Down-regulation of MDR1 by Ad-DKK3 via Akt/NFκB pathways augments the anti-tumor effect of temozolomide in glioblastoma cells and a murine xenograft model

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Abstract

Background: Glioblastoma multiforme (GBM) is the most malignant of brain tumors. Acquired drug resistance is a major obstacle for successful treatment. Earlier studies reported that expression of the multiple drug resistance gene (MDR1) is regulated by YB-1 or NFκB via the JNK/c-Jun or Akt pathway. Over-expression of the Dickkopf (DKK) family member DKK3 by an adenovirus vector carrying DKK3 (Ad-DKK3) exerted anti-tumor effects and led to the activation of the JNK/c-Jun pathway. We investigated whether Ad-DKK3 augments the anti-tumor effect of temozolomide (TMZ) via the regulation of MDR1.

Methods: GBM cells (U87MG and U251MG), primary TGB105 cells, and mice xenografted with U87MG cells were treated with Ad-DKK3 or TMZ alone or in combination.

Results: Ad-DKK3 augmentation of the anti-tumor effects of TMZ was associated with reduced MDR1 expression in both *in vivo* and *in vitro* studies. The survival of Ad-DKK3-treated U87MG cells was inhibited and the expression of MDR1 was reduced. This was associated with the inhibition of Akt/NFκB but not of YB-1 via the JNK/c-Jun- or Akt pathway.

Conclusions: Our results suggest that Ad-DKK3 regulates the expression of MDR1 via Akt/NFκB pathways and that it augments the anti-tumor effects of TMZ in GBM cells.

Keywords: DKK3, MDR1, Akt, glioblastoma, temozolomide

Introduction

Glioblastoma multiforme (GBM), a World Health Organization (WHO) grade IV astrocytoma, is the most malignant, most common primary brain tumor in adults. It is invasive and strongly resistant to radio- and chemotherapy. The DNA alkylating agent temozolomide (TMZ) has been approved for chemotherapy. Although the combination of surgery, TMZ, and radiation is the frontline treatment for GBM, the prognosis of GBM patients remains poor and their median survival is less than 15 months [1]. Also, the therapeutic efficacy of TMZ has been reported to be limited in patients with methylated O^6-methylguanine transferase (MGMT) [2,3].

Drug resistance acquired in the course of therapy is a major obstacle to the successful treatment of many cancers. The mechanisms contributing to chemoresistance remain unclear. Multidrug resistance protein 1 (MDR1, also known as P-glycoprotein), a 170-kDa transmembrane glycoprotein encoded by the *mdr1* gene, is a well-characterized drug efflux pump. It is a member of the ATP-binding cassette transporter family [4]. The overexpression of MDR1 confers multidrug resistance in cultured cells and is implicated in clinical multidrug resistance [5,6]. MDR1 expression was observed in human brain tumor cells and intratumoral capillary endothelial-, including GBM cells [7,8]. The transcriptional activation of the MDR1 gene has been studied in the presence of different stressors including chemotherapy [9]. Munoz et al. [10] attributed the increase in MDR1 in TMZ-resistant GBM cells to an increase in EGFR signaling, which induced MDR1 transcription.

Elsewhere we demonstrated that a Dickkopf (DKK) family member, DKK-3, was down-regulated in GBM cells and that its over-expression using plasmid vector degraded β-catenin inhibited cell growth via caspase-dependent apoptosis [11]. We also showed that an increase in DKK-3 inhibits the interaction between Wnt proteins and their co-receptors, leading to the inhibition of β-catenin-dependent and -independent Wnt signaling pathways, thereby affecting the Wnt downstream cascade such as the c-Jun NH2-teminal kinase (JNK)/c-Jun pathway [12]. Others demonstrated that JNK1/2 regulated MDR1 expression via c-Jun in human multidrug-resistant gastric and pancreatic cell lines [13] and that JNK/c-Jun signaling inhibited Y-box binding protein 1 (YB-1)-dependent MDR1 expression [14]. Akiyama et al. [15] found that tumor endothelial cells (ECs) are more chemoresistant than normal ECs due to the up-regulation of MDR1. They suggested that the drug resistance acquired by MDR1 up-regulation via VEGF signaling in the tumor microenvironment is associated with YB-1 via Akt activation. According to Kuo et al. [16], phosphoinositide 3-kinase (PI3K)-Akt activation induces MDR1 via nuclear factor (NF) κB. On the other hand, inhibition of the PI3K/Akt/NFκB signaling pathway decreases MDR1 expression [17,18].

