YKL-40 downregulation is a key factor to overcome temozolomide resistance in a glioblastoma cell line

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Abstract. The frequent recurrence of glioblastoma multiforme (GBM) after standard treatment with temozolomide (TMZ) is a crucial issue to be solved in the clinical field. O^6-methylguanine-DNA methyltransferase (MGMT) is considered one of the major mechanisms involved in TMZ resistance. However, some important mechanisms for TMZ resistance other than MGMT have recently been identified. In the present study, we established a TMZ-resistant (TMZ-R) U87 glioblastoma cell line *in vitro* and *in vivo* and investigated novel targeting molecules other than MGMT in those cells. The TMZ-R U87 glioblastoma cell line was established *in vitro* and *in vivo*. TMZ-R U87 cells showed a more invasive activity and a shorter survival time *in vivo*. Gene expression analysis using DNA microarray and quantitative PCR (qPCR) demonstrated that YKL-40, MAGEC1 and MGMT mRNA expression was upregulated 100-, 83- and 6-fold, respectively in the TMZ-R U87 cell line. Western blot analysis and qPCR demonstrated that STAT3 phosphorylation, STAT3 target genes and stem cell and mesenchymal marker genes were upregulated to a greater extent in the TMZ-resistant cell line. Notably, short hairpin (sh)RNA-based inhibition against the YKL-40 gene resulted in moderate growth inhibition in the resistant cells *in vitro* and *in vivo*. Additionally, YKL-40 gene inhibition exhibited significant suppression of the invasive activity and particularly partially restored the sensitivity to TMZ. Therefore, YKL-40 may be a novel key molecule in addition to MGMT, that is responsible for TMZ resistance in glioblastoma cell lines and could be a new target to overcome TMZ resistance in recurrent glioblastomas in the future.

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Abbreviations: GBM, glioblastoma multiforme; TMZ, temozolomide; STAT, signal transducer and activator of transcription; SH, Src homology; DMSO, dimethyl sulfoxide; JAK, Janus kinase; T/C, tumor/control; siRNA, small interfering RNA; shRNA, small hairpin RNA

Key words: TMZ resistance, STAT3, YKL-40, shRNA, recurrent glioblastoma

Introduction

Glioblastoma multiforme (GBM) is the most malignant and aggressive tumor, and GBM patients have an extremely poor prognosis and a mean survival time of less than 2 years, even following treatment with recent concomitant chemoradiation (1,2).

Temozolomide (TMZ) is an alkylating agent that induces DNA methylation of guanine at the O^6 position and triggers mismatch repair, which leads to arrest of the cell cycle and apoptosis (3). O^6-methylguanine-DNA methyltransferase (MGMT) is well known for removing methyl groups from the O^6 position of guanine and contributing to TMZ resistance induction (4). Several clinical studies have demonstrated that high MGMT expression through the methylation of the MGMT promoter is one of the genuine mechanisms responsible for TMZ resistance. Thus, novel therapeutics that aim to suppress TMZ resistance by deleting MGMT have been pursued, and O^6-benzylguanine has been considered to be a promising therapeutic candidate. However, clinical trials did not show the restoration of TMZ resistance (5,6). Considering that no tools presently exist to treat TMZ-resistant (TMZ-R) recurrent glioblastoma, novel therapeutic approaches that regulate MGMT expression and restore the sensitivity to TMZ are highly required.

Moreover, it has been suggested based on multi-omic analysis that mechanisms other than MGMT may trigger TMZ resistance. Several novel biomarkers that are linked to MGMT expression and methylation status, such as the HOX signature and EGFR expression (7), somatic mutations of the mismatch repair gene MSH6 (8), prolyl 4-hydroxylase, β polypeptide (P4HB) (9), mutated EGFR (EGFRvIII) (10) and CD74 (11), have been reported. Regarding novel approaches to overcome MGMT-related resistance to TMZ, bortezomib as a proteasome inhibitor (12), telomerase inhibition (13), a combination of

interleukin (IL)-24 with TMZ (14) and inactivation of MGMT by gene therapy (15) have been demonstrated to show moderate effects on MGMT downregulation and tumor cell death.

According to a recent molecular classification study of various glioblastomas, it is known that a mesenchymal signature expressing STAT3 and the C/EBPβ genes is closely linked to poor prognosis and TMZ resistance with tumor recurrence (16-19). These genes could be new possible targets for overcoming TMZ resistance in GB. In contrast, MGMT is not classified into any specific subtype marker, including the mesenchymal signature (17). In the present study, we identified the YKL-40 and MAGEC1 genes as TMZ resistance-associated biomarkers in the TMZ-R U87 cell line, which showed an obvious resistance *in vivo*. Furthermore, we focused on the YKL-40 and STAT3 mechanisms and proposed a restoration model of TMZ resistance.

