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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 (TMZ) concomitant with surgical resection and/or irradiation. However, a number of cases are resistant to TMZ-induced DNA damage due to elevated expression of the DNA repair enzyme O⁶-methylguanine-DNA methyltransferase (MGMT). Here we show that upregulation of both MGMT and STAT3 was accompanied with acquisition of TMZ resistance in the GBM cell line U87. Inactivation of STAT3 by inhibitor or shRNA downregulated MGMT expression in GBM cell lines. MGMT upregulation was not observed by the treatment of 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 demonstrated significant positive correlation between expression levels of MGMT and phosphorylated STAT3 (pSTAT3) ($p < 0.001$, $r = 0.58$). Importantly, the levels of both MGMT and pSTAT3 were increased in the recurrence compared to the primary lesion in paired identical tumors of 12 cases. Finally, we demonstrated that STAT3 inhibitor or STAT3 knockdown potentiated TMZ efficacy in TMZ-resistant GBM cell lines. Therefore, STAT3 inhibitor might be one of the candidate reagents for combination therapy with TMZ for TMZ-resistant GBM patients.

Introduction

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

Temozolomide (TMZ) is an alkylating agent applied to malignant glioma including GBM (3). TMZ induces DNA methylation of guanine at O⁶ position and O⁶-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). O⁶-methylguanine methyltransferase (MGMT) removes methylation from O⁶ position of guanine and induces TMZ 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 TMZ treatment together with irradiation (7). Despite pseudosubstrates of MGMT such as O⁶-benzylguanine were expected to suppress resistance by depleting MGMT (8-10), clinical trials did not show significant restoration of TMZ sensitivity in patients with TMZ-resistant GBM (11). Therefore, other therapeutic agents which suppress MGMT expression and sensitize the efficacy of TMZ are highly desired (12, 13).

It is well known that receptor tyrosine kinases including the epidermal growth factor receptor (EGFR), platelet derived growth factor receptor α (PDGFR α), and the vascular endothelial growth factor receptor (VEGFR), contribute to the growth of GBM through the activation of Ras/ERK- or phosphatidylinositide-3 kinase (PI3K)/AKT-dependent signalling pathways (14-16). These diverse signals converge at specific transcription factors, including signal transducer and activator of transcription-3 (STAT3) (17). STAT3 was initially identified as a signal mediator for interferon stimulation with various gene targets 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 >74% of the mesenchymal gene expression signature gene (24). Further, 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). Further, 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 TMZ 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 TMZ, we generated TMZ-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 TMZ-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 (USA). KMG4 cell line was kindly provided by Dr. Kazuo Tabuchi (Saga University, Japan) (30). LN382, LN308, and LN235 cell lines were kindly provided by Dr. Erwin G. Van Meir (Emory University School of Medicine, Atlanta, Georgia) (31, 32). All cell lines were cultured in Dulbecco's modified minimal essential medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS). Cell line authentication was not carried out by the authors within the last 6 months.

Reagents

STAT3 inhibitor III WP1066 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), STAT3 inhibitor V Stattic (Santa Cruz Biotechnology, Santa Cruz, CA, USA), STAT3 inhibitor VI NSC 74859 (Calbiochem, La Jolla, USA), JAK inhibitor I (Calbiochem, La Jolla, USA), MET inhibitor PHA 665752 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Src-family kinase inhibitor dasatinib (LC Laboratories, Woburn, MA, USA) 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 IL-6 (Sigma, St. Louis, MO, USA) 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, three sequences were designed by the BLOCK-It RNAi Designer (Invitrogen, Carlsbad, CA, USA). The preparation of lentiviral vectors expressing human STAT3 short hairpin RNA (shRNA) was performed using the BLOCK-It Lentiviral RNAi Expression System (catalog no. K4934-00; Invitrogen, Carlsbad, CA, USA), following the manufacturer's instruction. Briefly, three 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, Carlsbad, CA, USA) containing an optimized mixture of three packaging plasmids: pLP1, pLP2, and pLP/VSVG) into 293FT cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 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).

BrdU Assays

Bromodeoxyuridine (BrdU) labeling enzyme linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) were used to examine the effects of TMZ on the proliferation of glioma cell lines. BrdU is a thymidine nucleotide analog 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/well and were incubated with

DMEM supplemented with 10% FBS in the presence or absence of TMZ for 5 days. The rate of BrdU incorporation was measured by ELISA technique, and the percentage of cells in S-phase quantitated by colorimetric evaluation ($\lambda = 450 \text{ nm}$). The absorbance of cells treated with 10% FBS without TMZ was assumed to be 100%, and TMZ-induced decreases in S-phase were calculated as a percentage of the control FBS treated cells. Each sample was performed 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, MI, USA). Cells were plated in 96-well plates at a density of 2,500 cells/well. The cells were cultured in 100 μl of the medium with indicated concentration of TMZ. After 5 days, 10 μl 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 μl 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, Richmond, CA, USA) at a wavelength of 570 nm. Each sample was performed in triplicate, and the entire experiment was repeated twice.

