

Combination of a STAT3 Inhibitor and an mTOR Inhibitor Against a Temozolomide-resistant Glioblastoma Cell Line

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Abstract. *Background: Temozolomide-resistant (TMZ-R) glioblastoma is very difficult to treat, and a novel approach to overcome resistance is needed. Materials and Methods: The efficacy of a combination treatment of STAT3 inhibitor, STX-0119, with rapamycin was investigated against our established TMZ-resistant U87 cell line. Results: The growth-inhibitory effect of the combination treatment was significant against the TMZ-R U87 cell line (IC₅₀: 78 μM for STX-0119, 30.5 μM for rapamycin and 11.3 μM for combination of the two). Western blotting analysis demonstrated that the inhibitory effect of STX-0119 on S6 and 4E-BP1 activation through regulation of YKL-40 expression occurred in addition to the inhibitory effect of rapamycin against the mTOR pathway. Conclusion: These results suggest that the STAT3 pathway is associated with the mTOR downstream pathway mediated by YKL-40 protein, and the combination therapy of the STAT3 inhibitor and rapamycin could be worth developing as a novel therapeutic approach against TMZ-resistant relapsed gliomas.*

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Abbreviations: GB: Glioblastoma, TMZ: temozolomide, MGMT: O⁶-methylguanine-O⁶-methylguanine-DNA methyltransferase, STAT: signal transducer and activator of transcription, mTOR: mammalian target of rapamycin, shRNA: small hairpin RNA.

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Glioblastoma (GB) is one of the most malignant and aggressive tumors and has a very poor prognosis, with a mean survival time of less than 2 years even with the recent development of temozolomide (TMZ)-based intensive treatment (1, 2). Once recurrence develops, there are few therapeutic approaches to control the growth of glioblastoma. Therefore, TMZ-resistant GB is very difficult to treat, and a novel approach to overcome resistance is needed.

With regard to the mechanism of TMZ resistance, O⁶-methylguanine-DNA -methyltransferase (MGMT) removes methylation from the O⁶ position of guanine and contributes to TMZ resistance induction (3). It is generally accepted that high MGMT expression through the methylation of the MGMT promoter is one of the mechanisms responsible for TMZ resistance. Alternatively, several novel biomarkers linked to MGMT expression and the methylation status such as the HOX signature and epidermal growth factor receptor (EGFR) expression (4), somatic mutation of mismatch repair gene mutS homolog (MSH)6 (5), prolyl 4-hydroxylase, beta polypeptide (P4HB), EGFR mutation (EGFRvIII) (6), CD74 and signal transducer and activator of transcription (STAT)3 signaling have been reported. Kohsaka *et al.* reported the association of STAT3 expression with MGMT expression level using small interfering (si)RNA-mediated STAT3 gene inhibition (7).

Additionally, mammalian target of rapamycin (mTOR) signaling is activated in TMZ-resistant glioma cells as a result of EGFR, phosphoinositide 3 kinase (PI3K) and Akt signaling activation. mTOR is a Ser/Thr kinase that belongs to the phosphoinositide kinase-related family of protein kinases (PIKKs). mTOR acts as an essential integrator of growth-factor-activated and nutrient-sensing pathways to control and coordinate various cellular functions, including survival, proliferation, differentiation, autophagy and metabolism (8-11). More interestingly, Johnson *et al.* identified a novel mTOR-specific single nucleotide variant

(SNV) in a clinical tumor specimen from TMZ-resistant glioma patients (12), which can contribute to mTOR signal activation in TMZ resistance.

Previously, we identified a novel inhibitor of STAT3 dimerization, STX-0119, that exhibited a potent anti-tumor effect on a TMZ-resistant U87 glioma cell line, and demonstrated that anti-tumor activity was partly mediated by a down-regulation of YKL-40 (13-15). In the present study, we focused on cancer signaling in the TMZ-resistant glioma cell line, in which the STAT3 and PI3K/Akt/mTOR pathways were highly activated, and investigated the effect of a combination therapy of the STAT3 inhibitor (STX-0119) and mTOR inhibitor (rapamycin) on a TMZ-resistant glioblastoma cell line *in vitro* and *in vivo*. We demonstrated that combination therapy effectively inhibited the proliferation of even a TMZ-resistant cell line through the possible association of the STAT3 and mTOR signaling pathways.