Materials and methods

Cell lines. The human glioblastoma cell lines LN18, T98G and U87 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), penicillin and streptomycin.

Antibodies and reagents. Antibodies against STAT3, phospho-specific STAT3 (Tyr705), MGMT and β-actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and Becton-Dickinson (BD) Biosciences (Franklin Lakes, NJ, USA) for western blotting (WB). A mouse anti-human YKL-40 monoclonal antibody (MoAb) was purchased from Abcam (Cambridge, MA, USA) for immunohistochemical (IHC) studies. Short hairpin (sh)RNAs specific for the human STAT3 or YKL-40 genes were purchased from Qiagen GmbH (Hilden, Germany). TMZ was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was suspended in sterile 0.5% w/v methyl cellulose 400cp solution (Wako, Tokyo, Japan).

Establishment of the TMZ-resistant U87 cell line. The U87 parental cell line, which is sensitive to TMZ, was first maintained in low doses of TMZ (5 μM) and then successively exposed to incremental doses of TMZ (up to 150 μM). After the killing of a majority of the cells, the surviving cells were maintained until a normal rate of growth was obtained. The IC_{50} value of TMZ was evaluated using the WST-1 assay.

Cell proliferation assay. Cell proliferation was examined using the WST-1 assay (Dojindo Laboratories, Kumamoto, Japan) as described previously (20). Briefly, 1-2x10^4 human glioma cells were seeded into each well of a 96-well microculture plate (Corning, NY, USA). After 4 days, the WST-1 substrate was added to the culture and the optical density (OD) was measured at 450 and 620 nm using an immunoreader (Immuno Mini NJ-2300, Nalge Nunc International, Roskilde, Denmark). The IC_{50} value was defined as the dose required for a 50% reduction in the OD calculated from the survival curve. Percent survival was calculated as follows: (mean OD of test wells - mean OD of background wells)/(mean OD of control wells - mean OD of background wells).

DNA microarray analysis. Total RNA from the U87 parental and TMZ-R cell lines was extracted using the NucleoSpin RNA II kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. One microgram of total RNA, which was qualified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), was amplified to 100 μg of cRNA and hybridized to a high-density oligonucleotide array (GeneChip Human Genome U133 Plus 2.0 array; Affymetrix, Santa Clara, CA, USA). The intensity for each feature of the array was calculated using the GeneSpringGX ver11 (Agilent) software. To calculate the change in the average intensity, normalization for all probe sets was performed. Genes whose expression was significantly altered by >5-fold at the 5th and 20th passages of the TMZ-R U87 cell line compared to the U87 parental cell line were analyzed.

Inhibition of STAT3 or YKL-40 gene expression using shRNA transfection of the TMZ-resistant U87 cells. shRNA gene transfection into the TMZ-R U87 cell line was performed using a lipofection FreeStyle MAX reagent (Life Technologies, Carlsbad, CA, USA). Four micrograms of plasmid at 1 mg/ml containing STAT3 or YKL-40 shRNA (SureSilencing shRNA vector; Qiagen) and the same dose of FreeStyle MAX reagent were suspended in 100 μl of Opti-MEM I reduced-serum medium (Life Technologies), which was then mixed and incubated for 15 min at room temperature (RT). The solution was added to 2x10^6 TMZ-R U87 cells and incubated at 37˚C for 1 h. After washing, the cells were incubated in DMEM + 10% FBS, harvested on day 3 of culture and utilized for *in vitro* and *in vivo* experiments.

Cell invasion assay. Invasion assays using parental U87 and TMZ-R U87 cells were performed using Matrigel-coated (0.33 mg/ml) Transwell inserts with a 8-μm pore size (BD Biosciences, Franklin Lakes, NJ, USA). Cells at 1x10^5/ml (500 μl) were added to Transwells in triplicate, and 750 μl of DMEM containing 10% FBS was added to the lower wells. After 12-18 h of incubation, the cells that invaded through the membrane were fixed and stained with Diff-Quik II solution (Dade Behring AG, Germany). Migrated cells were counted using microscopy.

Quantitative polymerase chain reaction (qPCR) analysis. Real-time PCR analysis of 10 genes that were rated as significantly changed at the expression level (>10-fold, $P<0.05$) in the TMZ-R U87 cells compared to the parental cells was performed using the 7500 Real Time PCR System (Applied Biosystems, Foster, CA, USA) as described previously (20). Additionally, other stem cell and neuronal markers, GB mesenchymal type markers and STAT3 target genes were analyzed. Briefly, all PCR primers (CD24, YKL-40, GDF15, HLA-DQA1, MAGEC1, MGMT, MMP1, AMIGO2, NMU and RFC2 for expression-altered genes; ABCB1, ALDH1A1, CD44, EGFR, ESA, GFAP, KLF4, NANOG, NES, OLIG2, Oct3/4, CD133, SOX2, TGFBR2, TUBB3 and VIM for GB stem cell markers; CDH2, CDH11, COL1A2, FN1, FOXC2, MMP2, MMP3, SNAIL1, SNAIL2, TCF4, TWIST1, WNT5A, WNT5B, KRT19, CTNNB1, GSK3B, NOTCH1, PTK2, SIP1, SMAD2 and ZEB1 for EMT-associated genes; STAT3, C/EBP, bHLH-B2, RUNX1, FOSL2 and ZNF238 for GB