Clonogenic assay

Clonogenic survival assays were performed by seeding 500 cells in six well plates and exposing them to TMZ (10–1000 μM) for 48 h, followed by further observation for 7–14 days. Cell density or colonies were assessed using 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 using a Boyden chamber transwell assay (8 μm pore size; Corning Costar, Cambridge, MA, USA) using uncoated filters. 5×10^4 cells were trypsinized and introduced into the upper chamber. After incubation for 4 h 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 five random fields (20 $\times$) of each membrane. Experiments were done in triplicate.

Immunoblotting

Immunoblotting was performed by the method described elsewhere. Cells were lysed with buffer containing 0.5% NP40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM PMFS, 1 mmol/L Na_3VO_4 . Proteins were subjected to SDS-PAGE, and separated proteins were transferred to a polyvinylidene difluoride filter (Immobilon-P; Millipore, Billerica, MA, USA). Filters were probed with antibodies obtained from the following sources: anti-STAT3 monoclonal antibodies (MAb) (Cell Signaling Technology, Beverly, MA, USA); anti- MGMT (MT3.1) MAb and anti-actin MAb (Chemicon, Temecula, CA, USA); anti-phospho-STAT3 (Tyr705) polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA). 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, Freiburg, Germany).

Reverse Transcription-PCR analysis

Total RNA was isolated from cells using TRI-Reagent (Sigma, St. Louis, MO, USA) and resuspended in RNA secure resuspension solution (Ambion, Austin, TX, USA). Reverse transcription was carried out with Superscript II RT (Invitrogen, Carlsbad, CA, USA).

Resulting first-strand cDNA was used as a template and amplified by PCR using KOD-Plus DNA polymerase (Toyobo, Japan). 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 MGMT; 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, Temecula, CA, USA) and anti-phospho-STAT3 (Tyr705) polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA). 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

1. Establishment of TMZ-resistant U87 GBM cell line.

First, the expression levels of MGMT and TMZ sensitivity in seven GBM cell lines were investigated, and three of them T98G, U138, and LN382, were found to highly express MGMT compared to the other four 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 TMZ (Fig.1B, Supplementary Fig. 1). To identify the responsible molecules for TMZ resistance, comparatively TMZ-sensitive U87 cell line was treated with a low dose of TMZ in culture media for three weeks and established TMZ-resistant cells designated as U87R. IC₅₀ for the growth inhibition of TMZ to U87 was less than 40 μ M, but it was more than 150 μ M in U87R (Supplementary Fig. 2A). To ensure the TMZ-resistance of U87R cells, we employed clonogenic survival assay (35), and found that the IC₅₀ of U87 was less than 10 μ M and that of U87R was more than 400 μ M, suggesting that TMZ sensitivity was altered more than 40-folds decrease in resistant cells (Supplementary Fig. 2B). According to the increase of resistance, protein levels of MGMT were elevated in U87R cells (Fig.1C).

As U87R seemed to exhibit morphological changed as cuboidal cells (Fig.1D), we examined biological 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 to U87 cells (Fig.1F). These results suggested that U87R cells possess the mesenchymal signature of glioblastoma (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).

2. STAT3 inhibitor downregulated MGMT expression in GBM cell lines

As concomitant upregulation of STAT3 with MGMT was observed with acquisition of TMZ 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 demonstrated (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).

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 and Supplementary Fig. 3).

3. 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 non-transcriptional role for STAT3 dependent expression of MGMT, we investigated whether ectopically expressed MGMT could be downregulated by STAT3 inhibitor, and observed the reduction of MGMT protein levels in LN308 cells stably expressing MGMT after STAT3 inhibitor VI treatment (Fig. 3D).

4. Correlation between pSTAT3 and MGMT in malignant glioma by immunohistochemical analysis

To investigate the presence of STAT3-dependent regulation of the levels of MGMT, immunohistochemical analysis was performed by using 44 surgical specimens of human malignant glioma (Supplementary table 1). Positivities of MGMT and pSTAT3 were evaluated as 0 to 3+ (Fig. 4A), and significant correlation between the score of pSTAT3 and MGMT was demonstrated (Fig. 4B). Proliferation marker KI-67/MIB-1 index did not significantly correlate with MGMT levels (Supplementary table 2). 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 pseudopalisading 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 pSTAT3 staining was confined to the nucleus and positive for lymphocytes and vascular endothelial cells (40), we carefully scored MGMT and pSTAT3 of tumors excluding non-neoplastic brain components.

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, TMZ treatment was performed, and later on, recurrent tumors appeared and they were surgically removed (Supplementary table 3). In these sets, the scores of both MGMT and pSTAT3 were increased in the recurrent tumors (Fig. 4C).