Materials and Methods

TMZ-resistant U87 cell line and antibodies. TMZ-resistant (R) U87 cells were established using a TMZ-dose-escalation method up to 150 μ M, were maintained at a dose of 100 μ M TMZ and were used for *in vitro* and *in vivo* experiments (14). Antibodies against STAT3, phospho-STAT3 (Y705), cleaved caspase-3, EGFR, phospho-EGFR (Y845, Y1173), Ras, PI3 kinase p85, phospho-PI3 kinase p85 (Y458), Akt, phospho-Akt1 (S473), mTOR, phospho-mTOR (S2448), S6, phospho-S6 (S235, S236), 4E-BP1, phospho-4E-BP1 (T37, T46), B-Raf, phospho-B-Raf (S445), extracellular signal-regulated kinase (ERK)1/2, mitogen-activated protein kinase (MAPK), phospho-ERK1/2 pMAPK (T202, Y204), p38 MAPK and phospho-p38 MAPK (T180, Y182) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and Becton-Dickinson (BD) Biosciences (Franklin Lakes, NJ, USA) for Western blotting (WB). Mouse anti-human YKL-40 antibody was purchased from Abcam (Cambridge, MA, USA).

Chemicals. STX-0119 was supplied by the Center for Drug Discovery, University of Shizuoka (Shizuoka, Japan). These compounds were suspended and diluted in a sterile 0.5% w/v methyl cellulose 400cp solution (Wako, Tokyo, Japan) for use in animal experiments. TMZ and rapamycin were purchased from SIGMA-ALDRICH (St. Louis, MO, USA) and Selleck chemicals (Houston, TX, USA).

Cell proliferation assay. Cell proliferation was examined using the WST-1 assay (Dojin Kagaku Corp., Kumamoto, Japan) described previously (14). Briefly, 1×10^4 parental U87 or TMZ-R U87 cells were seeded into each well of a 96-well micro-culture plate (Corning, NY, USA) and compounds ranging from 0-200 μ M were added alone or combination. After 4 days, the WST-1 substrate was added to the culture and 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 needed for a 50% reduction in OD calculated from the survival curve. Percent survival was calculated as follows: (mean O.D. of test wells – mean O.D. of background wells)/(mean O.D. of control wells – mean O.D. of background wells) $\times 100$.

Western blotting. TMZ-R U87 cells were treated with STX-0119 or rapamycin at the determined doses for 24 h. The cells were lysed using RIPA buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) containing protease inhibitors and phosphatase inhibitors and used for western blotting as described previously (13). Briefly, cell lysate was 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 various primary antibodies in blocking solution. After washing, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-mouse IgG. Membranes were treated with ECL plus reagent (GE Healthcare, Piscataway, NJ, USA) and analyzed on a chemiluminescence scanner (LAS-4000; GE Healthcare). Apoptosis induction in TMZ-R U87 cells treated with chemical reagents for 24 h was investigated using a Caspase-3 Western detection kit including the primary antibody against cleaved caspase-3 (Cell Signaling).

Inhibition of YKL-40 gene expression using shRNA transfection into TMZ-resistant U87 cells. The shRNA gene transfection into the TMZ-R U87 cell line was performed using a lipofection FreeStyle MAX reagent (Life Technology, Carlsbad, CA, USA) as reported previously (15). Briefly, YKL-40 shRNA-containing plasmid (SureSilencing shRNA vector; Qiagen GmbH, Hilden, Germany) and FreeStyle MAX reagent was suspended in opti-MEM I reduced-serum medium (Life Technologies), that was then mixed and incubated for 15 min at room temperature (RT). The solution was added to 2×10^6 TMZ-resistant U87 cells, which were incubated in DMEM plus 10% FBS and utilized for *in vitro* experiments on day3. Similarly, plasmid pcDNA3.1 (Life Technologies) containing YKL-40 cDNA was transfected using lipofection into the parental U87 cell line, and transiently obtained U87 cells producing a high amount of YKL-40 protein were used for western blot analysis.

Animal experiments. Male nude mice (BALB/cA-*nu/nu*, 5-6 weeks old) were obtained from Nippon Clea (Tokyo, Japan). All of the animals were cared for and used humanely according to the guidelines for the welfare and use of animals in cancer research published in Br J Cancer in 2010, and the procedures were approved by the Animal Care and Use Committee of Shizuoka Cancer Center Research Institute.

TMZ-R U87 cells (1×10^6) were inoculated into the flank of BALB/cA-*nu/nu* mice. To evaluate the anti-tumor activity against subcutaneous (*s.c.*) inoculated tumors, tumor volume was calculated based on the National Cancer Institute formula as follows: tumor volume (mm^3) = length (mm) \times [width (mm)]² \times 1/2.

STX-0119 was administered orally daily from day 0 to day 4 followed by 2 day of rest (15 administrations at 40 mg/kg for 18 day). Rapamycin was administered intraperitoneally every other day followed by 2 days of rest (9 administrations at 4 mg/kg for 18 days). The efficacy of the treatment was expressed as the mean V/V_0 value or tumor/control ratio, where V is the tumor volume on the day of evaluation and V_0 is the tumor volume on the day of treatment.

