolving solution was added to each well to solubilize the formazan crystals. The absorbance of the plates was measured on a microplate reader (Bio-Rad) at a wavelength of 570 nm. Each sample was carried out in triplicate, and the entire experiment was repeated twice.

### Clonogenic assay

Clonogenic survival assays were conducted by seeding 500 cells in 6-well plates and exposing them to temozolomide (10–1,000  $\mu$ mol/L) for 48 hours, followed by further observation for 7 to 14 days. Cell density or colonies were assessed with crystal violet staining. Colonies of more than 50 cells were counted. In some clonogenic survival assays, the cells were cotreated with STAT3 inhibitor VI.

### Transwell migration assay

Chemotactic migration was quantified by a Boyden chamber Transwell assay (8- $\mu$ m pore size; Corning Costar) using uncoated filters. A total of  $5 \times 10^4$  cells were trypsinized and introduced into the upper chamber. After incubation for 4 hours, using a 10% serum stimulus in the lower chamber, the remaining cells were removed from the upper side of the membrane by wiping, and the cells that had passed through to the lower side of the insert were fixed with 100% methanol and stained with 0.04% crystal violet. The number of cells that had migrated was then quantified by counting those in 5 random fields ( $\times 20$ ) of each membrane. Experiments were done in triplicate.

### Immunoblotting

Immunoblotting was carried out by the method described elsewhere. Cells were lysed with buffer containing 0.5% NP-40, 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L PMFS, and 1 mmol/L  $\text{Na}_3\text{VO}_4$ . Proteins were subjected to SDS-PAGE, and separated proteins were transferred to a polyvinylidene difluoride filter (Immobilon-P; Millipore). Filters were probed with antibodies obtained from the following sources: anti-STAT3 monoclonal antibodies (mAb; Cell Signaling Technology); anti-MGMT (MT3.1) mAb and anti-actin MAb (Chemicon); anti-phospho-STAT3 (Tyr705) polyclonal antibody (Cell Signaling Technology). Bound antibodies were detected with peroxidase-labeled goat antibody to mouse IgG or goat

antibody to rabbit IgG, and visualized by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

### Reverse transcriptase PCR analysis

Total RNA was isolated from cells with TRI Reagent (Sigma) and resuspended in RNA secure resuspension solution (Ambion). Reverse transcription was carried out with Superscript II RT (Invitrogen). Resulting first-strand cDNA was used as a template and amplified by PCR using KOD-Plus DNA polymerase (Toyobo). The following primer sets were used. For STAT3, 5'-CAC CAA GCG AGG ACT GAG CAT-3' (sense) and 5'-GCC AGA CCC AGA AGG AGA AGC-3' (antisense); for MG1MT, 5'-ATG GAC AAG GAT TGT GAA-3' (sense) and 5'-GTT TCG GCC AGC AGG-3' (antisense); for IL-6, 5'-TTC AAT GAG GAG ACT TGC CTG-3' (sense) and 5'-ACA ACA ACA ATC TGA GGT GCC-3' (antisense); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-CTC ATG ACC ACA GTC CAT GC-3' (sense) and 5'-TTA CTC CTT GGA GGC CAT GT-3' (antisense).

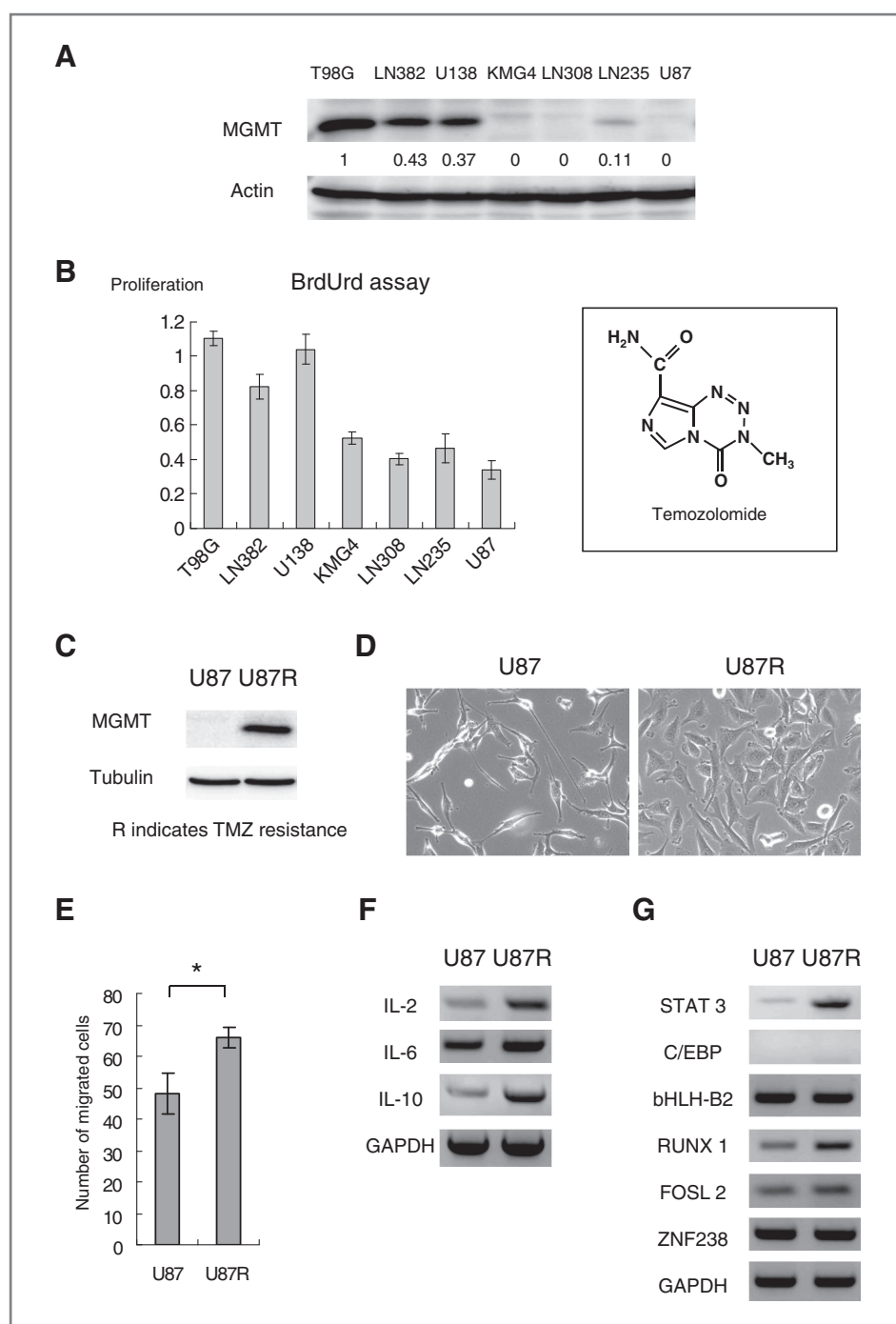
### Immunohistochemical analysis

Formalin-fixed paraffin-embedded tissues were sectioned and stained using anti-MGMT (MT3.1; Chemicon) and anti-phospho-STAT3 (Tyr705) polyclonal antibody (Cell Signaling Technology). Scoring was as follows: 0 (negative), 1+ (<10%–25% of cells weakly positive), 2+ (>25% of cells weakly positive or <10%–25% of cells strongly positive), and 3+ (>25% of cells strongly positive).