5. STAT3 inhibitor potentiated TMZ efficacy in TMZ-resistant GBM cell lines

To evaluate the possible combined therapy of TMZ with STAT3 inhibitor, we treated the TMZ-resistant cell line, T98G and U87R with TMZ alone or with a combination of TMZ 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 μ M of TMZ was more than 80%, indicating the resistance to TMZ. In case of U87R cells, no significant growth inhibition was observed in range of 50-200 μ M of TMZ. In both T98G and U87R cells, combined treatment of TMZ with STAT3 inhibitor was effective compared to TMZ or STAT3 inhibitor alone (Fig. 5A). Furthermore, to ensure these results, we employed the clonogenic survival assay and found that combination treatment with TMZ and STAT3 inhibitor showed synergistic effect of these chemicals (Fig 5B). Especially, TMZ combined with 150 μ M or 200 μ M 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. TMZ did not change the expression levels of MGMT, pSTAT3 and STAT3 (data not shown). These results suggest that STAT3 inhibitor attenuates TMZ resistance in T98G cells by the decrease of protein levels of MGMT. For confirmation, we investigated whether STAT3 knockdown potentiates the therapeutic efficacy of TMZ, and found that U138 cells in which

STAT3 was depleted by shRNA were proved to be susceptible to TMZ treatment compared to wild type U138 cells exhibiting resistance to TMZ (Fig 5C).

Discussion

In this study, we demonstrated 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 pSTAT3 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 microRNA (miR) raised the possibility that STAT3 may regulate microRNAs which inhibit translation of MGMT. It has been reported that STAT3-regulated miR-17 played a critical role in 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 microRNA such as miR-21 or miR-195 and TMZ resistance were reported (44, 45). We should address whether microRNAs 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, 3D). 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 paper, 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). Down-regulation of STAT3 by PDGFR 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 biological and clinical significance of possible STAT3-mediated regulation of MGMT, correlation between the elevated levels of MGMT and pSTAT3 was demonstrated by using human malignant glioma specimens. Cellular proliferative marker KI67 index was not correlated to MGMT or pSTAT3, thus enhancement of cell cycle is not essential factor for MGMT resistance. Although the double staining of MGMT and pSTAT3 is not technically available, the distributions of positivities of MGMT and pSTAT3 were carefully analyzed by using serial sections of the human GBM specimens. Further clinical significance of our data is demonstrated by the increase of the levels of both MGMT and pSTAT3 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 TMZ with unknown mechanism (26). In this study, STAT3 seems to regulate MGMT post-transcriptionally, thus it is strongly suggested that STAT3 is a therapeutic target for TMZ resistance in GBM.

For the evaluation of possible combination therapy for TMZ-resistant GBM with TMZ and STAT3 inhibitor, we examined the efficacy of several STAT3 inhibitors and found that STAT3 inhibitor VI potentiated TMZ efficacy in TMZ-resistant glioma cell lines *in vitro*. In this study, we established TMZ resistant cell line by using U87 which is relatively sensitive

for TMZ. Considering the clinical condition, the maximum plasma concentration of the patients administrated TMZ with daily dose of 150 mg/m², was 8.22 µg/ml (42 µM) (50). The concentration of TMZ in this experiment was approximately 40 to 150 µM, the alteration of the sensitivity to TMZ 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 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 TMZ and STAT3 inhibitor VI was effective for the regression of GBM *in vivo*. In fact, even after STAT3 inhibitor treatment, neither inhibition of pSTAT3 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 TMZ *in vivo* as about 2 hours (52, 53), continuous supply of inhibitors for both TMZ 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 interferon-β 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 interferon-β and STAT3 inhibitor might further potentiate TMZ efficacy hopefully applied to GBM cases in the future.

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Figure legend

Fig. 1 Establishment of TMZ-resistant U87 GBM cell line

(A) Immunoblot analysis of MGMT in seven glioblastoma cell lines, T98G, LN382, U138, KMG4, LN308, LN235, and U87 (upper panel). Actin is shown as a loading control (lower panel). (B) Evaluation of TMZ resistance by BrdU assay. Seven glioblastoma cell lines were cultured for 5 days in the presence of 60 μ M of TMZ. BrdU incorporation indicates proliferation of cells. Error bars represent standard deviation (SD) of three independent experiments. Right panel indicates the structure of TMZ. (C) Immunoblot analysis of MGMT in U87 and U87R cells (upper panel). Tubulin is shown as a loading control (lower panel). (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 μ m pores after 4 hours incubation was counted in five random fields ($\times 20$) of each membrane. Error bars represent SD of three independent experiments. * $p < 0.01$ U87 vs. U87R. (F) 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 panel). (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 panel).

Fig. 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 panel). Dose of the STAT3 inhibitor is indicated at the top. The level of pSTAT3 and STAT3 were also evaluated (2nd and 3rd from the top panel). Actin is shown as a loading control (bottom panel). (B) Immunoblot analysis of MGMT in T98G treated with 200 μ M of STAT3 inhibitor VI. Duration of the treatment is indicated at the top as 0 to 48hr.