Whole-exome sequencing (WES) analysis of the TMZ-R U87 cell line using next-generation sequencing. WES was performed using an Ion Proton system with the AmpliSeq Exome kit as reported previously (16). Briefly, raw data were processed, filtered and

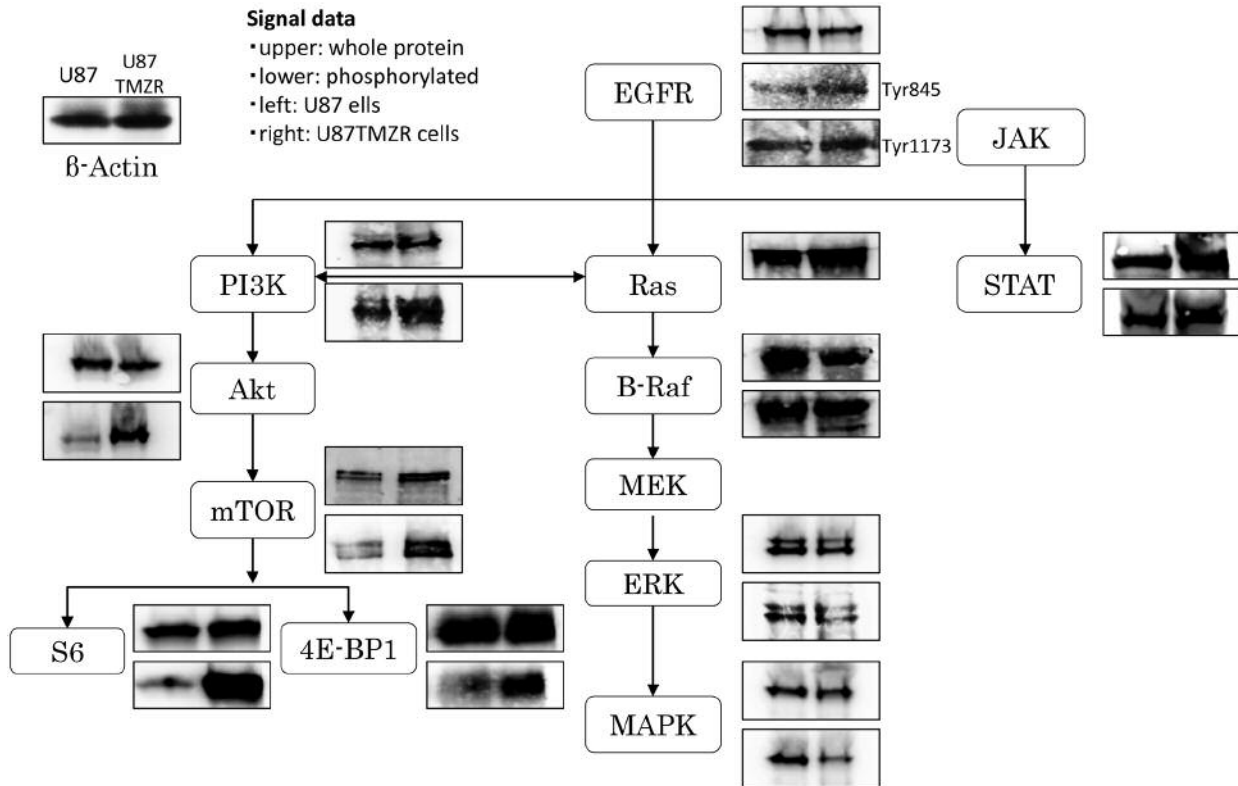


Figure 1. Cancer cell signaling profile in the TMZ-R U87 cell line. Cancer cell-associated signal molecules, such as EGFR, PI3K, Akt, mTOR, S6, 4E-BP1, Ras, Raf, ERK, MAPK and STAT3, were investigated using western blotting analysis. For each blot: Left, U87 parental cell line; right, TMZ-R U87 cell line; upper, whole protein; lower, phosphorylated protein.

converted to sequence reads by Torrent Suite Software and sequenced reads were mapped to the reference human genome (UCSC hg19) by tmap. Variant call was performed for each cell line separately by using Torrent Variant Caller. Quality <30, depth of coverage <20 and variant allele frequency <10% were applied to filter out low quality mutations. The IonReporter tumor-normal workflow was applied to identify those mutations that were observed in the TMZ-R cell line only.

Statistical analysis. Statistical differences were analyzed using Student's *t*-test. Values of $p < 0.05$ were considered statistically significant. For the *in vivo* experiment, statistical analysis was performed with corrected *p*-values to compare with the untreated control using Mann-Whitney's rank-sum test.

Results

Cancer signal pathway profiling of the TMZ-R U87 cell line. Phospho-EGFR, phospho-PIK3, phospho-Akt, phospho-mTOR (S6 and 4E-BP1), and phospho-STAT3 signaling molecule protein levels were found up-regulated in TMZ-R U87 cells compared to the parental U87 cells (Figure 1). In contrast, the levels of the RAS downstream molecules phospho-raf, phospho-ERK, and phospho-MAPK were down-regulated.