## Results

### Establishment of temozolomide-resistant U87 GBM cell line

First, the expression levels of MGMT and temozolomide sensitivity in 7 GBM cell lines were investigated, and 3 of them T98G, U138, and LN382, were found to highly express MGMT than the other 4 cell lines KMG4, LN308, LN235, and U87 (Fig. 1A). Consistent with previous reports, MGMT-expressing cell lines exhibited a growth advantage in the presence of temozolomide (Fig. 1B, Supplementary Fig. S1). To identify the responsible molecules for temozolomide resistance, comparatively temozolomide-sensitive U87 cell line was treated with a low dose of temozolomide in culture media for 3 weeks and established temozolomide-resistant cells designated as U87R. Half maximal inhibitory concentration ( $\text{IC}_{50}$ ) for the growth inhibition of temozolomide to U87 was less than 40  $\mu$ mol/L, but it was more than 150  $\mu$ mol/L in U87R (Supplementary Fig. S2A). To ensure the temozolomide resistance of U87R cells, we used clonogenic survival assay (35), and found that the  $\text{IC}_{50}$  value of U87 was less than 10  $\mu$ mol/L and that of U87R was more than 400  $\mu$ mol/L, suggesting that temozolomide sensitivity was altered more than 40-fold decrease in resistant cells (Supplementary Fig. S2B). According to the increase of



**Figure 1.** Establishment of temozolomide (TMZ)-resistant U87 GBM cell line. **A**, immunoblot analysis of MGMT in 7 glioblastoma cell lines, T98G, LN382, U138, KMG4, LN308, LN235, and U87 (top). Actin is shown as a loading control (bottom). **B**, evaluation of temozolomide resistance by BrdUrd assay. Seven glioblastoma cell lines were cultured for 5 days in the presence of 60  $\mu\text{mol/L}$  of temozolomide. BrdUrd incorporation indicates proliferation of cells. Error bars represent SD of 3 independent experiments. Right, the structure of temozolomide. **C**, immunoblot analysis of MGMT in U87 and U87R cells (top). Tubulin is shown as a loading control (bottom). **D**, phase-contrast microscopy of U87 cells (left) U87R cells (right) with  $\times 400$  magnification. **E**, evaluation of migration ability of U87 and U87R by Transwell migration assay. The number of migrated cells through the Transwell membranes with 8.0- $\mu\text{m}$  pores after 4-hour incubation was counted in 5 random fields ( $\times 20$ ) of each membrane. Error bars represent SD of 3 independent experiments. \*,  $P < 0.01$  U87 versus U87R. **F**, reverse transcriptase PCR (RT-PCR) analysis of cytokine. The mRNA levels of IL-2, IL-6, and IL-10 were evaluated. GAPDH is shown as a loading control (bottom). **G**, RT-PCR analysis of molecules related to mesenchymal type of GBM. The mRNA levels of STAT3, C/EBP, bHLH-B2, RUNX1, FOSL2, and ZNF238 were evaluated. GAPDH is shown as a loading control (bottom).

resistance, protein levels of MGMT were elevated in U87R cells (Fig. 1C).

As U87R seemed to exhibit morphologic change as cuboidal cells (Fig. 1D), we examined biologic phenotype such as migration by Transwell assay, and found that after 4 hours, increased number of U87R cells (1.38-fold increase) passed the filter compared with U87 cells (Fig. 1E). We also investigated the expression of several cytokines, which are previously reported to promote

invasion or angiogenesis in glioma (36, 37). The expression of IL-2, IL-6, and IL-10 were upregulated in U87R cells compared with U87 cells (Fig. 1F). These results suggested that U87R cells possess the mesenchymal signature of GBM. Therefore, we further investigated the expression levels of STAT3, C/EBP, bHLH-B2, RUNX1, FOSL2, and ZNF238, which were reported to regulate the mesenchymal signature of GBM (24), and among them the mRNA level of STAT3 was found to be dominantly

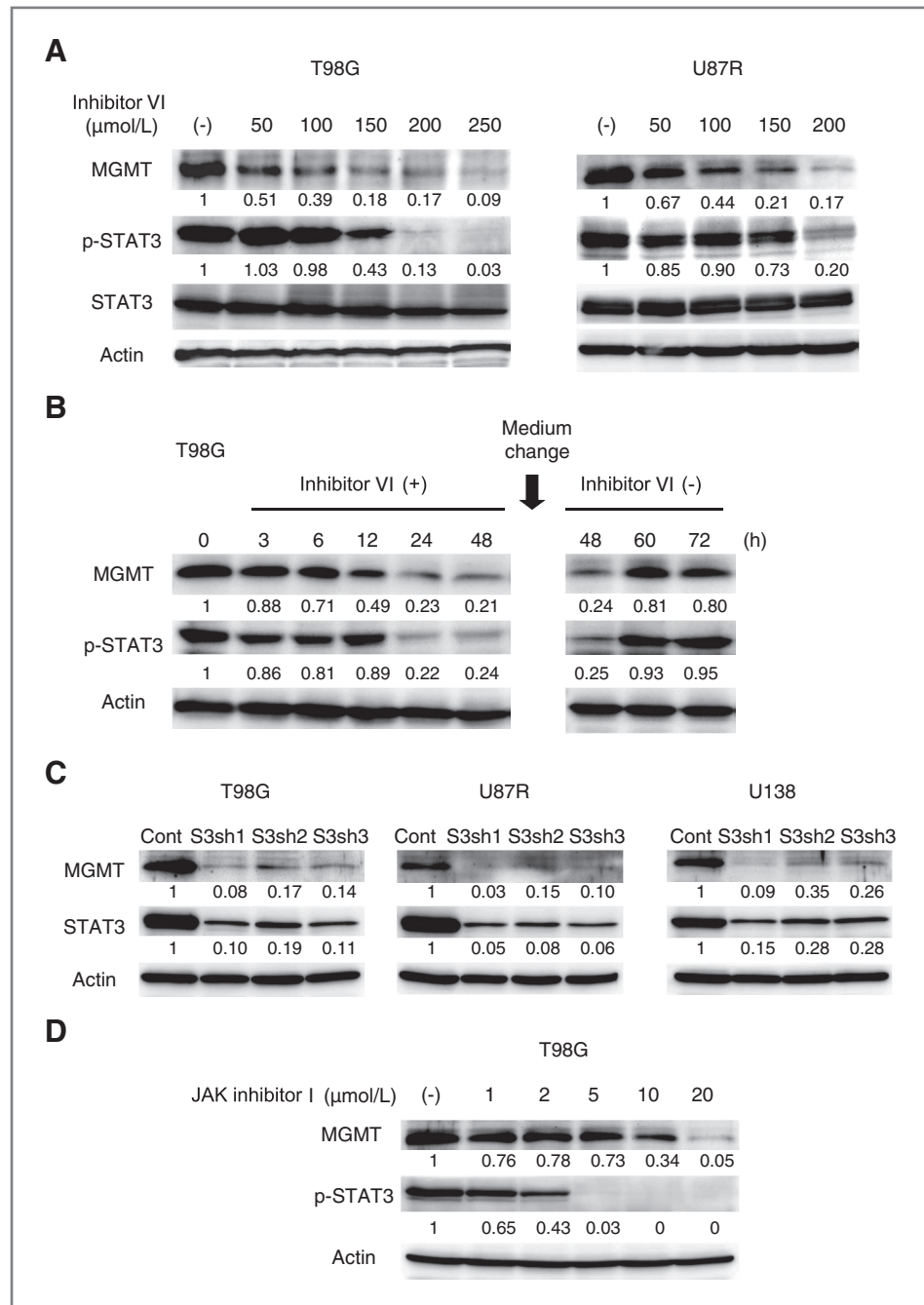
upregulated in U87R cells. Small increase of RUNX1 and almost no significant alteration of expression of others were observed (Fig. 1G).