Medium change indicated removal of STAT3 inhibitor. The level of pSTAT3 was also evaluated (middle panel). Actin is shown as a loading control (bottom panel). (C) Immunoblot analysis of MGMT and STAT3 in T98G, U87R and U138 expressing STAT3 shRNA as S3sh1, S3sh2 and S3sh3 (upper and middle panel). Cont indicates negative control. Actin is shown as a loading control (bottom panel). (D) Immunoblot analysis of MGMT (top panel) and pSTAT3 (middle panel) 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 panel).

Fig. 3 Posttranscriptional regulation of MGMT by STAT3

(A) RT-PCR analysis of MGMT in T98G cells treated with 100 μ M and 200 μ M of STAT3 inhibitor VI for 24 hours (upper panel). GAPDH is shown as a loading control (lower panel). (B) (i) Immunoblot analysis of MGMT and pSTAT3 in KMG4, U138, and LN308 treated with 10 μ M of IL-6 for 24 hours (upper and middle panels). Actin is shown as a loading control (bottom panel). (ii) RT-PCR analysis of MGMT and IL-6 in KMG4, U138, and LN308 cells introduced MGMT or IL-6 by retroviral vector (upper and middle panels). GAPDH is shown as a loading control (bottom panel). (iii) Immunoblot analysis of MGMT and pSTAT3 in KMG4, U138, and LN308 cells introduced IL-6 or MGMT by retroviral vector (upper and middle panels). Actin is shown as a loading control (bottom panel). (C) Immunoblot analysis of MGMT by STAT3 inhibitor and its recovery by MG132 (top panel). MG132 was absent (-), or present at 20 μ M for 8 hours (+). STAT3 inhibitor VI was absent (-), or present at 100 μ M (+) or at 200 μ M (++) for 24 hours. The level of STAT3, pSTAT3 were also evaluated (2nd and 3rd from the top panel). Actin is shown as a loading control (bottom panel). (D) Immunoblot analysis of MGMT and pSTAT3 in LN308 and LN308 cells constituting expressing MGMT by the treatment of STAT3 inhibitor VI for 24 hours (upper and middle panel). Dose of STAT3 inhibitor VI is indicated at the top. Actin is

shown as a loading control (bottom panel).

Fig. 4 Correlation between pSTAT3 and MGMT in immunohistochemical analysis in malignant glioma

(A) Representative photographs of immunohistochemical analysis for pSTAT3 and MGMT in surgical specimens of malignant glioma as score 0 to 3+ (left panel). Each scoring was determined by combination of both intensity and proportion of immunohistochemical positivity schematically demonstrated as right panel, and also described in Materials and Methods. (B) Correlation between pSTAT3 and MGMT in 44 cases of malignant glioma specimens. x and y axes indicate score of positivity of pSTAT3 and MGMT, respectively. 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 pSTAT3 in primary or recurrent tumor was shown as graph. Photographs of pSTAT3 and MGMT in the representative case (No.#7) in which primary tumor with no pSTAT3 and recurrent tumor with pSTAT3 positivity together with MGMT expression are shown.

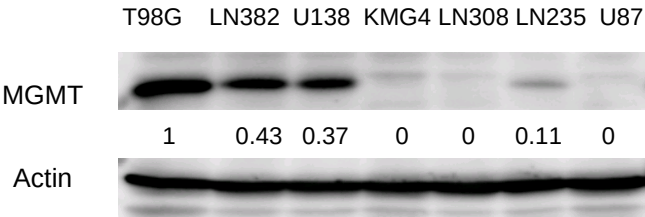
Fig. 5 STAT3 inhibitor and STAT3 knockdown potentiate TMZ efficacy in TMZ-resistant glioma cell lines

(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 TMZ for 5 days. Error bars represent SD of three independent experiments. * $p < 0.01$ vs. TMZ(-), # $p < 0.01$ vs. TMZ 100 μ M, † $p < 0.01$ vs. TMZ 150 μ M. (B) Clonogenic assay of GBM treated by STAT3 inhibitor. 500 cells of T98G and U87R were seeded in six well plates and exposed to TMZ (0-200 μ M) and STAT3 inhibitor VI (0-200 μ M) for 48 hours. After 7 days, colonies of more than 50 cells were counted. Error bars represent SD of three independent

experiments. * $p < 0.01$ vs. TMZ 100 μM , † $p < 0.01$ vs. TMZ (-). (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 TMZ for 5 days. Error bars represent SD of three independent experiments. * $p < 0.01$ vs. U138 control.