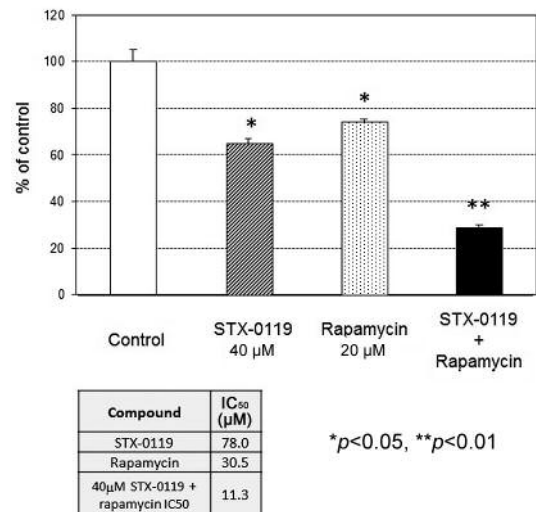


Figure 2. Effect of STX-0119 and/or rapamycin on the proliferation of the TMZ-R U87 cell line. The proliferation of the TMZ-R U87 cell line without treatment was designed 100 as a control, and the growth-inhibitory effect of the drug was expressed as % control. Each column shows the mean \pm SD of triplicate samples. Open column: Control, shaded column: STX-0119, hatched column: rapamycin, closed column: STX-0119 and rapamycin. * $p < 0.05$, ** $p < 0.01$, statistically significant.

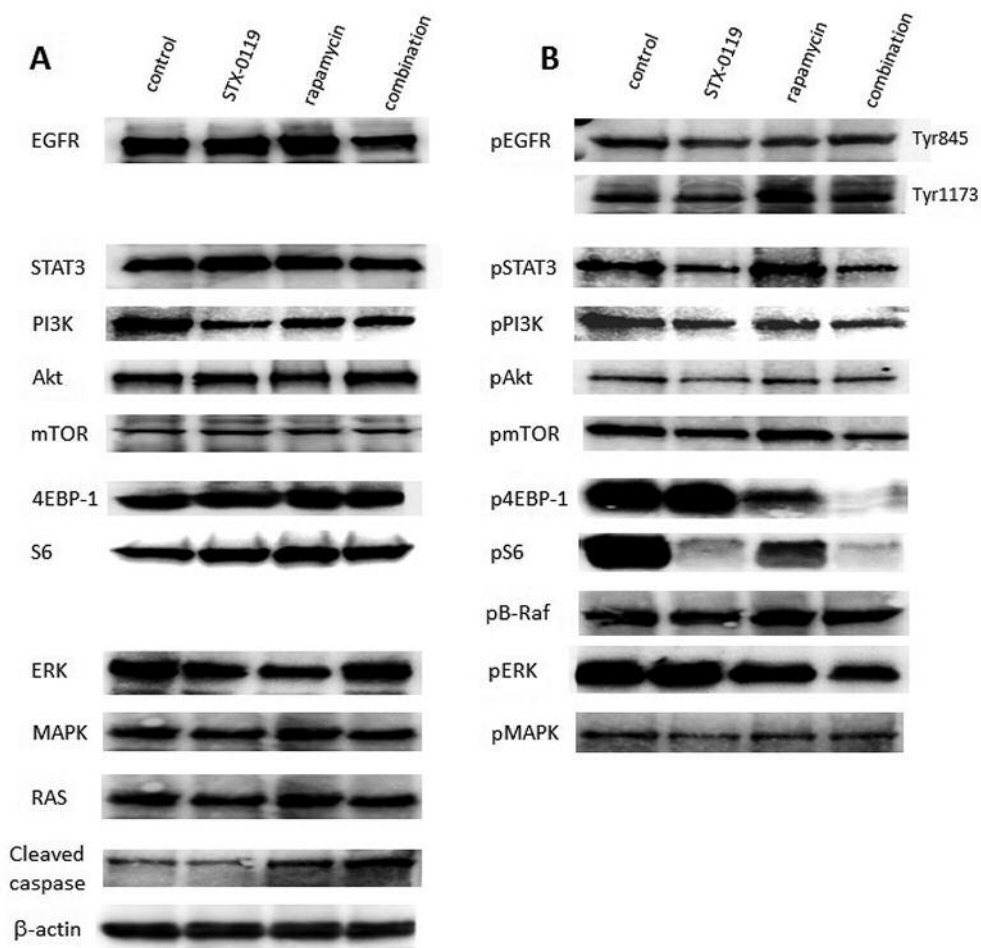


Figure 3. Effect of a combination treatment of STX-0119 and rapamycin on cancer cell signaling in the TMZ-R U87 cell line. The cells were treated with STX-0119 and/or rapamycin for 24 h and then used for WB analysis of cancer cell signaling molecules, such as EGFR, PI3K, Akt, mTOR, S6, 4E-BP1, HIF-1, Ras, Raf, ERK, MAPK and STAT3. (A) Whole proteins, (B) phosphorylated proteins.