**STAT3 inhibitor downregulated MGMT expression in GBM cell lines**

As concomitant upregulation of STAT3 with MGMT was observed with acquisition of temozolomide resistance in U87 cells, we investigated whether STAT3 regulates MGMT expression in GBM. STAT3 inhibitor VI,

NSC 74859, reduced MGMT levels in a dose-dependent manner in T98G and U87R cells (Fig. 2A). The efficacy of STAT3 inhibitor was validated by the reduction of phosphorylated levels of STAT3 (Fig. 2A). Furthermore, time-dependent suppression and recovery of MGMT levels by STAT3 inhibitor VI were also showed (Fig. 2B). We also examined effect of other STAT3 inhibitors such as inhibitor III and inhibitor V; however, these inhibitors were cytotoxic and specific inhibition of STAT3 was not observed (data not shown).

Figure 2. STAT3 inhibition downregulated MGMT expression in GBM cell lines. A, immunoblot analysis of MGMT in T98G and U87R treated with STAT3 inhibitor VI (top). Dose of the STAT3 inhibitor is indicated at the top. The level of p-STAT3 and STAT3 were also evaluated (second and third from the top). Actin is shown as a loading control (bottom). B, immunoblot analysis of MGMT in T98G treated with 200  $\mu\text{mol/L}$  of STAT3 inhibitor VI. Duration of the treatment is indicated at the top as 0 to 48 hours. Medium change indicated removal of STAT3 inhibitor. The level of p-STAT3 was also evaluated (middle). Actin is shown as a loading control (bottom). C, immunoblot analysis of MGMT and STAT3 in T98G, U87R, and U138 expressing STAT3 shRNA as S3sh1, S3sh2, and S3sh3 (top and middle). Cont indicates negative control. Actin is shown as a loading control (bottom). D, immunoblot analysis of MGMT (top) and p-STAT3 (middle) in T98G treated with JAK inhibitor I. Dose of the JAK inhibitor I is indicated at the top. Actin is shown as a loading control (bottom).



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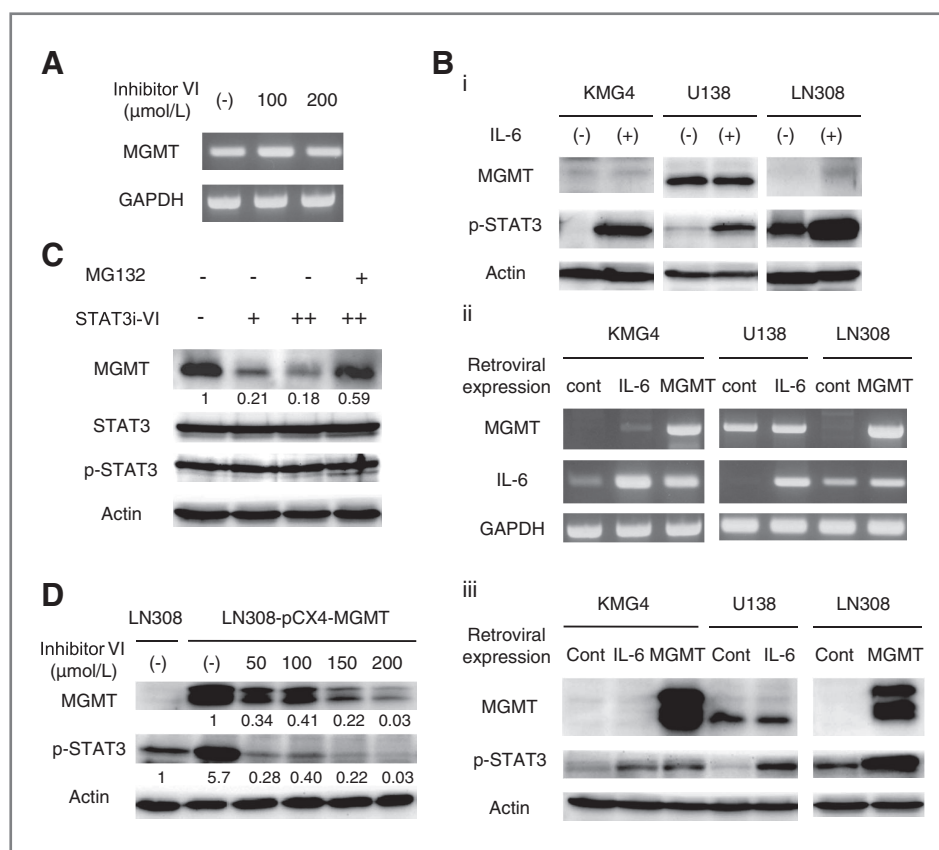
To exclude the possible but unpredictable effect of STAT3 inhibitor VI rather than STAT3 inactivation on the reduction of MGMT level, we used shRNA for STAT3 by using lentiviral vector system and found that depletion of STAT3 diminished the expression of MGMT in representative GBM cell lines as T98G, U87R, and U138 cells (Fig. 2C). For further confirmation of the involvement of STAT3, we examined the effect of JAK inhibitor which is upstream of STAT3, together with the inhibitors for Src-family kinase (dasatinib), MET (PHA665752), and MEK (U0126), and among them only JAK inhibitor could inhibit phosphorylation of STAT3 and decreased the expression of MGMT (Fig. 2D, Supplementary Fig. S3).