mesenchymal type markers; and BCL2, Bcl-XL, Survivin, cyclin D1, c-Myc, CXCL10, VEGFR2, MMP9, TGFB1, P53, VEGFA, VEGFC and HIF-1α for STAT3 target genes) and TaqMan probes were purchased from Applied Biosystems. Total RNA was extracted from parental U87 and TMZ-R U87 cells. Complementary DNA was synthesized from 100 ng of total RNA, and qPCR was carried using a TaqMan RNA-to-Ct 1-Step kit (Applied Biosystems).

Western blotting (WB). TMZ-R U87 cells transfected with or without STAT3 and YKL-40 shRNAs were lysed using RIPA buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing protease and phosphatase inhibitors and used for WB as described previously (21). Briefly, the cell lysates were subjected to SDS-PAGE with a 7.5% polyacrylamide separating gel and then transferred to PVDF membranes. After blocking, the membranes were incubated at 4˚C overnight with a primary antibody against STAT3, phospho-specific STAT3, MGMT, YKL-40 or β-actin (1:200-1:2,000) in blocking solution. After washing, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5,000). Membranes were treated with ECL Plus reagent (GE Healthcare) and analyzed using a chemiluminescence scanner (LAS-3000; Fujifilm, Tokyo, Japan).

ELISA for human YKL-40. YKL-40 levels in the supernatant of parental U87 or TMZ-R U87 cells were measured using human YKL-40-specific ELISA. Cells were suspended in DMEM + 2% FBS medium and plated in 96-well microplates (Corning Inc., Corning, NY, USA) at $4x10^4$ cells (200 μl cells at $2x10^5$/ml)/well. The supernatants at 24, 48, 72 and 96 h of the culture were collected and YKL-40 levels were measured.

Animal experiments. Male nude mice (BALB/cA-*nu/nu*, 5-6 weeks of age) were obtained from Nippon Clea (Tokyo, Japan), housed in a separate experimental room and given sterilized food and water *ad libitum*. All animals were cared for and used humanely according to Guidelines for the Welfare and Use of Animals in Cancer Research (22), and all procedures were approved by the Animal Care and Use Committee of Shizuoka Cancer Center Research Institute.

U87 ($1x10^6$) and U87/TMZ-R cells ($1x10^6$) were inoculated into the flanks of BALB/cA-*nu/nu* mice. To evaluate the antitumor activity against subcutaneous (s.c.) inoculated tumors, the tumor volume (V) was calculated based on the National Cancer Institute formula as follows: V (mm^3) = length (mm) x [width (mm)]^2 x 1/2.

TMZ was administered orally daily from day 0 to 4 at the dose of 5 mg/kg. The efficacy of TMZ against the human tumor cells that were inoculated into the nude mice was expressed as the mean V/V_0 value, where V is the tumor volume on the day of evaluation and V_0 is that on the day of treatment. The tumor-control (T/C) value was calculated as the mean V/V_0 value of the treated group vs. that of the untreated group.

For the *in vivo* experiments using YKL-40 gene inhibition, mock or YKL-40 shRNA-4-transfected TMZ-R U87 cells were harvested 3 days after gene transfection and inoculated into 5 nude mice per shRNA group.