Cell proliferation assay. STX-0119 and rapamycin displayed a moderate inhibitory effect on TMZ-R U87 cells (STX-0119 IC_{50} =87 μ M, rapamycin IC_{50} =30.5 μ M) (Figure 2). Remarkably, a combination of a suboptimal dose of STX-0119 (40 μ M) and rapamycin (20 μ M) significantly suppressed the proliferation of TMZ-R U87 cells by more than 70% compared to a single reagent (IC_{50} =11.3 μ M).

Effect of a combination treatment on signaling pathways of the TMZ-R U87 cell line. STX-0119 alone moderately inhibited the expression of both STAT3 and mTOR signaling molecules; however, rapamycin alone inhibited only mTOR. Remarkably, a combination of STX-0119 and rapamycin at 40 μ M and 20 μ M, respectively, significantly suppressed STAT3 and PI3K/Akt/mTOR signaling molecule levels (Figure 3).

Apoptosis induction by a combination of STX-0119 with rapamycin in TMZ-R U87 cell line. Cleaved caspase-3 expression increased in TMZ-R U87 cells treated with rapamycin. Additionally, a combination of STX-0119 and rapamycin demonstrated the highest increase of cleaved caspase-3 expression in TMZ-R U87 cells (Figure 3).

Impact of the regulation of YKL-40 gene expression on PI3K/Akt/mTOR signaling. YKL-40 mRNA inhibition by shRNA inhibited mTOR and 4E-BP1, but not S6. Alternatively, YKL-40 overexpression in the parental U87 cells using YKL-40 cDNA transduction induced a significant up-regulation of the S6 signaling molecule. In contrast, mTOR phosphorylation was inhibited (Figure 4).

Effect of the combination of STX-0119 with rapamycin on TMZR U87 tumor growth in vivo. TMZ-R U87 cell-