### Posttranscriptional regulation of MGMT expression by STAT3

To investigate whether STAT3 regulates MGMT transcription, we examined the mRNA levels of MGMT after treatment of STAT3 inhibitor in T98G cells. mRNA levels

of MGMT were unchanged by the treatment of STAT3 inhibitor VI in T98G cells (Fig. 3A). As STAT3 is one of the downstream effectors of IL-6 (38, 39), we investigated whether IL-6 stimulation could activate STAT3 and induce MGMT expression in GBM cells. While IL-6 successfully phosphorylated STAT3, induction of MGMT could not be observed in any GBM cell line (Fig. 3B). mRNA expression levels of MGMT was mildly increased by IL-6 overexpression in KMG4 cells whereas IL-6 overexpression did not increase the level of MGMT in U138 cells (Fig. 3B).

As transcriptional regulation did not seem to be involved in STAT3-dependent MGMT expression, involvement of the protein degradation system was analyzed and partial recovery of MGMT was observed in T98G treated by proteasome inhibitor, MG132 (Fig. 3C). To confirm nontranscriptional role for STAT3-dependent expression of MGMT, we investigated whether ectopically expressed MGMT could be downregulated by STAT3



**Figure 3.** Posttranscriptional regulation of MGMT by STAT3. **A**, RT-PCR analysis of MGMT in T98G cells treated with 100 and 200 μmol/L of STAT3 inhibitor VI for 24 hours (top). GAPDH is shown as a loading control (bottom). **B**, **i**, immunoblot analysis of MGMT and p-STAT3 in KMG4, U138, and LN308 treated with 10 nmol/L of IL-6 for 24 hours (top and middle). Actin is shown as a loading control (bottom). **ii**, RT-PCR analysis of MGMT and IL-6 in KMG4, U138, and LN308 cells introduced MGMT or IL-6 by retroviral vector (top and middle). GAPDH is shown as a loading control (bottom). **iii**, immunoblot analysis of MGMT and p-STAT3 in KMG4, U138, and LN308 cells introduced IL-6 or MGMT by retroviral vector (top and middle). Actin is shown as a loading control (bottom). **C**, immunoblot analysis of MGMT by STAT3 inhibitor and its recovery by MG132 (top). MG132 was absent (–) or present at 20 μmol/L for 8 hours (+). STAT3 inhibitor VI (STAT3i-VI) was absent (–) or present at 100 μmol/L (+) or at 200 μmol/L (++) for 24 hours. The level of STAT3, p-STAT3 were also evaluated (second and third from the top). Actin is shown as a loading control (bottom). **D**, immunoblot analysis of MGMT and p-STAT3 in LN308 and LN308 cells constituting expressing MGMT by the treatment of STAT3 inhibitor VI for 24 hours (top and middle). Dose of STAT3 inhibitor VI is indicated at the top. Actin is shown as a loading control (bottom).

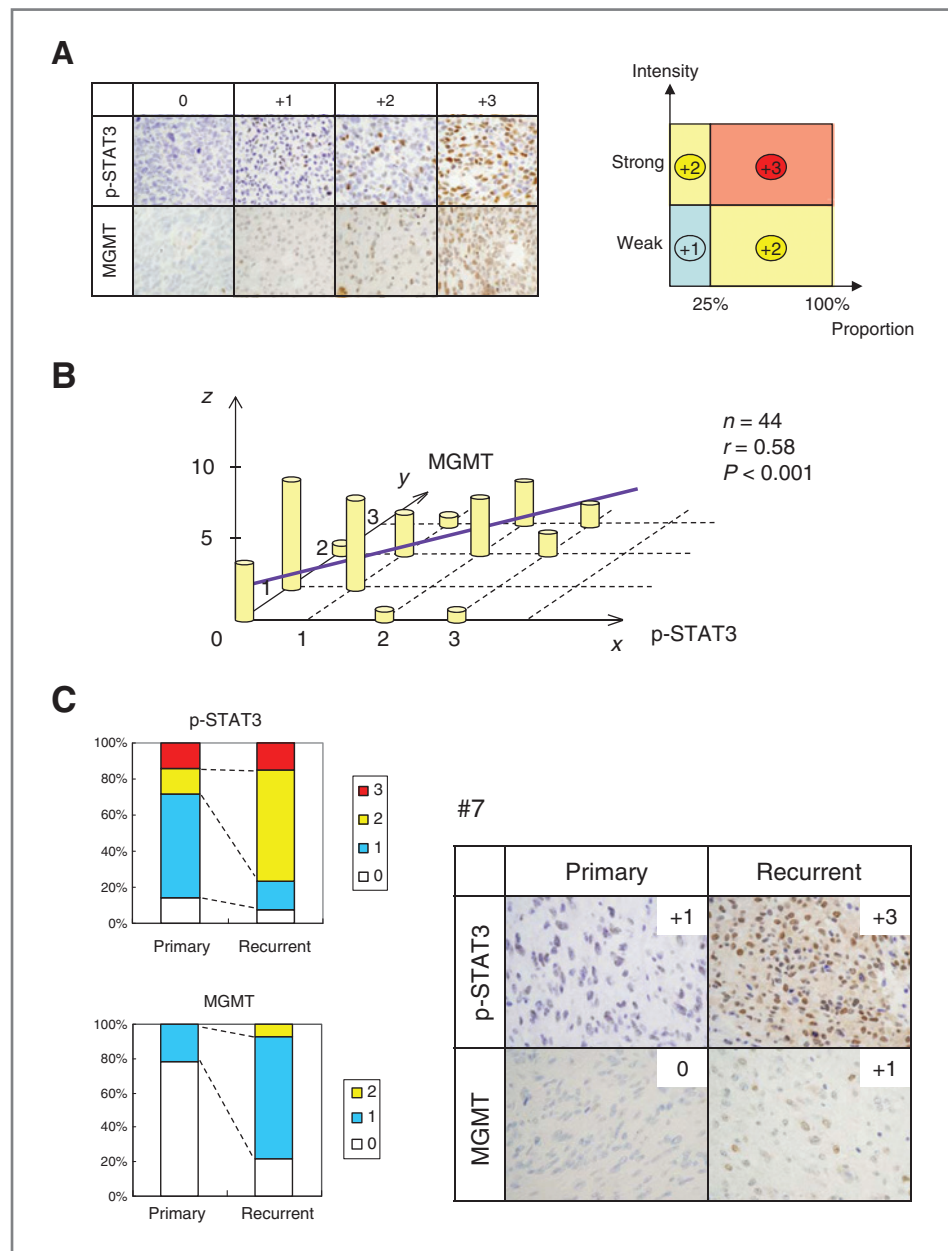
inhibitor, and observed the reduction of MGMT protein levels in LN308 cells stably expressing MGMT after STAT3 inhibitor VI treatment (Fig. 3D).