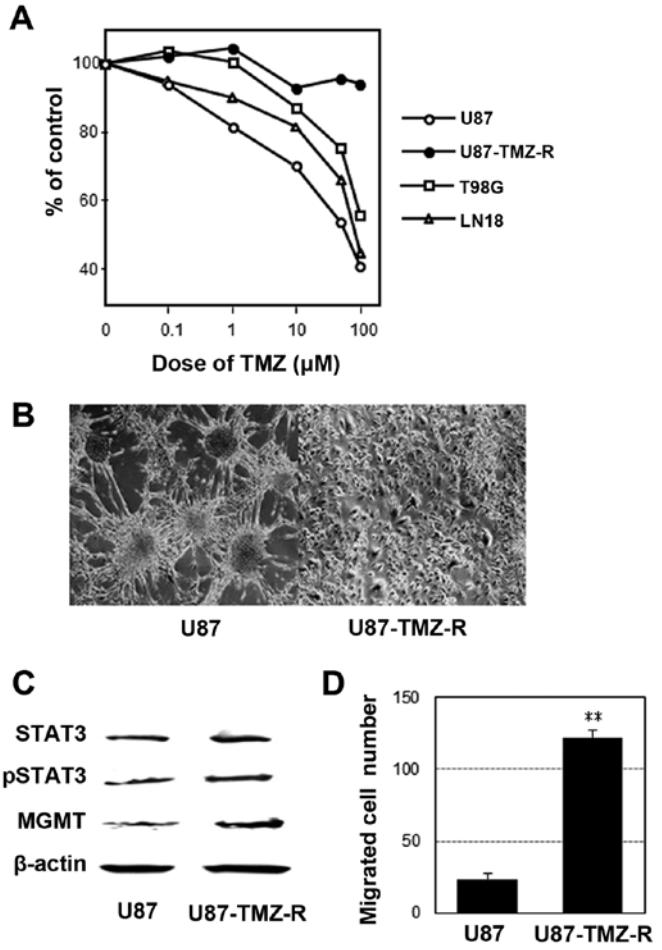

Figure 1. Characterization of the TMZ-resistant U87 cell line. (A) Sensitivity of U87 parental, TMZ-R U87 and other glioma cell lines resistant to TMZ. (B) Morphological features of the U87 parental and TMZ-resistant cell lines. Magnification, x100. (C) STAT3 phosphorylation and MGMT expression in the TMZ-R U87 cell line. (D) Promotion of invasive activity in the TMZ-R U87 cell line. Each data point shows the mean of triplicate experiments. **P<0.01, statistically significant. TMZ, temozolomide.

Statistical analysis. Significant differences were analyzed using the Student's t-test. Values of P<0.05 were considered to indicate statistically significant results.

Results

Establishment and characterization of the TMZ-resistant U87 cell line. The IC_{50} values of the U87 and TMZ-R U87 cells were 45 and >500 μM, respectively (Fig. 1A). The LN18 and T98G cells were more chemoresistant to TMZ than the U87 parental cells. Morphologically, TMZ-R U87 cells did not show a tendency for aggregation when they reached subconfluency (Fig. 1B). The activation (phosphorylation) of STAT3 and upregulation of MGMT were identified in the TMZ-R U87 cell line compared with the U87 cell line using WB analysis (Fig. 1C). Additionally, the TMZ-R U87 cell line exhibited a greater invasive activity compared with the parental U87 cell line (Fig. 1D).

Characterization of U87-TMZR cell-derived tumor xenografts in nude mice. The U87 parental and TMZ-R U87 cell

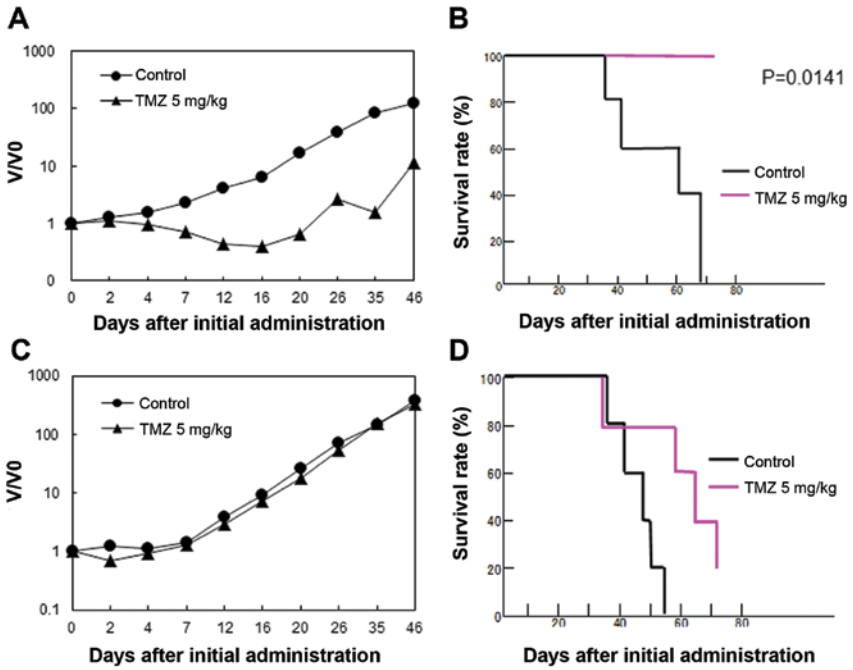

Figure 2. Characterization of TMZ-R U87 cell-derived tumor xenografts in nude mice. The U87 parental and TMZ-R U87 cell lines were transplanted into nude mice, and the sensitivity of the tumors to TMZ was investigated. The effect of TMZ on the proliferation of the parental U87 tumor (A) and on the overall survival of U87 tumor-bearing mice (B). The effect of TMZ on the proliferation of TMZ-R U87 tumors (C) and on the overall survival of TMZ-R U87 tumor-bearing mice (D). Each data point in A and C indicates the mean of 5 mice. V is the tumor volume on the day of evaluation and V_0 is that on the day of treatment. TMZ, temozolomide.