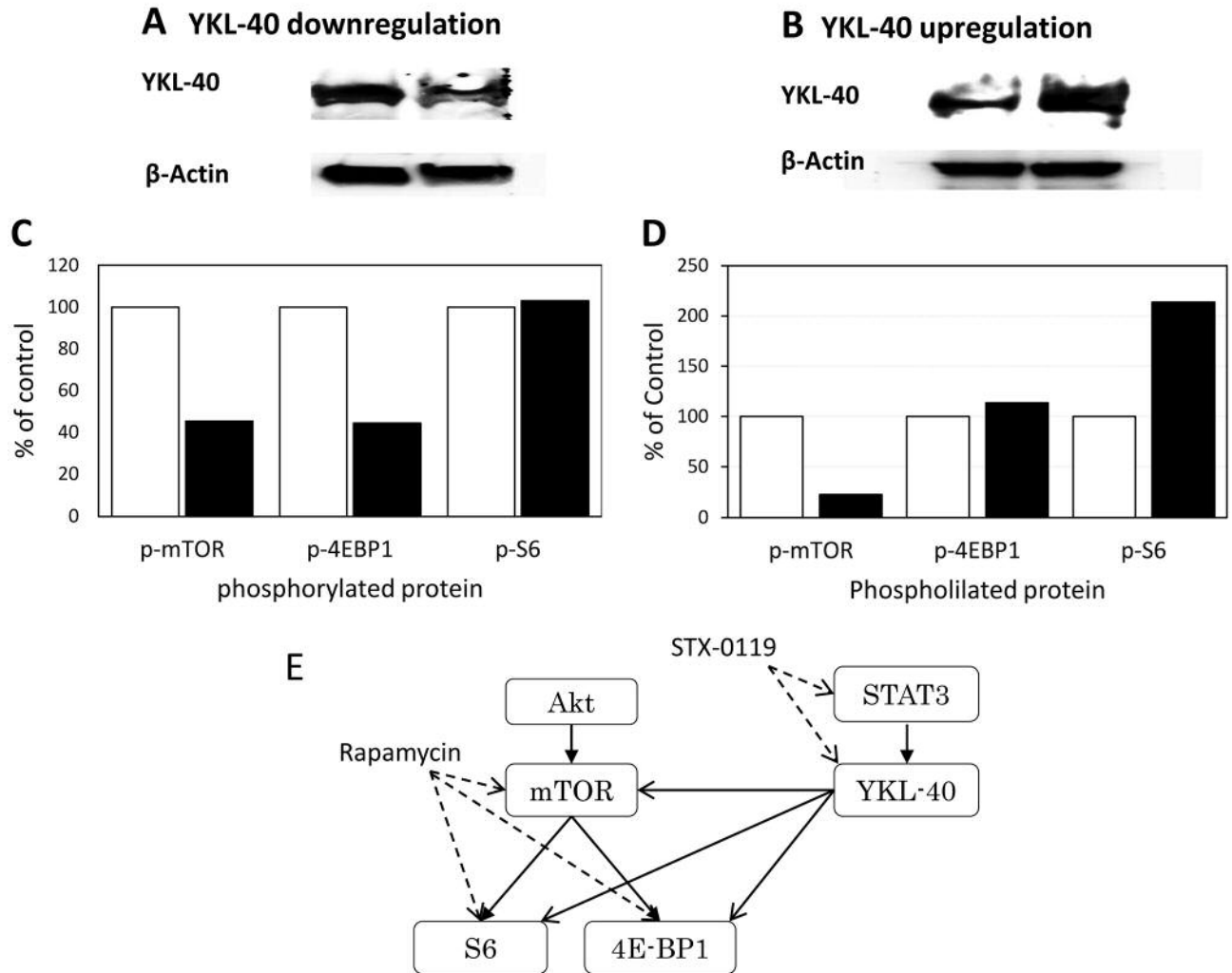


Figure 4. Impact of YKL-40 expression level on mTOR pathway signals. The expression levels of mTOR, S6 and 4E-BP1 phosphoproteins were investigated in the case of (A) YKL-40-downregulation (TMZ-R U87 cells) or (B) YKL-40-upregulation (U87 parental cells). At the bottom, each phosphoprotein level is shown as % of control (C) Open column: Control, closed column: YKL-40 down-regulated by shRNA, (D) Open column: control, closed column: YKL-40 overexpressed by gene transfection. (E) A speculated pathways between STAT3-YKL-40 and mTOR signaling. Solid line: Stimulating signal, dashed line: inhibiting signal.

transplanted mice showed significant resistance to TMZ and a shorter survival time *in vivo* compared to the parental U87 cell line, as shown in our previous study (14). STX-0119 alone showed a moderate inhibitory effect on TMZ-R U87 tumor growth in nude mice. In contrast, rapamycin alone exhibited improved growth inhibition of TMZ-R U87 tumors. Therefore, a combination of STX-0119 with rapamycin did not show a significant additive effect on TMZ-R U87 tumor growth (Figure 5).

Whole-exome sequencing (WES) analysis of the TMZ-R U87 cell line. The number of mutated genes and SNVs per cell line was 9,533 and 22,824 in the U87 parental cell line and 11,837

and 30,872 in the U87 TMZ-R cell line, respectively. U87 TMZ-R cell-specific SNVs related to cancer signaling pathway-associated genes are shown in Table I. The number of non-synonymous SNVs detected per cell line was 389 in the U87 parental and 686 in the U87 resistant cell line. U87 TMZ-R cell-specific SNVs were found in the EGFR, PIK3C3, STAT1, STAT2, STAT6 and YKL-40 (CHI3L1) genes. Interestingly, a somatic mutation of the EGFR C-terminal domain (E1004K) was verified. Additionally, an adjacent mutation (E1005-1006KD) was reported to induce spontaneous activation of EGFR phosphorylation (17). Alternatively, a somatic mutation of the YKL-40 gene (G292R) was also found, but it was unlikely to be functional in signal transduction.