**Correlation between p-STAT3 and MGMT in malignant glioma by immunohistochemical analysis**

To investigate the presence of STAT3-dependent regulation of the levels of MGMT, immunohistochemical analysis was conducted by using 44 surgical specimens of human malignant glioma (Supplementary Table S1). Positivities of MGMT and p-STAT3 were evaluated as 0 to 3+ (Fig. 4A), and significant correlation between the score of p-STAT3 and MGMT was showed (Fig. 4B). Prolifer-

ation marker KI-67/MIB-1 index did not significantly correlate with MGMT levels (Supplementary Table S2). No specific localization of positive tumor cells was observed, in terms of the tumor cells in the perivascular region, leading edge of the lesion, or vicinity of pseudo-palisading necrosis. As we have previously reported that lymphocytes, vascular endothelial cells, and macrophages/microglia exhibited positivity of MGMT (13), and it was also reported that p-STAT3 staining was confined to the nucleus and positive for lymphocytes and vascular endothelial cells (40), we carefully scored MGMT and p-STAT3 of tumors excluding nonneoplastic brain components.

Figure 4. Correlation between p-STAT3 and MGMT in immunohistochemical analysis in malignant glioma. A, representative photographs of immunohistochemical analysis for p-STAT3 and MGMT in surgical specimens of malignant glioma as score 0 to 3+ (left). Each scoring was determined by combination of both intensity and proportion of immunohistochemical positivity schematically showed (right), and also described in Materials and Methods. B, correlation between p-STAT3 and MGMT in 44 cases of malignant glioma specimens. The x- and y-axes indicate score of positivity of p-STAT3 and MGMT, respectively. The z-axis indicates the number of cases.  $n = 44$ , correlation coefficient  $r = 0.58$ ,  $P < 0.001$ . C, immunohistochemical analysis of 12 cases of recurrent malignant glioma. The score of MGMT and p-STAT3 in primary or recurrent tumor was shown as graph. Photographs of p-STAT3 and MGMT in the representative case (no. #7) in which primary tumor with no p-STAT3 and recurrent tumor with p-STAT3 positivity together with MGMT expression are shown.



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To ensure clinical significance, we analyzed the 12 sets of malignant glioma as primary and recurrent tumors of identical cases. These primary tumors were surgically resected, temozolomide treatment was carried out, and later on, recurrent tumors appeared and they were surgically removed (Supplementary Table S3). In these sets, the scores of both MGMT and p-STAT3 were increased in the recurrent tumors (Fig. 4C).

### STAT3 inhibitor potentiated temozolomide efficacy in temozolomide-resistant GBM cell lines

To evaluate the possible combined therapy of temozolomide with STAT3 inhibitor, we treated the temozolomide-resistant cell line, T98G and U87R with temozolomide alone or with a combination of temozolomide and STAT3 inhibitor VI. As T98G cells possess a remarkable amount of MGMT, the viability of T98G cells after 5 days of treatment with 200  $\mu\text{mol/L}$  of temozolomide was more than 80%, indicating the resistance to temozolomide. In case of U87R cells, no significant growth inhibition was observed in range of 50 to 200  $\mu\text{mol/L}$  of temozolomide. In both T98G and U87R cells, combined treatment of temozolomide with STAT3 inhibitor was effective compared with temozolomide or STAT3 inhibitor alone (Fig. 5A). Furthermore, to ensure these results, we used the clonogenic survival assay and found that combination treatment with temozolomide and STAT3 inhibitor showed synergistic effect of these chemicals (Fig. 5B). Especially, temozolomide combined with 150 or 200  $\mu\text{mol/L}$  of STAT3 inhibitor exhibited a strong synergistic effect. These concentrations of STAT3 inhibitor are enough to decrease the protein levels of MGMT as shown in Fig. 2A. Temozolomide did not change the expression levels of MGMT, p-STAT3, and STAT3 (data not shown). These results suggest that STAT3 inhibitor attenuates temozolomide resistance in T98G cells by the decrease of protein levels of MGMT. For confirmation, we investigated whether STAT3 knock-down potentiates the therapeutic efficacy of temozolomide, and found that U138 cells in which STAT3 was depleted by shRNA were proved to be susceptible to temozolomide treatment compared with wild-type U138 cells exhibiting resistance to temozolomide (Fig. 5C).

### Discussion

In this study, we showed a novel role for STAT3 in regulation of cellular protein levels of MGMT. STAT3 activation was found to be necessary for elevated levels of MGMT in GBM. Although STAT3 is known as a transcriptional factor, STAT3-mediated regulation of MGMT did not depend on its transcriptional activity. Recently, it has been reported that active STAT3 is involved in protein stabilization through inhibition of the ubiquitylation of the target molecule (41), and consistent with this report, we observed that MGMT downregulation by STAT3 inhibitor was recovered partially by the proteasome inhibitor MG132 treatment. However, it should be noted that ubiquitylated levels of MGMT were unchanged after

STAT3 inhibitor treatment (data not shown). Complex formation of p-STAT3 and MGMT was not detectable by immunoprecipitation in regular cell lysis condition (data not shown). Therefore, other than protein degradation such as translational regulation of MGMT might be regulated by STAT3. Especially, recent advance in research for miRNA (miR) raised the possibility that STAT3 may regulate miRNAs which inhibit translation of MGMT. It has been reported that STAT3-regulated miR-17 played a critical role in MAP-ERK kinase (MEK) inhibitor resistance (42), as well as that STAT3 directly increased the levels of miR-21 and miR-181b-1 (43). Currently only correlation of some specific miRNA such as miR-21 or miR-195 and temozolomide resistance were reported (44, 45). We should address whether miRNAs are involved in the regulation of MGMT in the future.

In MGMT-overexpressed GBM cells, the increased levels of IL-6 and enhanced phosphorylation of STAT3 were observed (Fig. 3B and D). It was reported that IL-6 decreased the levels of MGMT by induction of the methylation of MGMT promoter in glioblastoma (46). Thus, overexpressed MGMT induces IL-6 possibly to decrease the MGMT levels as negative feedback mechanism to maintain constant MGMT levels in the cell. As a result, STAT3 is phosphorylated by IL-6. Further analysis should be required for the mechanism of MGMT-induced IL-6 expression.

For the relation between STAT3 status and representative molecular abnormalities for glioma such as amplification/mutation of EGFR, PDGFR, or PTEN, it was previously reported that constitutive active STAT3 frequently coexpressed with EGFR in high-grade gliomas (26). In this article, we used T98G and U87 cells and EGFR status is wild-type in U87 cells and the expression level of STAT3 was low. In T98G cells, EGFR is known to be amplified and STAT3 was highly expressed (47). In case of PTEN, both U87 and T98G have been shown to be mutated (48). Downregulation of STAT3 by PDGFR inhibitor was reported in mesangium cells in kidney (49), but there is no report for the correlation of PTEN/PDGFR and phosphorylation status of STAT3.