To test our hypothesis that Ad-DKK3 contributes to the attenuation of chemoresistance to TMZ in GBM cells we used a U87MG cell-xenograft model and GBM cell lines. Our *in vivo* and *in vitro* studies showed that Ad-DKK3 augments the anti-tumor effects of TMZ via the attenuation of MDR1 expression. We also demonstrate that the reduction of MDR1 by Ad-DKK3 is mediated by Akt/NF□B-signaling in GBM cells.

Materials and Methods

Tissue samples

This study was approved by our institutional Ethics Committee. Tissue samples were provided by the Department of Neurosurgery of Tokushima University Graduate School. We studied 16 human samples, 5 were from GBM-, 3 from anaplastic astrocytoma (AA)-, 2 from diffuse astrocytoma (DA)-, and 6 from non-neoplastic brain tissues. One group of samples was fixed in 4% formalin in phosphate-buffered saline (PBS) and processed for paraffin embedding; the other was fresh-frozen in liquid nitrogen and stored at –70°C. All samples were classified by neuropathologists according to the WHO classification of brain tumors.

Cell lines

The human GBM cell lines U87MG and U251MG were purchased from American Type Culture Collection (Manassas, VA, USA) and the Health Science Research Resources Bank (Osaka, Japan), respectively. The TGB105 cells were primary GBM cells from a patient who granted prior informed consent for their use in this study. All GBM cell lines were cultured in RPMI-1640 medium (Invitrogen, NJ, USA) with 10% fetal bovine serum (GIBCO-BRL, NY, USA) at 37°C in an atmosphere of 5% CO_2 and air.

Adenovirus vectors

For DKK3 over-expression, cells were infected with an adenovirus vector carrying DKK3 (Ad-DKK3) as described elsewhere [19]. An adenovirus vector carrying LacZ (Ad-LacZ) was used to monitor infection efficiency. Ad-DKK3 and Ad-LacZ were prepared by Kumon et al. at Okayama University and kindly gifted to us. Ad-DKK3 was produced according to the protocol of the adeno-X adenoviral system 3 (Clontech Laboratories Inc., CA, USA), purified, and assessed with the titer assay using the adeno-X^{TM} rapid titer kit (Clontech).

Cell viability assay

The cells were plated in 96-well tissue culture plates (1×10^3 cells/well); 24 hr later they were infected with Ad-DKK3 or Ad-LacZ at a multiplicity of infection (MOI) of 30, the same volume of PBS was added in the controls. Cells were treated with TMZ (100 μM/day; LKT Laboratories Inc., MN, USA) 96-, 120-, and 144 hr post-infection. The delivered dose was based on our previous studies [12,20]. The number of viable cells 168 hr post-infection was determined with the WST-8 reagent (Dojindo, Osaka, Japan). The conversion of WST-8 to formazan by metabolically active cells was measured on a microplate reader (Infinit F200 PRO®, TECAN) at an absorbance of 450 nm. To determine the percent viability of TMZ-treated cells we considered PBS-treated cells to represent 100% viability.

Nuclear extraction

Nuclear extract was separated from the cytoplasmic fraction of U87MG cells using the nuclear/cytosol fractionation kit (BioVision Inc., Milipitas, CA, USA) according to the manufacturer's instructions. Nuclear fractions were analyzed by Western blotting for YB-1 and phospho-NFκB antibodies.

Inhibition of Y box binding protein-1 or MDR1 by small interfering RNA transfection

Transfection was conducted using the Lipofectamine RNAi MAX reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's protocol. Briefly, the cells were seeded, incubated for 24 hr, and transfected with small interfering RNA (siRNA,12.5 nM) for Y box binding protein 1 (YB-1, Life Technologies) or MDR1(Sigma-Aldrich). The sequence for YB-1 siRNA was (sense) 5'-CGAAGGUUUUGGGAACAGU-3', (antisense) 5'-ACUGUUCCCAAAACCUUCG-3'. The sequence for MDR-1 siRNA was (sense) 5'-GACCAUAAAUGUAAGGUUUTT-3', (antisense) 5'-AAACCUUACAUUUAUGGUCTT-3'. Non-targeting siRNA was used as the negative control. The mRNA level of YB-1 or MDR1 was validated after incubating transfected cells for 24 and 48 hr.