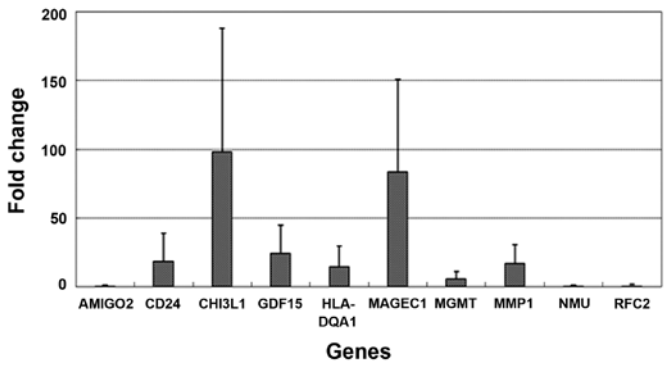

Figure 3. Changes in the mRNA gene expression levels in the TMZ-R U87 cell line compared with that of the parental U87 cell line. DNA microarrays showed that the expression of 10 genes were changed >5-fold at passages 5 and 20 in the TMZ-R U87 cell line. The mRNA levels of the 10 genes were analyzed using real-time PCR. Each column shows the mean value of four samples.

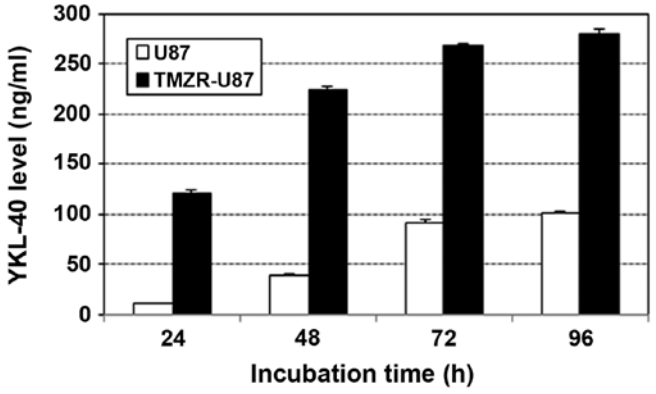

Figure 4. Upregulation of YKL-40 production in the TMZ-R U87 cell line. YKL-40 levels in the supernatant of parental U87 or TMZ-R U87 cells were measured using human YKL-40-specific ELISA. The supernatants at 24, 48, 72 and 96 h of the culture were collected, and YKL-40 levels were measured.

lines were transplanted into nude mice, and the sensitivity of the tumors to TMZ was investigated. TMZ administration for 5 days showed a significant inhibition on the parental U87 tumor growth and a beneficial effect on the survival of tumor-bearing mice (Fig. 2A and B). In contrast, TMZ-R U87 cell-transplanted mice showed significant resistance to TMZ and a shorter survival time *in vivo* (Fig. 2C and D).

Genetic profile and analysis of the TMZ-resistant U87 cell line. Gene Chip microarray analysis demonstrated that the expression of 10 genes was significantly altered (>5-fold) at the 5th and 20th passages of the U87-TMZR cell line (upregulated: CD24, YKL-40, GDF15, HLA-DQA1, MAGEC1, MGMT and MMP1; downregulated: AMIGO2, NMU and RFC2) (Table I). qPCR showed that YKL-40 and MAGEC1 were the top-2 upregulated genes (98- and 83-fold, respectively) and 4 and 3 genes were >10-fold upregulated and downregulated, respectively (Fig. 3). Meanwhile, as expected, MGMT expression was found to be increased 6-fold, however its level was extremely low.

YKL-40 production in the TMZ-resistant U87 cell line. The YKL-40 level in the supernatant of the TMZ-R U87 cell line was ~100 ng/ml at 24 h and reached >200 ng/ml at 48 h, which