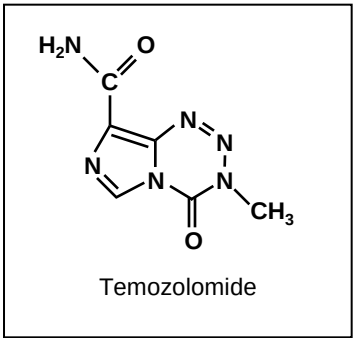
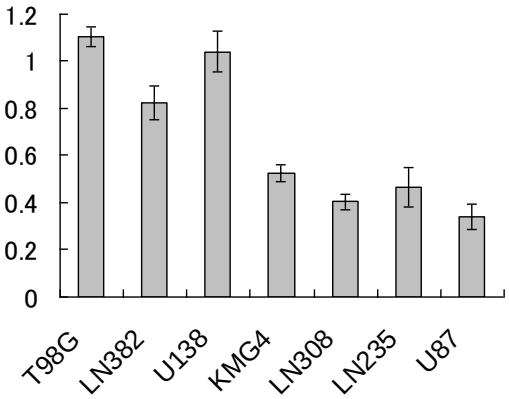
Fig. 1

A

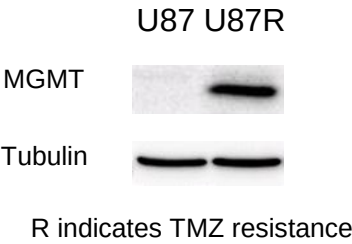


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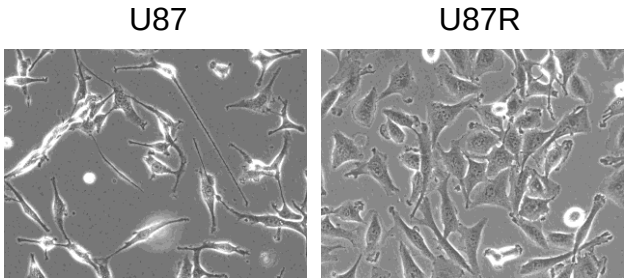
Proliferation BrdU assay



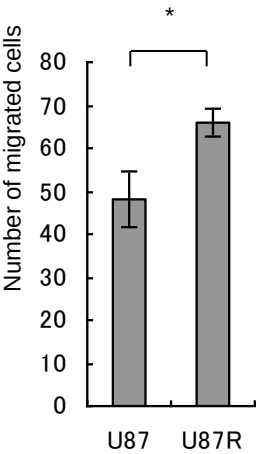
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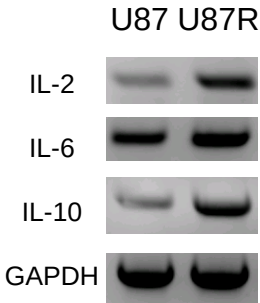
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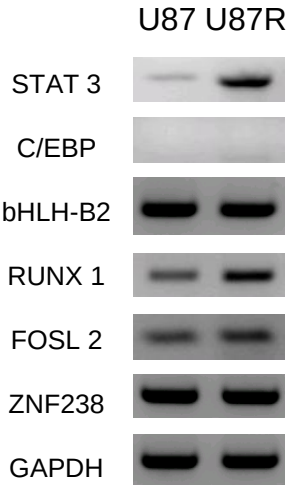
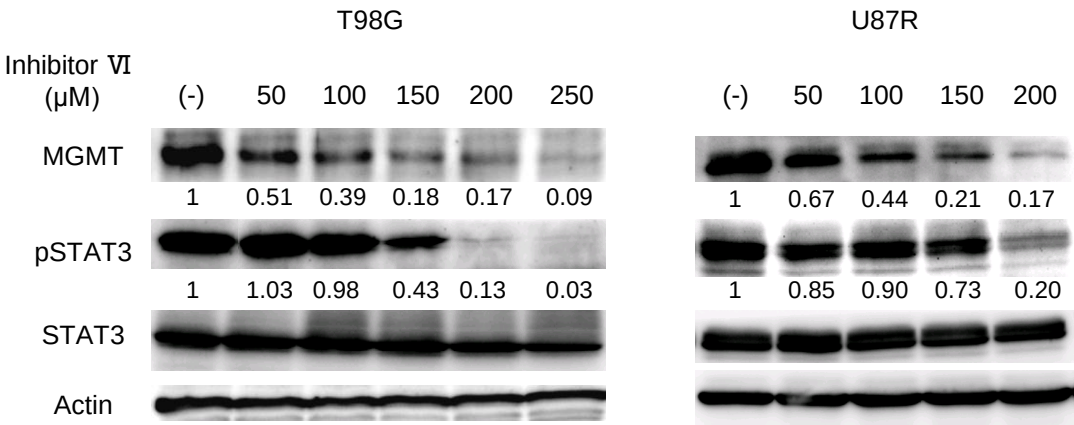
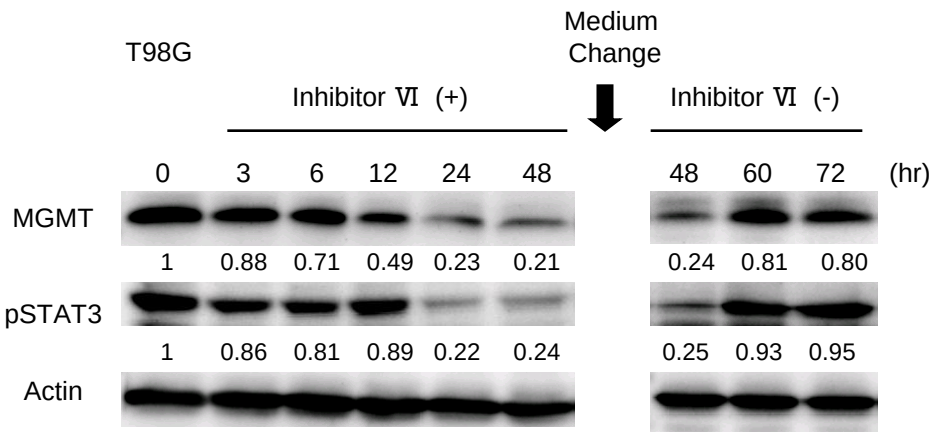