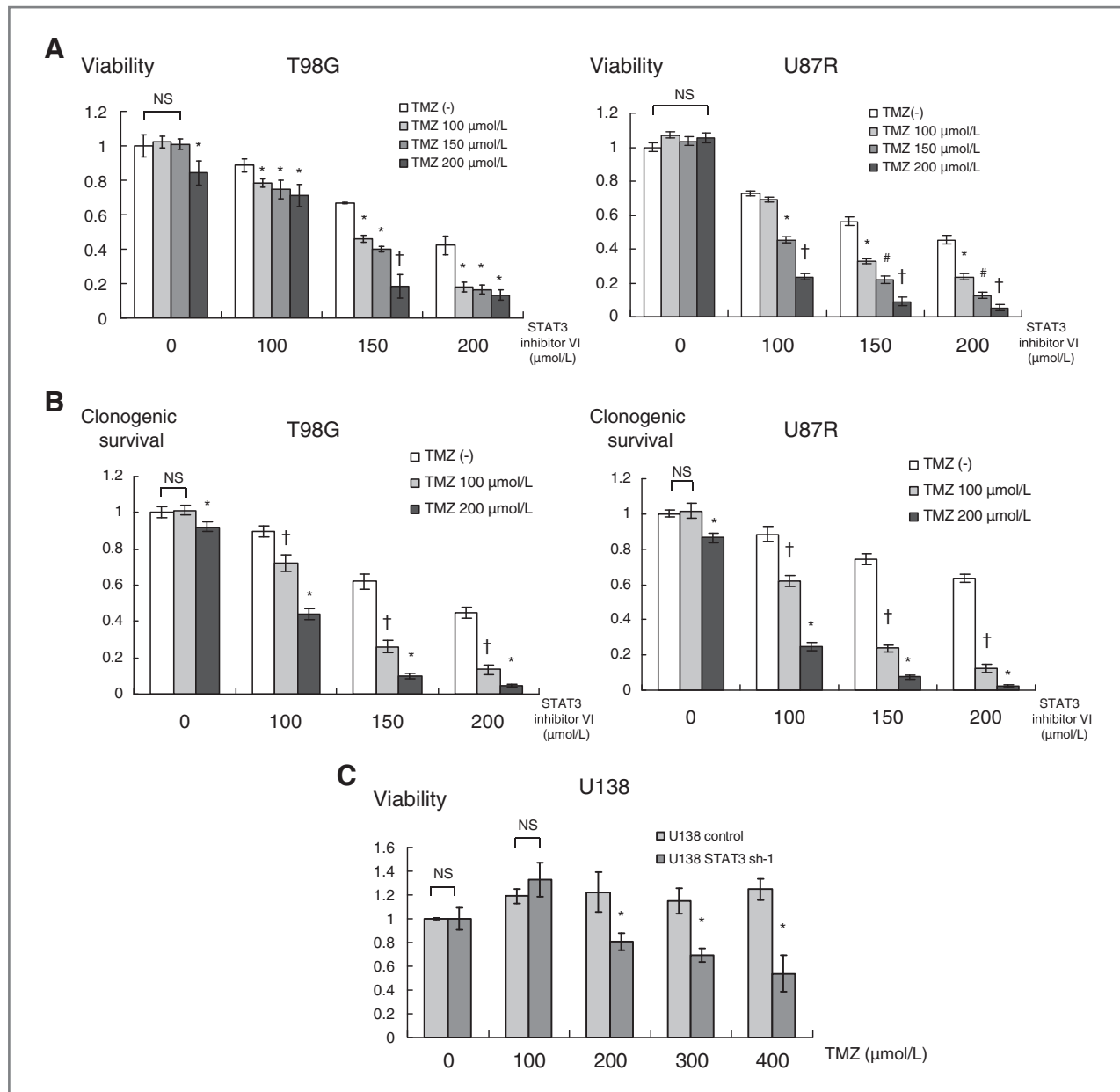
To ensure the biologic and clinical significance of possible STAT3-mediated regulation of MGMT, correlation between the elevated levels of MGMT and p-STAT3 was showed by using human malignant glioma specimens. Cellular proliferative marker KI-67 index was not correlated to MGMT or p-STAT3, thus enhancement of cell cycle is not essential factor for MGMT resistance. Although the double staining of MGMT and p-STAT3 is not technically available, the distributions of positivities of MGMT and p-STAT3 were carefully analyzed by using serial sections of the human GBM specimens. Further clinical significance of our data is shown by the increase of the levels of both MGMT and p-STAT3 observed in recurrent tumor comparing to the primary GBM of identical patients. To date, it was reported that targeting STAT3/JAK2 sensitizes GBM to treatment of temozolomide with unknown mechanism (26). In this study, STAT3 seems to regulate MGMT posttranscriptionally, thus it is



strongly suggested that STAT3 is a therapeutic target for temozolomide resistance in GBM.

For the evaluation of possible combination therapy for temozolomide-resistant GBM with temozolomide and STAT3 inhibitor, we examined the efficacy of several STAT3 inhibitors and found that STAT3 inhibitor VI potentiated temozolomide efficacy in temozolomide-

resistant glioma cell lines *in vitro*. In this study, we established temozolomide-resistant cell line by using U87 which is relatively sensitive for temozolomide. Considering the clinical condition, the maximum plasma concentration of the patients administrated temozolomide with daily dose of 150 mg/m<sup>2</sup>, was 8.22 µg/mL (42 µmol/L; ref. 50). The concentration of temozolomide in



**Figure 5.** STAT3 inhibitor and STAT3 knockdown potentiate temozolomide (TMZ) efficacy in temozolomide-resistant glioma cell lines. **A**, MTT assay of GBM treated by STAT3 inhibitor. T98G and U87R cells were cultured in the presence or absence of indicated concentration of STAT3 inhibitor and temozolomide for 5 days. Error bars represent SD of 3 independent experiments. \*,  $P < 0.01$  versus temozolomide (-); #,  $P < 0.01$  versus temozolomide 100 µmol/L; †,  $P < 0.01$  versus temozolomide 150 µmol/L. **B**, clonogenic assay of GBM treated by STAT3 inhibitor. Five hundred cells of T98G and U87R were seeded in 6-well plates and exposed to temozolomide (0–200 µmol/L) and STAT3 inhibitor VI (0–200 µmol/L) for 48 hours. After 7 days, colonies of more than 50 cells were counted. Error bars represent SD of 3 independent experiments. \*,  $P < 0.01$  versus temozolomide 100 µmol/L; †,  $P < 0.01$  versus temozolomide (-). **C**, MTT assay of GBM in which MGMT was suppressed by shRNA. U138 and U138-shSTAT3 cells were cultured in the presence or absence of indicated concentrations of temozolomide for 5 days. Error bars represent SD of 3 independent experiments. \*,  $P < 0.01$  versus U138 control. NS, not significant.

this experiment was approximately 40 to 150  $\mu\text{mol/L}$ , the alteration of the sensitivity to temozolomide of U87 and U87R might closely mimic the clinical concentrations. Comparing the several genes expression between U87 and U87R, we discovered upregulation of STAT3 expression in U87R.