In vivo experiments

All animal experiments were approved by the Ethics Committee of Tokushima University Graduate School

and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

U87MG cells (2.0×10^6 in 50 µl PBS) were mixed with 50 µl Matrigel (BD Biosciences, Tokyo, Japan) and subcutaneously injected into the right flank of 8-week-old male BALB/C nude mice (n = 12) (Charles River Laboratories Japan Inc., Yokohama, Japan). One week later, when the tumor diameter reached 5 mm, the mice were randomly divided into 4 groups (each group n=3): Ad-LacZ-, Ad-DKK3-, Ad-LacZ plus TMZ (50 mg/kg/day)-, and Ad-DKK3 plus TMZ (50 mg/kg/day) groups. On day 0 we injected small aliquots of Ad-DKK3 or Ad-LacZ in 100 µl PBS [total 2.4×10^8 plaque-forming units (pfu)] into different tumor sites. On days 7 - 11, the mice received an intraperitoneal injection of TMZ (50 mg/kg/day); the delivered dose was based on our previous studies [12,20]. The tumor size was measured every other day for 28 days and the tumor volume was calculated using an empirical formula where

$$V = 1/2 \times [(\text{the shortest diameter})^2 \times (\text{the longest diameter})].$$

The experiments were repeated twice. All mice were sacrificed at the end of the 28-day observation period. We used 2 mice from each group for immunostaining and Western blot analysis.

Statistical analysis

Survival rates were analyzed by the Kaplan-Meier method. All data (mean ± SD) were subjected to the *t*-test for 2-group comparisons and analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons. Statistical analyses were performed with IBM SPSS Statistics 20.0 (IBM Corp., NY, USA). Differences of $p < 0.05$ were considered statistically significant.

Immunofluorescence staining, immunohistochemistry, Western blot analysis, and quantitative real-time PCR

See Supplementary Methods.

Results

MDR1 is highly expressed in the blood vessels and tumor cells of GBM patients

First we examined the expression profile of MDR1 in human non-neoplastic brain (NNB) tissues and in tissues from patients with diffuse astrocytoma (DA, WHO grade II), anaplastic astrocytoma (AA, WHO grade III),

and GBM (WHO grade IV). The MDR1 protein level was higher in glioma- than NNB tissues (Fig. 1A). As the WHO tumor grade increased, the expression of MDR1 increased while the expression of DKK3 decreased. Immunohistochemically, MDR1 expression was increased in the blood vessels and tumor cells of AA and GBM patients (Fig. 1B). These findings agreed with the results of Western blot analysis and suggest that the increase in MDR1 is associated with the chemoresistance of malignant gliomas.

In U87MG-xenografted mice, treatment with Ad-DKK3 plus TMZ extends survival and is associated with decreased MDR1 expression

To assess whether the induction of DKK3 protein reduces chemoresistance in xenografted mice, we treated their tumors with Ad-DKK3 plus TMZ (50 mg/kg/day) and recorded their survival during a 28-day observation period. Compared to mice treated with Ad-LacZ, mice subjected to combination therapy survived significantly longer and none died (Fig. 2A). The efficacy of TMZ alone was limited. On days 14 and 21, the tumor volume was significantly smaller in mice treated with Ad-DKK3 plus TMZ than in Ad-LacZ- and Ad-DKK3-treated mice (Figs. 2B and 2C). On day 21 it was also significantly smaller in mice treated with TMZ plus Ad-LacZ than in mice treated with Ad-LacZ alone (Figs. 2B and 2C). There was no difference in the body weight of all mice (Fig. 2D).

Immunohistochemical evaluation showed that the expression of DKK3 was low in Ad-LacZ-treated mice while the expression of MDR1 was high. In contrast, in Ad-DKK3-treated mice, DKK3 was highly expressed while the expression of MDR1 was reduced (Fig. 2E). Our observations suggest that the elevation of DKK3 protein may result in the down-regulation of MDR1.

Viability and MDR1 expression are reduced by in vitro treatment of GBM cells with Ad-DKK3 and TMZ

To further address the regulation of MDR1 expression by Ad-DKK3 we treated GBM cells with TMZ plus Ad-DKK3 at doses reported elsewhere [12, 20]. Each GBM cell line was exposed to Ad-DKK3 or Ad-LacZ at 30 MOI. At 24 hr post-infection, the mRNA level of MDR1 was reduced in Ad-DKK3-infected U87MG cells (Fig. 3A). At 120 hr post-infection, the expression of DKK3 was increased while the expression of MDR1 was decreased in U87MG- and TGB105 cells that had or had not also been treated with TMZ (Fig. 3B).