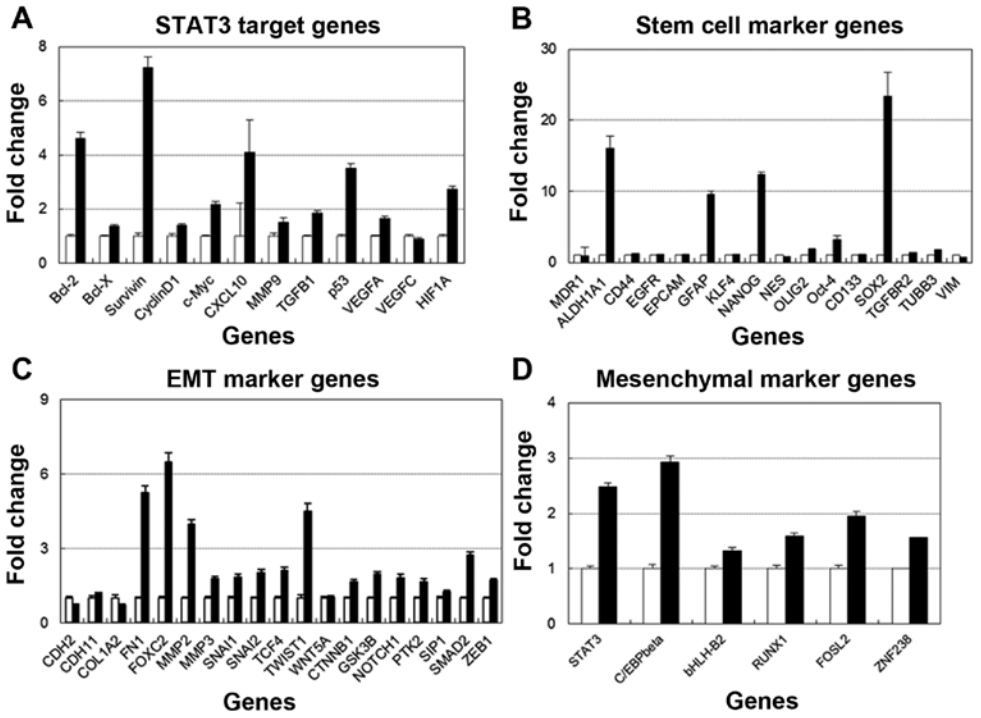

Figure 5. Expression of various mRNAs in the TMZ-R U87 cell line using qPCR. (A) STAT3 target genes, (B) stem cell marker genes, (C) EMT marker genes and (D) mesenchymal marker genes. The expression level of each gene in the parental U87 cell line was rated as 1. The vertical axis shows the fold change in mRNA expression in the TMZ-R U87 cell line compared with that of the U87 cells.

Table I. Fold-change of gene expression in the TMZ-R U87 cell line compared to the U87 parental cell line.

Gene symbol	ProbeSet ID	TMZ-R U87 (P5)	TMZ-R U87 (P20)
RFC2	1053_at	-5.1	-5.5
MMP-1	204475_at	5.4	7.4
NMU	206023_at	-5.9	-14.5
MAGEC1	206609_at	22.8	5.6
YKL-40	209395_at	12.6	6.6
CD24	209771_x_at	23.2	19.3
HLA-DQA1/A	2212671_s_at	8.9	7.2
GDF15	221577_x_at	6.2	8.6
AMIGO2	222108_at	-9.4	-6.3

Each value shows the mean of 3 gene chip data. A negative value indicates downregulation of expression compared to the U87 cells. P5 and P20 indicate data on cell passage number 5 and 20, respectively.

were more than several fold upregulated compared with the level of the parental U87 cell line (Fig. 4).

STAT3 target genes, glioma-associated genes and EMT gene expression in the TMZ-resistant cell line. A >2-fold upregulation of many genes was identified in the TMZ-R U87 cell line compared with the U87 parental cell line as follows: BCL2, Survivin, cMYC, p53 and HIF1A as STAT3-target genes; ALDH1, GFAP, NANOG and SOX2 as stem cell markers; FN-1, FOXC2, MMP2, SNAIL2, TCF4, TWIST1 and SMAD2 as EMT-associated genes and STAT3 and C/EBPβ as mesenchymal genes (Fig. 5).

Impact of YKL-40 inhibition on cell proliferation, invasive activity and in vivo tumorigenesis in the TMZ-resistant cell line. The YKL-40 protein levels in the shRNA-3 and shRNA-4-transfected TMZ-R U87 cells were significantly reduced (Fig. 6A). shRNA-mediated YKL-40 gene inhibition significantly suppressed the cell proliferation and invasive activity of the TMZ-R U87 cells (Fig. 6B and C). Nude mice transplanted with YKL-40 shRNA-4-transfected TMZ-R U87 cells showed significant growth suppression compared with the mock gene-transfected TMZ-R U87-transplanted mice after the 10th day of transplantation (Fig. 6D).

Effect of YKL-40 or STAT3 gene inhibition on the TMZ-resistance of TMZ-R U87 cells. TMZ-R U87 cells transfected with shRNA-4 exhibited recovered sensitivity to TMZ at a dose of ~250 μM, which was considered a partial effect compared with the parental U87 sensitivity to TMZ (Fig. 7A). In contrast, TMZ-R U87 cells transfected with the mock gene showed no sensitivity to TMZ, even at 1 mM. Additionally, STAT3 gene inhibition by shRNA demonstrated a partially restorative effect on TMZ-R U87 cells as well as YKL-40 gene inhibition (Fig. 7B).