Fig. 2

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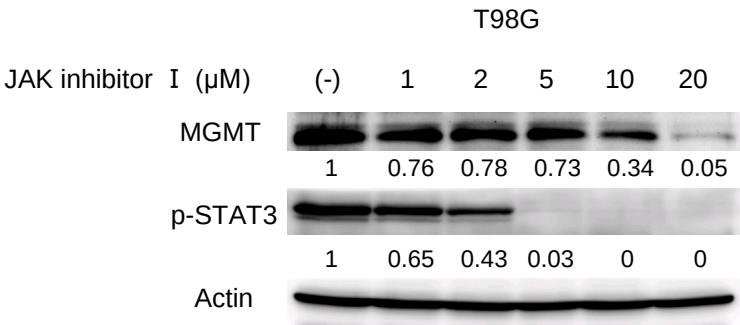


Fig. 3

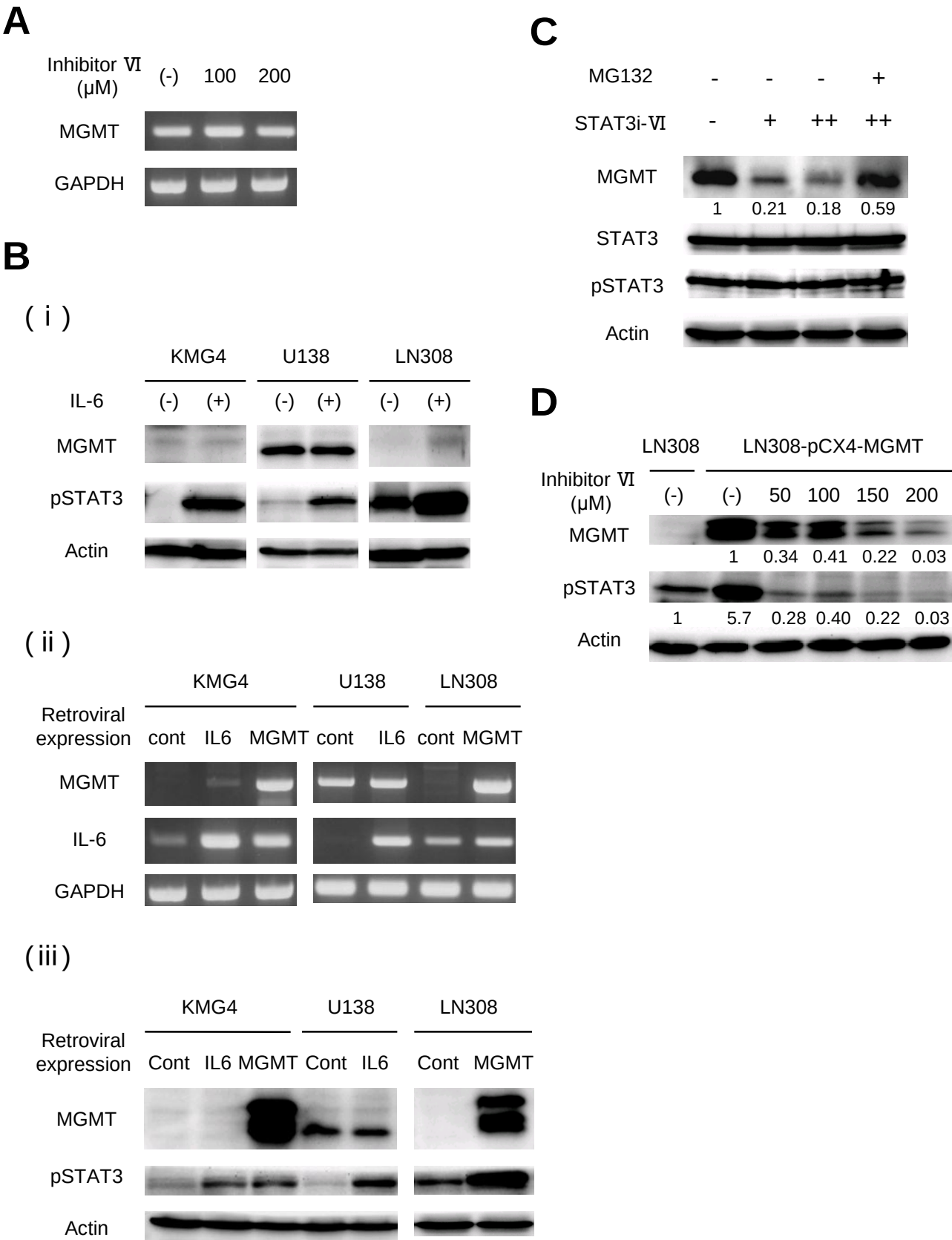