The observations made by immunofluorescence staining of cells treated with Ad-DKK3 or Ad-LacZ agreed with our Western blot findings (Fig. 3C). At 168 hr post-infection, viability was lowest in U87MG-, U251MG-, and

TGB105 cells exposed to Ad-DKK3 plus TMZ (Fig. 3D); it was significantly lower than in cells treated with Ad-LacZ with or without TMZ, or with Ad-DKK3 alone. This indicates that Ad-DKK3 enhanced the effect of TMZ and that the Ad-DKK3-induced reduction in the expression of MDR1 may contribute to the beneficial effect of TMZ.

Inhibition of NFκB but not of YB-1 via the JNK/c-Jun- or Akt pathway by Ad-DKK3 is attributable to the down-regulation of MDR thereby enhancing the anti-tumor effects of TMZ

Choi et al. [14] demonstrated that JNK/c-Jun signaling inhibited YB-1-dependent MDR1 expression. To identify the mechanisms underlying the down-regulation of MDR1 by Ad-DKK3 we first focused on YB-1. In U87MG cells treated with Ad-DKK3 or Ad-LacZ (100 MOI), the expression of MDR1 and of phosphorylated YB-1 (pYB-1) was significantly decreased; phosphorylated c-Jun (p-c-Jun) was significantly increased without affecting JNK (Fig. S1). While the JNK inhibitor SP600125 abrogated the increase in p-c-Jun by Ad-DKK3, it did not affect the expression of MDR1 and pYB-1. This suggests that the activation of p-c-Jun and the regulation of YB-1 have no effect on the regulation of MDR1 in U87MG cells.

The drug resistance acquired by MDR1 up-regulation is associated with YB-1 via Akt activation [15] and the PI3K downstream targets Akt and NFκB interact with the MDR1 promoter [16,18]. Next we examined whether the regulation of MDR1 by Ad-DKK3 is mediated through the PI3K/Akt/YB-1 or the Akt/NFκB pathway. In Ad-DKK3-treated cells (100 MOI), the expression of phosphorylated Akt (p-Akt), pYB-1, and NFκB/p65 was significantly decreased and associated with the reduction in MDR1 (Fig. 4A). Furthermore, the nuclear expression of pYB-1 and NFκB/p65 was significantly reduced 96 hr post-infection in U87MG cells treated with Ad-DKK3 (Fig. 4B). To further examine the role of YB-1 on MDR1 down-regulation, U87MG cells treated with siYB-1or siCont were exposed to TMZ (100 μM/day). Although the mRNA level of YB-1 and the protein expression of pYB-1 were significantly decreased by siYB-1 transfection, the viability of U87MG cells was significantly higher than of cells treated with siCont or TMZ (Fig. 4C). On the other hand, in U87MG cells transfected with siMDR1, the expression of MDR1 was reduced and their viability was significantly lower than of U87MG cells treated with siCont or TMZ alone (Fig. 4D). These results suggest that the downregulation of YB-1 may not increase the effectiveness of TMZ mediated by the down-regulation in MDR1. We further examined other pathways regulating MDR1 expression.

Using LY249002, an inhibitor of PI3K/Akt, we found that, as in Ad-DKK3-treated U87MG cells, the

expression of MDR1 and NFκB/p65 was significantly decreased (Fig. 5A). At 96 hr after the administration of LY249002, the viability of U87MG cells treated with or without TMZ was significantly decreased. There was no significant difference between these groups (Fig. 5B), suggesting that the rate of cell death was not increased by TMZ via the inhibition of Akt.

To further examine the role of NFκB on MDR1 down-regulation, we used MG132, a proteasome inhibitor. The expression of MDR1 and NFκB was significantly decreased in U87MG cells treated with MG132 (Fig. 5C). U87MG cells exposed to TMZ (100 μM/day) plus MG132 exhibited the lowest viability; it was significantly lower than of cells treated with TMZ or MG132 alone (Fig. 5D). These observations suggest that DKK3 treatment down-regulates MDR1 expression by mediating the inhibition of NFκB, thereby at least partly contributing to the decreased resistance of GBM cells to TMZ.