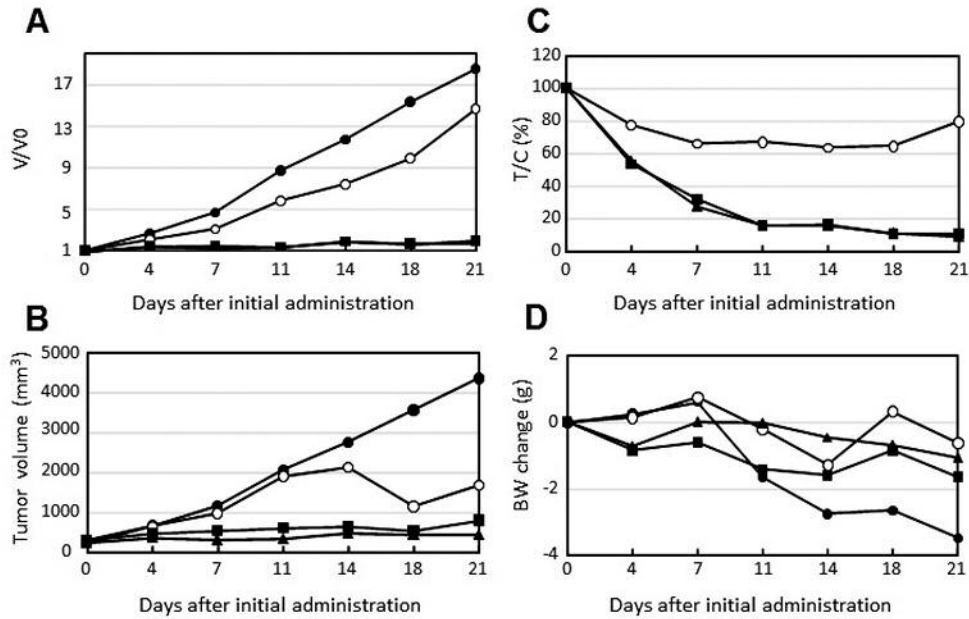


Figure 5. Inhibitory effect of STX-0119 and/or rapamycin on in vivo tumor growth of TMZ-R U87 cells. Nude mice transplanted with TMZ-R U87 cells were used. (●) Control, (○) STX-0119, (■) rapamycin, (▲) STX-0119 and rapamycin. The tumor growth was indicated as V/V_0 value (A), actual tumor volume (B) or tumor/control ratio (%) (C). To evaluate adverse effects, the change in body weight is shown in (D). Each point shows the mean value of 5 mice.

Table I. U87-TMZR signaling-associated SNV gene list.

Chrom	Position	Ref	Var	Gene ID	UniProt acc	Gene symbol	Snpeff prediction
U87 TMZR cell-associated mutation							
chr7	55268944	G	A	1956	P00533	EGFR	missense:Gaa/Aaa:E1004K
chr18	39595517	G	A	5289	Q8NEB9	PIK3C3	missense:gGc/gAc:G468D
chr3	178937024	C	T	5290	P42336	PIK3CA	missense:Ctt/Ttt:L569F
chr10	102296366	C	T	55662	Q9NWT6	HIF1AN	missense:Cat/Tat:H126Y
chr9	5069121	G	A	3717	O60674	JAK2	missense:Gat/Aat:D476N
chr2	191839582	C	T	6772	P42224	STAT1	missense:Gtg/Atg:V738M
chr2	191855954	C	T	6772	P42224	STAT1	missense:aGa/aAa:R346K
chr12	56745161	C	T	6773	P52630	STAT2	missense:Gtt/Att:V282I,V286I
chr12	57496113	G	A	6778	P42226	STAT6	missense:gCc/gTc:A381V,A491V
chr1	203149618	C	T	1116	P36222	CHI3L1	missense:Ggg/Agg:G292R
chr22	51042359	C	T	23542	Q13387	MAPK8IP2	missense:Cct/Tct:P184S,P211S

Discussion

Glioblastomas are the most malignant and aggressive of tumors and have a very poor prognosis and a high recurrence rate, with a less than 5% survival rate at 2 years.

Frequent recurrence even after chemo-radiation treatment is a crucial problem in the clinical field, which must be overcome to extend the overall survival of GBM patients. Recently, genomic studies using exome-based clinical sequencing revealed tumor-specific genetic alterations

according to the tumor development stage from primary to relapse as follows; loss of heterozygosity (LOH) 10q (69%), EGFR amplification (34%), TP53 mutation (31%), p16 (INK4a) deletion (31%) and phosphatase and tensin homolog deleted on chromosome ten (PTEN) mutations (24%) in primary glioblastomas which account for 95% of all glioblastomas (18, 19).

PTEN is a phosphatase that was, originally identified as a tumor suppressor gene and is frequently altered in a variety of human cancers, such as brain, breast and prostate (20, 21);

these alterations lead to deregulation of protein synthesis, the cell cycle, migration, growth, DNA repair and survival signaling. PTEN inactivation by deletion and mutations occurs in approximately 40% of glioblastoma patients. PTEN inactivation is known to induce PI3K/Akt/mTOR signal activation in various tumors. Targeting these pathways is often complex and can result in pathway activation depending on the presence of upstream mutations (22, 23). In glioblastomas, PTEN inactivation-based mTOR signal activation is frequently seen together with PI3K mutation-based amplification; therefore, those tumors are more sensitive to mTOR inhibitors (24, 25). Additionally, as for mTOR activation in glioblastomas, Pelloski *et al.* showed that higher expression of phospho (p)-mTOR, p-70S6K and p-MAPK was associated with worse outcome, as shown by immunohistochemical staining in 268 cases of newly diagnosed glioblastomas. Importantly, Cloughesy *et al.* reported anti-tumor activity of the mTOR inhibitor rapamycin in a phase I trial for patients with recurrent PTEN-deficient glioblastomas; however, the result of the trial was disappointing (25).