We also observed that JAK inhibitor I downregulated the expression of MGMT. However, STAT3 phosphorylation and MGMT expression did not completely synchronize compared with STAT3 inhibitor VI. This might be because JAK inhibitor I also inhibited Jak1, Jak2, Jak3 and Tyk2 and blocked STAT5 (51), and these off-target effects possibly inhibited the decrease of MGMT.

In this study, we could not conclude that combination of temozolomide and STAT3 inhibitor VI was effective for the regression of GBM *in vivo*. In fact, even after STAT3 inhibitor treatment, neither inhibition of p-STAT3 nor MGMT was observed in mice xenograft (data not shown). One possible explanation why the STAT3 inhibitor did not function *in vivo* is the quick metabolism of STAT3 inhibitor VI *in vivo*. In addition, considering the short half-life of temozolomide *in vivo* as about 2 hours (52, 53), continuous supply of inhibitors for both temozolomide and STAT3 should be required for the appropriate evaluation of this combination therapy *in vivo*.

It has been reported that MGMT expression was suppressed by IFN- $\beta$  through the inactivation of transcriptional activity of p53 and suppressed the promoter of MGMT (54, 55). As our findings suggested the possible posttranscriptional suppression of MGMT protein levels by STAT3 inhibitor, one might hypothesized that combination of both IFN- $\beta$  and STAT3 inhibitor might further

potentiate temozolomide efficacy hopefully applied to GBM cases in the future.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Authors' Contributions

**Conception and design:** S. Kohsaka, M. Tanino, S. Tanaka

**Development of methodology:** S. Kohsaka, L. Wang, S. Tanaka

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S. Kohsaka, L. Wang, T. Narita, T. Itoh, M. Tanino, S. Tanaka

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S. Kohsaka, L. Wang, M. Tanino, S. Tanaka

**Writing, review, and/or revision of the manuscript:** S. Kohsaka, R. Mahabir, S. Tanaka

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** S. Kohsaka, K. Yachi, T. Narita, M. Tanino, H. Nishihara, S. Tanaka

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### References

- Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* 2007;114:97–109.
- Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. *CA Cancer J Clin* 2010;60:166–93.
- Mrugala MM, Chamberlain MC. Mechanisms of disease: temozolomide and glioblastoma—look to the future. *Nat Clin Pract Oncol* 2008;5:476–86.
- Cheng CL, Johnson SP, Keir ST, Quinn JA, Ali-Osman F, Szabo C, et al. Poly(ADP-ribose) polymerase-1 inhibition reverses temozolomide resistance in a DNA mismatch repair-deficient malignant glioma xenograft. *Mol Cancer Ther* 2005;4:1364–8.
- Trivedi RN, Almeida KH, Fornasaglio JL, Schamus S, Sobol RW. The role of base excision repair in the sensitivity and resistance to temozolomide-mediated cell death. *Cancer Res* 2005;65:6394–400.
- Kokkinakis DM, Bocangel DB, Schold SC, Moschel RC, Pegg AE. Thresholds of O6-alkylguanine-DNA alkyltransferase which confer significant resistance of human glial tumor xenografts to treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea or temozolomide. *Clin Cancer Res* 2001;7:421–8.
- Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med* 2005;352:997–1003.
- Ranson M, Middleton MR, Bridgewater J, Lee SM, Dawson M, Jowle D, et al. Lomeguatrib, a potent inhibitor of O6-alkylguanine-DNA-alkyltransferase: phase I safety, pharmacodynamic, and pharmacokinetic trial and evaluation in combination with temozolomide in patients with advanced solid tumors. *Clin Cancer Res* 2006;12:1577–84.
- Khan O, Middleton MR. The therapeutic potential of O6-alkylguanine DNA alkyltransferase inhibitors. *Expert Opin Investig Drugs* 2007;16:1573–84.
- Quinn JA, Desjardins A, Weingart J, Brem H, Dolan ME, Delaney SM, et al. Phase I trial of temozolomide plus O6-benzylguanine for patients with recurrent or progressive malignant glioma. *J Clin Oncol* 2005;23:7178–87.
- Quinn JA, Jiang SX, Reardon DA, Desjardins A, Vredenburgh JJ, Rich JN, et al. Phase II trial of temozolomide plus O6-benzylguanine in adults with recurrent, temozolomide-resistant malignant glioma. *J Clin Oncol* 2009;27:1262–7.
- Sasai K, Akagi T, Aoyanagi E, Tabu K, Kaneko S, Tanaka S. O6-methylguanine-DNA methyltransferase is downregulated in transformed astrocyte cells: implications for anti-glioma therapies. *Mol Cancer* 2007;6:36.
- Sasai K, Nodagashira M, Nishihara H, Aoyanagi E, Wang L, Katoh M, et al. Careful exclusion of non-neoplastic brain components is required for an appropriate evaluation of O6-methylguanine-DNA methyltransferase status in glioma: relationship between immunohistochemistry and methylation analysis. *Am J Surg Pathol* 2008;32:1220–7.
- Rao RD, James CD. Altered molecular pathways in gliomas: an overview of clinically relevant issues. *Semin Oncol* 2004;31:595–604.
- Alcantara Llaguno SR, Chen J, Parada LF. Signaling in malignant astrocytomas: role of neural stem cells and its therapeutic implications. *Clin Cancer Res* 2009;15:7124–9.

16. Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, et al. Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev* 2007;21:2683–710.
17. Brantley EC, Benveniste EN. Signal transducer and activator of transcription-3: a molecular hub for signaling pathways in gliomas. *Mol Cancer Res* 2008;6:675–84.
18. Yu CL, Meyer DJ, Campbell GS, Larner AC, Carter-Su C, Schwartz J, et al. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. *Science* 1995;269:81–3.
19. Bromberg J. Stat proteins and oncogenesis. *J Clin Invest* 2002;109:1139–42.
20. Yu H, Jove R. The STATs of cancer—new molecular targets come of age. *Nat Rev Cancer* 2004;4:97–105.
21. Dasgupta A, Raychaudhuri B, Haqqi T, Prayson R, Van Meir EG, Vogelbaum M, et al. Stat3 activation is required for the growth of U87 cell-derived tumours in mice. *Eur J Cancer* 2009;45:677–84.
22. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006;9:157–73.
23. Tso CL, Shintaku P, Chen J, Liu Q, Liu J, Chen Z, et al. Primary glioblastomas express mesenchymal stem-like properties. *Mol Cancer Res* 2006;4:607–19.
24. Carro MS, Lim WK, Alvarez MJ, Bollo RJ, Zhao X, Snyder EY, et al. The transcriptional network for mesenchymal transformation of brain tumours. *Nature* 2010;463:318–25.
25. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010;17:98–110.
26. Lo HW, Cao X, Zhu H, Ali-Osman F. Constitutively activated STAT3 frequently coexpresses with epidermal growth factor receptor in high-grade gliomas and targeting STAT3 sensitizes them to Iressa and alkylators. *Clin Cancer Res* 2008;14:6042–54.
27. Wei D, Le X, Zheng L, Wang L, Frey JA, Gao AC, et al. Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis. *Oncogene* 2003;22:319–29.
28. Niu G, Wright KL, Huang M, Song L, Haura E, Turkson J, et al. Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. *Oncogene* 2002;21:2000–8.
29. Sinibaldi D, Wharton W, Turkson J, Bowman T, Pledger WJ, Jove R. Induction of p21WAF1/CIP1 and cyclin D1 expression by the Src oncoprotein in mouse fibroblasts: role of activated STAT3 signaling. *Oncogene* 2000;19:5419–27.
30. Wang L, Tabu K, Kimura T, Tsuda M, Linghu H, Tanino M, et al. Signaling adaptor protein Crk is indispensable for malignant feature of glioblastoma cell line KMG4. *Biochem Biophys Res Commun* 2007;362:976–81.
31. Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC, et al. Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. *Brain Pathol* 1999;9:469–79.
32. Albertoni M, Daub DM, Arden KC, Viars CS, Powell C, Van Meir EG. Genetic instability leads to loss of both p53 alleles in a human glioblastoma. *Oncogene* 1998;16:321–6.
33. Kohsaka S, Sasai K, Takahashi K, Akagi T, Tanino M, Kimura T, et al. A population of BJ fibroblasts escaped from Ras-induced senescence susceptible to transformation. *Biochem Biophys Res Commun* 2011;410:878–84.
34. Akagi T, Sasai K, Hanafusa H. Refractory nature of normal human diploid fibroblasts with respect to oncogene-mediated transformation. *Proc Natl Acad Sci U S A* 2003;100:13567–72.
35. Hermisson M, Klumpp A, Wick W, Wischhusen J, Nagel G, Roos W, et al. O6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. *J Neurochem* 2006;96:766–76.
36. Wagner S, Stegen C, Bouterfa H, Huettner C, Kerkau S, Roggendorf W, et al. Expression of matrix metalloproteinases in human glioma cell lines in the presence of IL-10. *J Neurooncol* 1998;40:113–22.
37. Liu Q, Li G, Li R, Shen J, He Q, Deng L, et al. IL-6 promotion of glioblastoma cell invasion and angiogenesis in U251 and T98G cell lines. *J Neurooncol* 2010;100:165–76.
38. Grivennikov S, Karin M. Autocrine IL-6 signaling: a key event in tumorigenesis? *Cancer Cell* 2008;13:7–9.
39. Gao SP, Mark KG, Leslie K, Pao W, Motoi N, Gerald WL, et al. Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas. *J Clin Invest* 2007;117:3846–56.
40. Abou-Ghazal M, Yang DS, Qiao W, Reina-Ortiz C, Wei J, Kong LY, et al. The incidence, correlation with tumor-infiltrating inflammation, and prognosis of phosphorylated STAT3 expression in human gliomas. *Clin Cancer Res* 2008;14:8228–35.
41. Jung JE, Kim HS, Lee CS, Shin YJ, Kim YN, Kang GH, et al. STAT3 inhibits the degradation of HIF-1alpha by pVHL-mediated ubiquitination. *Exp Mol Med* 2008;40:479–85.
42. Dai B, Meng J, Peyton M, Girard L, Bornmann WG, Ji L, et al. STAT3 mediates resistance to MEK inhibitor through microRNA miR-17. *Cancer Res* 2011;71:3658–68.
43. Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Mol Cell* 2010;39:493–506.
44. Shi L, Chen J, Yang J, Pan T, Zhang S, Wang Z. MiR-21 protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase-3 activity. *Brain Res* 2010;1352:255–64.
45. Ujifuku K, Mitsutake N, Takakura S, Matsuse M, Saenko V, Suzuki K, et al. miR-195, miR-455-3p and miR-10a(\*) are implicated in acquired temozolomide resistance in glioblastoma multiforme cells. *Cancer Lett* 2010;296:241–8.
46. Piperi C, Themistocleous MS, Papavassiliou GA, Farmaki E, Levidou G, Korkolopoulou P, et al. High incidence of MGMT and RARbeta promoter methylation in primary glioblastomas: association with histopathological characteristics, inflammatory mediators and clinical outcome. *Mol Med* 2010;16:1–9.
47. Carrasco-Garcia E, Saceda M, Grasso S, Rocamora-Reverte L, Conde M, Gomez-Martinez A, et al. Small tyrosine kinase inhibitors interrupt EGFR signaling by interacting with erbB3 and erbB4 in glioblastoma cell lines. *Exp Cell Res* 2011;317:1476–89.
48. Sinn B, Tallen G, Schroeder G, Grassl B, Schulze J, Budach V, et al. Caffeine confers radiosensitization of PTEN-deficient malignant glioma cells by enhancing ionizing radiation-induced G1 arrest and negatively regulating Akt phosphorylation. *Mol Cancer Ther* 2010;9:480–8.
49. Hirai T, Masaki T, Kuratsune M, Yorioka N, Kohno N. PDGF receptor tyrosine kinase inhibitor suppresses mesangial cell proliferation involving STAT3 activation. *Clin Exp Immunol* 2006;144:353–61.
50. Beale P, Judson I, Moore S, Statkevich P, Marco A, Cutler DL, et al. Effect of gastric pH on the relative oral bioavailability and pharmacokinetics of temozolomide. *Cancer Chemother Pharmacol* 1999;44:389–94.
51. Lucet IS, Fantino E, Styles M, Bamert R, Patel O, Broughton SE, et al. The structural basis of Janus kinase 2 inhibition by a potent and specific pan-Janus kinase inhibitor. *Blood* 2006;107:176–83.
52. Hammond LA, Eckardt JR, Baker SD, Eckhardt SG, Dugan M, Forral K, et al. Phase I and pharmacokinetic study of temozolomide on a daily-for-5-days schedule in patients with advanced solid malignancies. *J Clin Oncol* 1999;17:2604–13.
53. Britten CD, Rowinsky EK, Baker SD, Agarwala SS, Eckardt JR, Barrington R, et al. A Phase I and pharmacokinetic study of temozolomide and cisplatin in patients with advanced solid malignancies. *Clin Cancer Res* 1999;5:1629–37.
54. Motomura K, Natsume A, Kishida Y, Higashi H, Kondo Y, Nakasu Y, et al. Benefits of interferon-beta and temozolomide combination therapy for newly diagnosed primary glioblastoma with the unmethylated MGMT promoter: a multicenter study. *Cancer* 2011;117:1721–30.
55. Natsume A, Ishii D, Wakabayashi T, Tsuno T, Hatano H, Mizuno M, et al. IFN-beta down-regulates the expression of DNA repair gene MGMT and sensitizes resistant glioma cells to temozolomide. *Cancer Res* 2005;65:7573–9.