Discussion

We first confirmed that in glioma patients, the expression of MDR1 was increased in the tumor blood vessels and cells and that the degree of its expression increases with the glioma grade. We document that combination treatment with Ad-DKK3 and TMZ enhanced the survival GBM xenografted mice. We also show that this combination treatment reduced MDR1 expression in both U87MG-xenograft mice and in human GBM cell lines and that the down-regulation of MDR1 by Ad-DKK3 is mediated by the inhibition of NFκB in GBM cell lines. Our findings suggest that the down-regulation of MDR1 may at least partly contribute to the enhanced anti-tumor effect of TMZ and the decreased drug resistance of GBM tumors.

GBM becomes chemoresistant to TMZ and the survival of GBM patients is limited. No strategies to overcome this resistance are currently available. Earlier studies [10,21] demonstrated that P-glycoprotein competes with TMZ and contributes to the chemoresistance of GBM cells. Molecular modeling identified 6 predicted binding sites for TMZ within the intracellular region of P-gp [21]. Riganti et al. [22,23] reported that TMZ down-regulates MDR1 expression by disrupting Wnt3 signaling. Ad-DKK3 down-regulates Wnt3 [12] and treatment with TMZ alone reduced the expression of MDR1. Therefore, treatment with TMZ plus Ad-DKK3 may further reduce the expression of MDR1 via Wnt3 signaling.

Elsewhere our group reported that the over-expression of DKK3 exerts anti-tumor effects in GBM patients

via β-catenin-dependent apoptosis [11] and that Ad-DKK3 also inhibited the β-catenin-independent Wnt signaling pathway [12] The transcriptional regulation of MDR-1 in breast cancer is thought to be associated with the β-catenin independent pathway downstream from the JNK/c-JUN pathway [24] and may be mediated by transcription via YB-1 [14]. As Ad-DKK3 activated the JNK/c-JUN signal pathway [12], we speculated that the Ad-DKK3-increased expression of p-c-Jun was associated with a reduction in the expression of YB-1. However, unexpectedly, the expression of MDR1 before and after treatment with the JNK inhibitor SP600125 was not markedly different despite the reduction in p-c-Jun. These findings suggest that the regulation of MDR1 mediated by YB-1 through the JNK/c-Jun pathway may be different and dependent on the tumor cell type.

On the other hand, Xi et al. [18] reported that in GBM cells, MDR1 is regulated through the PI3K/Akt/NFκB pathway. We also documented the high expression of Akt (s473) and NFκB/p65 in GBM cells and the decreased expression of these molecules in GBM cells treated with Ad-DKK3. We found that LY294002, an inhibitor of PI3K/Akt, attenuated the expression of MDR1. However, we observed that the viability of U87MG cells treated with LY294002 with or without TMZ was not significantly different. Therefore, Akt may not directly increase TMZ-induced cell death. In contrast, the inhibition of NFκB resulted in the down-regulation of MDR1 and acted synergistically with TMZ. The Ad-DKK3-induced reduction of MDR-1 may be mediated by the inhibition of NFκB, suggesting that Ad-DKK3 attenuates MDR1 expression by hampering the transcriptional activation of NFκB and that it thereby contributes to reducing the resistance of GBM to TMZ. Our studies on combination therapy with Ad-DKK3 plus TMZ may provide new insights for the establishment of a strategy to treat GBM and promote the development of new agents targeting the regulation of MDR1.

Treatment with Ad-DKK3 plus TMZ augmented anti-tumor effects in our *in vivo* and *in vitro* studies; these effects appeared to be greater in our murine xenograft model than in the human GBM cell lines. Our observation that in humans, the expression of MDR1 was higher in tumor blood vessels than in glioma tissues suggests that the combination of Ad-DKK3 plus TMZ was more effective *in vivo* than *in vitro*.

MGMT methylation and the presence of glioma stem cells may be involved in the resistance of GBM to drug treatment [25]. It has been shown that Chk1 and Chk2 checkpoint kinases are enhanced in glioma and in glioma stem cells [26,27]. The sonic hedgehog pathway and its receptor may be associated with resistance to TMZ and the high expression of MDR1 [28,29]. A GTPase-activating protein, RLIP76, a central regulator in the response to the redox status, controls the growth and apoptosis of cells in many cancers, it is also thought to be implicated in

the chemoresistance of MDR1 [30]. Therefore, we cannot rule out that other mechanisms underlie the regulation of MDR1 expression.