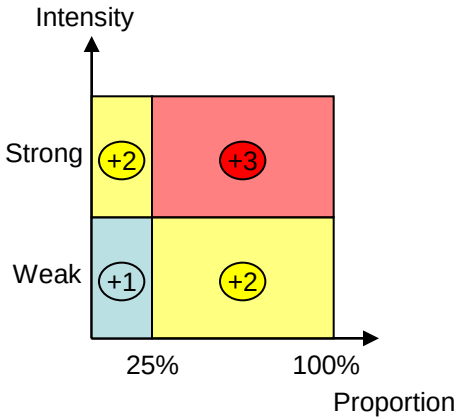
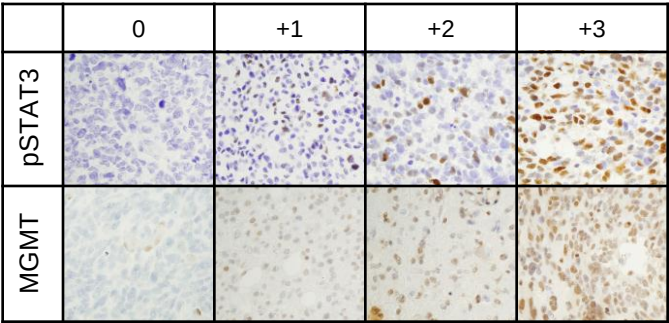
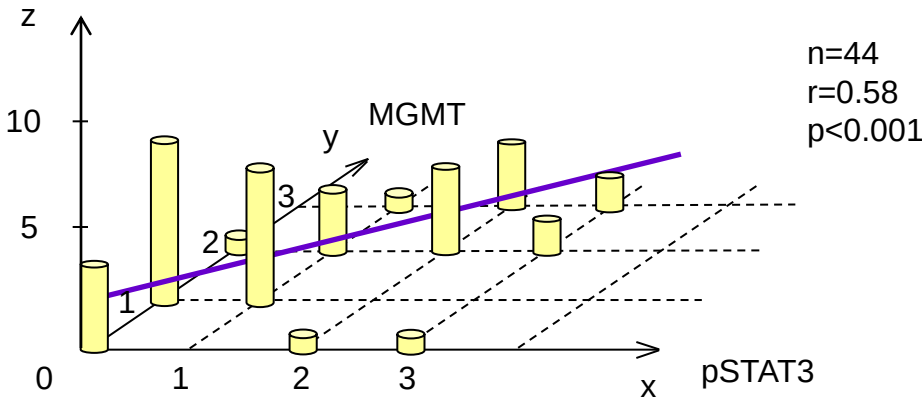