Cell signaling profiling of TMZ-R U87 cells using WB analysis demonstrated that p-EGFR, p-PIK3, p-Akt, p-mTOR (S6 and 4E-BP1), and phospho-STAT3 signaling protein levels were up-regulated in TMZ-R U87 cells compared to parental U87 cells. These results seemed to be similar to the observation verified in TMZ-resistant glioma cells or tissues (11, 23). The inhibitory effect of rapamycin seemed to be efficient but was restricted to p-mTOR and p-70S6, and did not affect the p-PIK3 or p-Akt proteins. STX-0119 was specific and potent for p-STAT3, and the inhibitory effect on p-mTOR and p-Akt protein was positive but moderate. Remarkably, a combination of rapamycin with STX-0119 diminished both PI3K/Akt/mTOR and STAT3 signaling and significantly suppressed the growth of TMZ-R U87 cells.

The signaling relationship between mTOR and STAT3 has been suggested in a few studies regarding mTOR inhibitor development (26-28). Specifically, Hu *et al.* (27) identified brain-expressed X-linked 2 (BEX2), a novel downstream molecule of the mTOR pathway; they found that mTOR signal activation can be mediated by BEX2 and transmitted to the STAT3 and NF- κ B pathways. Oroxylin, a natural monoflavonoid, reported by Zou *et al.*, inhibited Akt and ERK activation and the downstream phosphorylation of mTOR and STAT3, which was mediated by Beclin 1, a key autophagy-related protein (28). Our shRNA-mediated gene inhibition study showed that both STAT3 and YKL-40 gene inhibition suppressed mTOR signaling; however, inhibition of the mTOR signaling pathway was greater when YKL-40 was inhibited (data not shown); this result suggests that mTOR is located downstream of the STAT3/YKL-40 pathway, because STAT3 signal inhibition by STX-0119 resulted in a significant reduction of the YKL-40 protein level in our previous study (14).

Another important issue regarding mTOR signal activation in TMZ-R U87 cells is genetic alteration of the mTOR gene (29-31). Briefly, three studies regarding mTOR mutation research have been cited. Sato *et al.* identified two mTOR point mutations (S2215Y and R2505P) out of 750 cancer samples from the COSMIC library; however, these mutations were not functional (29). Gerlinger *et al.* identified a tumor-specific functional mTOR mutation (L2431P) from four cases of metastatic renal cancer that showed mTOR kinase activation with S6 and 4EBP phosphorylation (30). Finally, Johnson *et al.* reported that a novel mTOR mutation (S2215F) was identified in relapsed secondary glioblastoma tissue, which induced p-RPS6 and p-4EBP1 activation (12).

Our whole-exome sequencing of TMZ-R U87 cells revealed that tumor-specific SNVs (non-synonymous mutations) were identified, and some SNVs belonged to the Vogelstein cancer driver gene group. The list of genes that were specifically mutated in TMZ-resistant U87 cells contained EGFR, PIK3CA, MSH2, ARID1A, PRDM1, and SMARCA4, some of which had already been reported in relapsed glioblastoma tissue (data not shown). However, specific mTOR mutations were not recognized, as demonstrated by Johnson *et al.* (12). Interestingly, a novel mutation (E1004K) located in the C-terminal tail domain of EGFR was found in Table I. Considering that the N-terminal half of the EGFR C-terminal tail domain has an inhibitory function in EGFR signal activation (31) and that a neighboring mutation (ER1005-1006KD) was reported to induce spontaneous activation of EGFR phosphorylation (17), this mutation (E1004K) might be involved in EGFR signaling. Alternatively, a novel somatic mutation of the YKL-40 gene (G292R) was also found, but it likely does not function in signal transduction.

In contrast to the *in vitro* study, the *in vivo* study of a combination treatment of STX-0119 and rapamycin against TMZ-R U87 tumors did not show a significant additive inhibitory effect. There is a concern regarding the dose of rapamycin used in the *in vivo* study. Mukhopadhyay *et al.* (32) demonstrated the enigma of rapamycin dosage: a low dose of rapamycin inhibits mainly S6 phosphorylation, and a high dose inhibits both S6 and 4E-BP1 phosphorylation. Therefore, different doses of rapamycin can be investigated to identify the optimal combination for *in vivo* study.

The present study is the first report of a successful combination therapy of an mTOR inhibitor and a STAT3 inhibitor against TMZ-R glioblastoma cells *in vitro*. Additionally, we made the novel observation of an association between mTOR and STAT3 signaling that is mediated by the YKL-40 protein (Figure 4E). Considering that novel dual or triple inhibitor development against PI3K/Akt/mTOR signal activation has been in progress since the development of rapalogs, everolimus and temsirolimus (33-38), the novel

effective combination of an mTOR inhibitor and a STAT3 inhibitor shown in the present study deserves attention as a novel therapeutic approach against relapsed TMZ-resistant glioblastomas.

Conflicts of Interest

The Authors have no competing interests to declare.

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