Our study has some limitations. Although we attempted to induce murine intracranial tumors by stereotactic injection of GBM cells into the right brain hemisphere and three days later injected the Ad-DKK3 vector into the tumor site, we were unable to confirm that the vector was uniformly infected in the intracranial tumors. We were unable to determine the intracellular TMZ level after Ad-DKK3 treatment because TMZ was degraded in the culture medium and we did not examine the regulation of MDR1 by Ad-DKK3 in our murine brain tumor model. Intracranial tumor models using human glioma cells are not invasive [31], and the microenvironment is different between flank- and brain tumors. Nonetheless, we think that the down-regulation of MDR1, resulting in increased TMZ uptake by the tumor cells in our murine xenograft model, is reflective of its down-regulation in intracranial tumor models. We did not perform detailed experiments involving different TMZ doses for various levels of DKK3-induction in this study. Rather, we delivered doses of Ad-DKK3 and TMZ based on findings reported in our previous studies [12,20]. With respect to Ad-DKK3, IC_{50} for GBM cell viability was around 100 MOI [12]. To reduce the influence of Ad-DKK3 alone, in our cell viability assay we delivered Ad-DKK3 at 30 MOI. In our earlier study [20] we exposed cell lines to TMZ at 300 μM/day; because the viability of U87MG cells was approximately 80% at 100 μM TMZ, we used this dose in our current investigation. In preliminary experiments we confirmed the combination effects at these doses. Additional studies are underway to clarify these issues.

In conclusion, we first demonstrate that combination treatment with Ad-DKK3 plus TMZ enhances the anti-tumor effects of TMZ via the down-regulation of MDR1 due to the inhibition of the NFκB pathway by Ad-DKK3. Strategies to down-regulate MDR1 with combination therapies may hold promise for the treatment of patients with GBM.

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Figure Captions

Figure 1

Expression profile of MDR1 in human gliomas

(A) Representative expression of MDR1 and DKK3 in normal brain tissue and gliomas. The protein level of MDR1 was analyzed in 6 non-neoplastic regions (NNR), 2 diffuse astrocytomas (DA, WHO grade II), 3 anaplastic astrocytomas (AA, WHO grade III) and 5 glioblastomas (GBM, WHO grade IV) by Western blot analysis. The protein band density was calculated using Image-J software and normalized by β-actin. Bars are the mean. *$p < 0.05$ NNR vs GBM (ANOVA followed by the Tukey-Kramer test).

(B) Representative immunohistochemistry for MDR1 in a NNR, in DA, AA, and GBM.

Figure 2

Anti-tumor effect of the combination of Ad-DKK3 plus TMZ in U87MG-xenografted mice

(A) Survival rates by Kaplan-Meier analysis. The mice were injected with 2×10^6 U87MG cells 2 weeks before combination therapy and then divided into 4 groups of 3 mice each. They were treated with Ad-LacZ (vehicle control), Ad-DKK3, Ad-LacZ plus TMZ (50 mg/kg/day), or Ad-DKK3 plus TMZ (50 mg/kg/day). After the intratumoral injection of adenovirus vector (2.4×10^8 pfu) on day 0, TMZ (50 mg/kg/day) was injected intraperitoneally on days 7-11. The experiments were repeated twice. *$p<0.05$ (log-rank test).

(B) The tumor volume on the indicated days is shown. Data are the mean ± SD (n=6). *$p<0.05$ vs Ad-LacZ, $^{\dagger}p<0.05$ vs Ad-DKK3 (ANOVA followed by the Tukey-Kramer test).

(C) Photographs of tumor-bearing mice (day 21).

(D) The body weight of mice subjected to the different treatments was recorded on the indicated days. Data are the mean ± SD (n=6).

(E) Representative immunohistochemistry for DKK3 **(red)** and MDR1 **(red**) in the tumor of mice treated with Ad-LacZ or Ad-DKK3. The nuclei were counterstained with DAPI (blue). Bar: 100 μm.

Figure 3

Augmented anti-tumor effect associated with the Ad-DKK3-induced reduction in MDR1 in U87MG cells

(A) The mRNA level of MDR1 24 hr post-treatment with 30 MOI Ad-DKK3 was determined by qRT-PCR assay. Data are the mean ± SD (n=7). Western blots show the expression of DKK3 at 24 hr in Ad-DKK3-infected U87MG cells (30 MOI). *p<0.05 by Student's *t*-test.

(B) The expression level of DKK3 and MDR1 at 120 hr in Ad-DKK3-infected U87MG- and TGB105 cells (30 MOI) was analyzed on Western blots and compared to cells treated with or without TMZ (100 μM/day). Each analysis was repeated four times. The protein band density was calculated with Image-J software and normalized by β-actin. Data are the mean ± SD. *p<0.05 vs. Ad-LacZ, †p<0.05 vs. Ad-LacZ plus TMZ (ANOVA followed by the Tukey-Kramer test).