Fig. 4

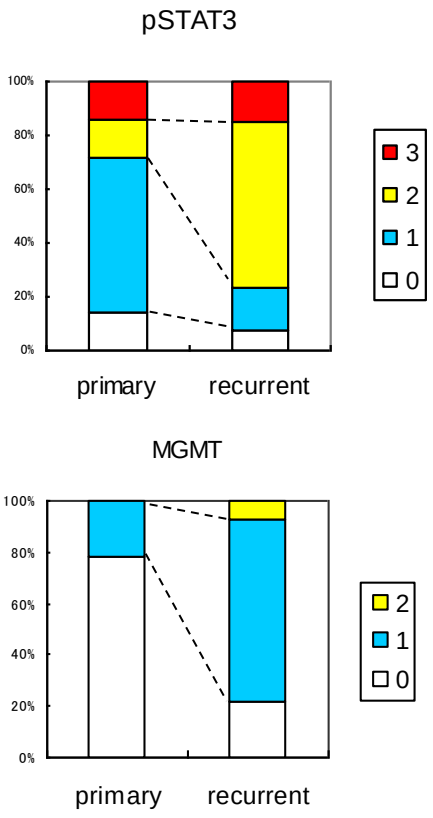
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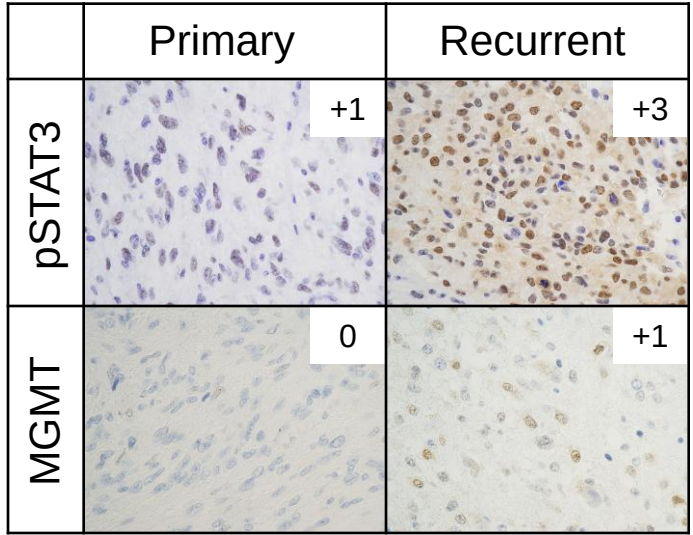
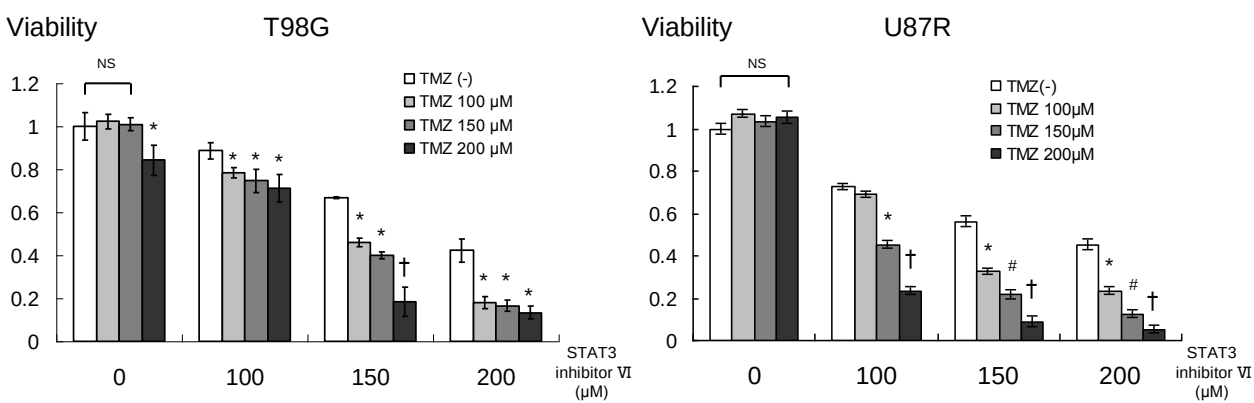
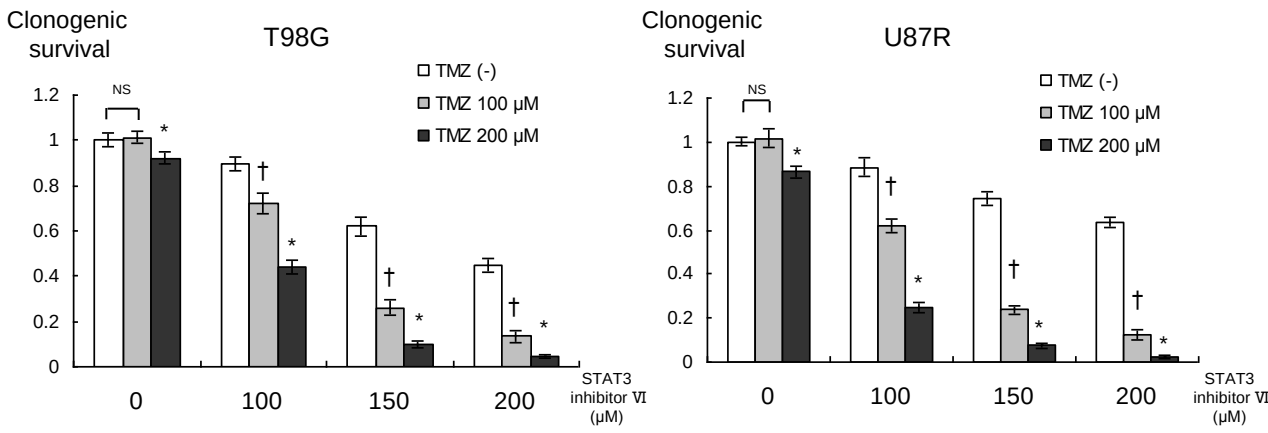


Fig. 5

A



B



C