Discussion

Glioblastoma multiforme (GBM) is one of the most malignant tumors and has an extremely poor prognosis. Despite recent therapeutic advances, the median survival of GBM patients is less than one year, mainly since most cases relapse after

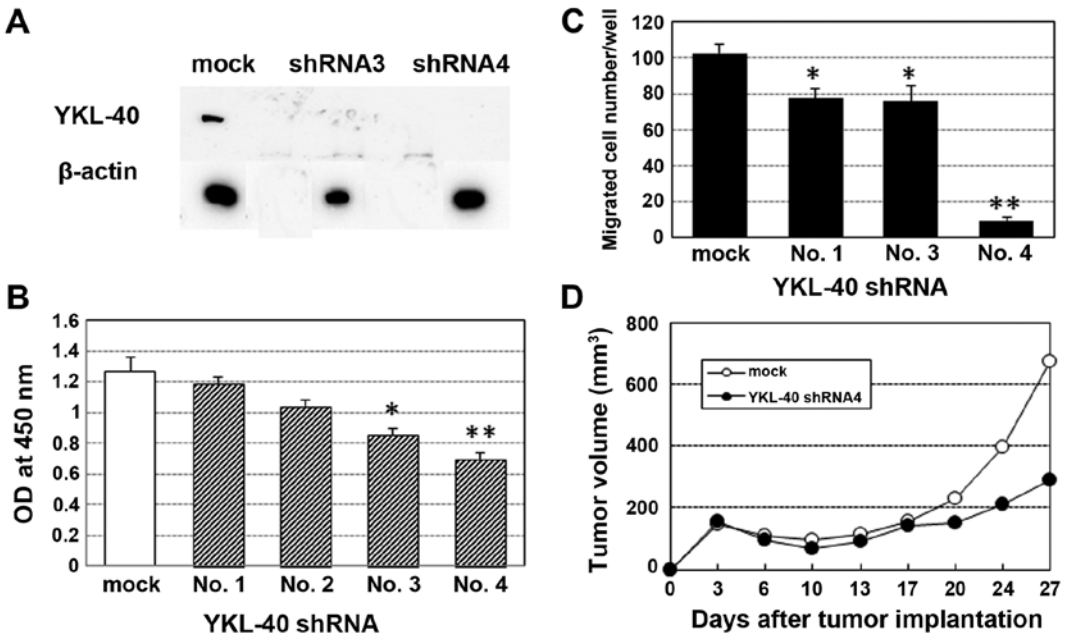

Figure 6. Effect of YKL-40 gene inhibition by shRNA on cell proliferation, invasive activity and tumorigenesis of TMZ-R U87 cells. (A) Inhibition of YKL-40 (CHI3L1) expression by shRNA-3 and -4 using WB analysis. (B) The inhibitory effect of YKL-40 downregulation on TMZ-R U87 cell proliferation. (C) The enhanced suppression of invasive activity in shRNA-4-transfected TMZ-R U87 cells. (D) Inhibition of shRNA-4-treated TMZ-R U87 tumorigenesis *in vivo*. Each data point shows the mean value for triplicate samples or mice. *P<0.05, **P<0.01, statistically significant.

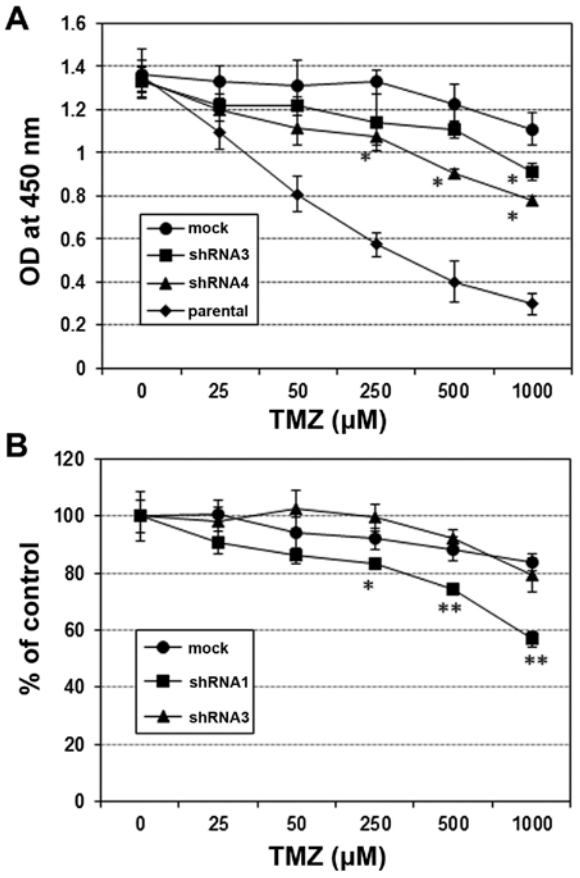

Figure 7. The effect of YKL-40 or STAT3 gene inhibition on the TMZ-resistance of TMZ-R U87 cells. YKL-40 or STAT3 shRNA-transfected TMZ-R U87 cells were harvested 3 days after gene transfection and used in a TMZ assay. (A) YKL-40 shRNA. (•)Mock gene, (■) shRNA-3, (▲) shRNA-4 and (♦) U87 parental cells. U87 parental cells were used as a positive control that was sensitive to TMZ. (B) STAT3 shRNA. (•) Mock gene, (■) shRNA-1 and (▲) shRNA-3. *P<0.05, **P<0.01, statistically significant.

concomitant chemoradiation (1,2). Thus, a novel therapeutic approach is urgently needed to control recurrence and overcome resistance to treatment.