(C) Representative immunohistochemical stains for DKK3 and MDR1 (red) in Ad-LacZ- and Ad-DKK3-treated U87MG cells. The nuclei were counterstained with DAPI (blue). Bar: 10 μm.

(D) The viability of GBM cell lines was determined by WST-8 assay 168 hr post-infection. Ad-DKK3 and Ad-LacZ were transfected at 30 MOI. TMZ treatment (100 μM/day) was 96-, 120-, and 144 hr post-infection. Data are the mean±SD. *p<0.05 vs. Ad-LacZ, †p<0.05 vs. Ad-LacZ plus TMZ, §p<0.05 vs. Ad-DKK3 (ANOVA followed by the Tukey-Kramer test).

Figure 4

YB-1-independent down-regulation of MDR1 augments the anti-tumor effects of TMZ in U87MG cells

(A) Representative Western blots showing the expression of MDR1, phosphorylated Akt (p-Akt), NFκB/p65, phosphorylated YB-1 (pYB-1), and β-actin in cells infected with Ad-LacZ- or Ad-DKK3 (100 MOI). Each analysis was repeated four times. The protein band density was calculated with Image-J software and normalized by β-actin. Data are the mean±SD. *p<0.05 vs. Ad-LacZ by Student's *t*-test.

(B) Reduced nuclear expression of NFκB/p65 and pYB-1 after treatment with Ad-DKK3 (100 MOI). The nucleus fraction in cell lysates was extracted according to the manufacturer's instructions. Each analysis was repeated four times. The protein band was calculated using Image-J software and normalized to Lamin B1. Data are the mean ± SD. *p<0.05 vs Ad-LacZ by Student's *t*-test.

(C) The mRNA level of YB-1 at 24 and 48 hr post-transfection with small interfering RNA for YB-1 (siYB-1) was determined by qRT-PCR assay (n = 4). Western blots show the expression of pYB-1 at 48 hr in

siYB-1-transfected U87MG cells. The viability of U87MG cells was determined by WST-8 assay 96 hr post-transfection with siYB-1. TMZ (100 μM/day) was delivered 24-, 48-, and 72 hr post-transfection.

(D) The mRNA level of MDR1 24- and 48 hr post-transfection with siMDR1 was determined by qRT-PCR assay (n = 4). Western blots show the expression of MDR1 at 48 hr in siMDR-1-transfected U87MG cells. The viability of U87MG cells was determined by WST-8 assay 96 hr post-transfection with siMDR1. TMZ (100 μM/day) was delivered 24-, 48-, and 72 hr post-transfection. Data are the mean ± SD. *p<0.05 vs. VC, †p<0.05 vs. TMZ alone, §p<0.05 vs. siYB-1 or siMDR1 alone (ANOVA followed by the Tukey-Kramer test).

Figure 5

Effect of the Akt/NFκB pathway on the down-regulation of MDR1 by Ad-DKK3 in U87MG cells

(A) Representative Western blots showing the expression of MDR1, p-Akt, NFκB/p65, pYB-1, and β-actin in the vehicle control (VC) and in LY249002-treated cells. LY249002, an inhibitor of PI3 kinase-dependent Akt phosphorylation was added 6 hr before analysis. Each analysis was repeated four times. The protein band was calculated using Image-J software and normalized to β-actin. Data are the mean ± SD. *p<0.05 vs VC by Student's *t*-test.

(B) The viability of U87MG cells was determined by WST-8 assay 96 hr after the administration of LY249002. TMZ (100 μM/day) was added 24-, 48-, and 72 hr post-administration. Data are the mean ± SD. *p<0.05 vs. VC, †p<0.05 vs. TMZ alone (ANOVA followed by the Tukey-Kramer test).

(C) Representative Western blots showing the expression of MDR1 and NFκB/p65 in the VC and in MG132-treated cells. To inhibit NFκB activation, the proteasome inhibitor MG132 was added 1 hr before analysis. Each analysis was repeated four times.

(D) The viability of U87MG cells was determined by WST-8 assay 1 hr after MG132 administration. TMZ (100 μM/day) was added 24-, 48-, and 72 hr post-seeding; MG132 was added at 96 hr. Data are the mean ± SD. * p<0.05 vs. VC, †p<0.05 vs. MG132 alone, §p<0.05 vs. TMZ (ANOVA followed by the Tukey-Kramer test).