MGMT is well known to remove the methyl group from the O^6 position of guanine and contribute to TMZ resistance, resulting in an important prognostic factor in the clinical field (4). However, novel therapeutic strategies that include O^6-benzylguanine to overcome the obtained TMZ resistance have been attempted in clinical studies, but none have been successful (5,6). Therefore, new approaches for regulating MGMT expression and restoring the sensitivity to TMZ are highly required.

In the present study, we demonstrated STAT3 activation, MGMT and YKL-40 upregulation in TMZ-R U87 cells, which formed an *in vivo* transplanted tumor with obvious TMZ resistance and YKL-40 high expression. The correlation of STAT3 with MGMT has been reported by Kohsaka *et al*, who demonstrated that TMZ-R U87 cells exhibited active STAT3 phosphorylation and that STAT3 inhibition reduces MGMT expression (23). These results suggest a possible mechanism for MGMT regulation; however, it has not been proven in *in vivo* experiments. On the other hand, Singh *et al* reported that knockdown of STAT3 inhibited active astrocyte migration through the reduction in YKL-40 production (24). These results suggest that a downstream pathway from STAT3 to MGMT through YKL-40 may exist and contribute to the gain of TMZ resistance in GB tumors.

Additionally, the comprehensive qPCR study demonstrated that many genes were upregulated in the TMZ-R U87 cell line compared with the parental cell line. In particular, the following gene groups appear to be involved in the malignant features of TMZ-R U87 tumors: BCL-2, Survivin, c-Myc, Sox2 and Nanog for tumorigenesis; CXCL10, FN1, MMP2,

TWIST1 and SMAD2 for invasive activity and ALDH1, STAT3 and C/EBPβ for TMZ resistance. Thus, such machineries for causing TMZ resistance and an invasive phenotype in clinical status will be challenging targets for overcoming TMZ resistance and developing novel therapeutics against TMZ-resistant GBM tumors.

YKL-40, also known as chitinase 3-like 1, human cartilage glycoprotein 39, is a secreted glycoprotein that belongs to the 18-glycosyl-hydrolase family of proteins. YKL-40 is produced by many cell types including macrophages, neutrophils, chondrocytes, smooth muscle and endothelial cells as well as several types of cancer cells such as breast cancer, osteosarcoma, ovarian cancer, lung cancer and GBM (25). YKL-40 is considered an angiogenic factor for tumor vessel formation through VEGF production and contributes to invasion and radio-resistance in *in vivo* tumors (26,27).

YKL-40 is also a well-known biomarker for predicting poor prognosis and a serum biomarker in GBM patients (28-33). YKL-40 was reported as a prognostic marker in GBM patients for the first time by Tanwar *et al*, who used DNA microarray analysis (28). Similarly, several studies (29-33) have demonstrated that YKL-40 is a potential serum biomarker and prognostic marker of high-grade glioma or other solid tumors such as ovarian and non-small cell lung cancers. Notably, Bernardi *et al* reported that postoperative YKL-40 level increases may be a negative prognostic index by comparing serum YKL-40 levels before and after tumor resection (34). However, the involvement of YKL-40 in TMZ resistance has yet to be clarified.

In the present study, we investigated the effect of YKL-40 gene inhibition on TMZ resistance using shRNA gene transfection into the TMZ-R U87 cell line. As a result, YKL-40 gene inhibition significantly suppressed cell proliferation, invasive activity and even tumorigenicity of TMZ-R U87 cells *in vivo*; however, the restorative effect on TMZ resistance seemed to be partial as well as STAT3 gene inhibition. Importantly, YKL-40 gene inhibition did not induce the downregulation of MGMT expression (data not shown), which may suggest a difference in the restoration mechanism for TMZ sensitivity between YKL-40 and STAT3 gene inhibition experiments.

YKL-40 is a good surrogate marker for STAT3 targeting since YKL-40 downregulation restores TMZ sensitivity and suppresses TMZ-R tumor growth, which is a mechanism for overcoming TMZ resistance. Therefore, YKL-40 is the next target by which therapeutics against TMZ-resistant GB tumors can be developed. The combination of a STAT3 inhibitor with an anti-YKL-40 antibody or other YKL-40 inhibiting reagents could be a promising new approach to overcome TMZ resistance in GB through suppression of angiogenesis and invasion.

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