Supplementary Table 1

List of antibodies used for Western blotting.

Supplementary Figure 1

Effect of the inhibition of the JNK/YB-1 pathway on the regulation of MDR1 by Ad-DKK3 in U87MG cells

MDR1, JNK, c-Jun, phosphorylated-c-Jun (p-c-Jun), phosphorylated YB-1 (pYB-1), and β-actin were subjected to Western blot analysis 96 hr after transfection with Ad-LacZ or Ad-DKK3 (100 MOI). The effect of the JNK inhibitor SP600125 was examined 6 hr after its addition. Each analysis was repeated four times. The protein band density was calculated with Image-J software and normalized by β-actin. Data are the mean ± SD. *$p<0.05$ vs. Ad-LacZ, $^{\dagger}p<0.05$, vs. Ad-DKK3 (ANOVA followed by the Tukey-Kramer test).

Supplementary Methods

Immunofluorescence staining

Tissues: Tissue samples from U87MG xenografted mice were fixed with 4% paraformaldehyde, 5-μm-thick frozen sections were mounted on Matsunami adhesive saline (MAS)-coated glass slides (Matsunami Glass, Tokyo, Japan), blocked with serum-free protein block (Dako Cytomation Japan, Tokyo, Japan), immunostained with polyclonal anti-human DKK3 antibody (R&D Systems Inc., Minneapolis, MN, USA, 1:100 dilution) in Can Get Signal immunostain (Toyobo, Osaka, Japan), with Mdr-1(D-11) antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, 1:100 dilution) in Can Get Signal immunostain, and incubated overnight in a 4°C humidity chamber. Residual antibody was washed off with PBS (three 5-min washes) and the samples were then reacted with secondary antibody-labeled Alexa Flour® 594 donkey anti-goat IgG antibody and goat anti-mouse IgG antibody (Invitrogen). Nuclear staining was with 4',6-diamidino-2-phenylindole (DAPI).

Cells: U87MG cells were plated (1×10^5 cells/well) in 35-mm tissue culture dishes. After 24 hr they were infected with 1×10^7 plaque-forming units (pfu) of Ad-DKK3 or Ad-LacZ in 400 μl of culture medium; one hour later we added culture medium to each dish. After 48 hr, the culture medium was aspirated, the dishes were rinsed with PBS, and 4% paraformaldehyde in PBS was added to fix the cells and extracellular proteins. Immunostaining was with polyclonal anti-human DKK3 antibody (R&D Systems Inc., 1:100 dilution) in PBS in the presence of 1% bovine serum albumin (BSA). The dishes were incubated for 1 hr at room temperature. After removing the residual antibody with three 5-min washes in PBS, the cells were reacted with secondary antibody-labeled Alexa Flour® 594 donkey anti-goat IgG antibody (Invitrogen). DAPI was used for nuclear staining.

Immunohistochemistry

Sections (5 μm) from formalin-fixed, paraffin-embedded tissue samples were placed on charged glass slides, deparaffinized with xylene, and rehydrated through a graded alcohol series. For antigen retrieval, deparaffinized sections were immersed in Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 9.0), heated at 95°C for 20 min, and allowed to cool to room temperature. The sections were soaked in 3% H_2O_2 in methanol for 10 min to inactivate endogenous peroxidase. After blocking with a serum-free protein block (Dako), they

were immunostained with rabbit monoclonal anti-MDR1 antibody (Abcam, Cambridge, UK; ab170904) at 1:100 dilution in Can Get Signal immunostain (Toyobo) and incubated overnight in a 4°C humidity chamber. Residual antibody was washed off with PBS (three 10-min washes) and the samples were reacted with secondary antibody using the avidin-biotin system (VECTASTAIN Elite ABC Rabbit IgG Kit, Vector Laboratories, Burlingame, CA, USA). The sections were incubated with 3,3'-diaminobenzidine (DAB, Liquid DAB+ Substrate Chromogen System, Dako). After washing and counterstaining with hematoxylin, the samples were mounted on glass slides.

Western Blot Analysis

Tissue samples and precipitates from harvested cells were homogenized in RIPA buffer (Thermo SCIENTIFIC, IL, USA) and protease inhibitors (complete, mini, EDTA-free®, Roche Diagnostics K.K., Tokyo, Japan). After 5-min centrifugation at 5,000 rpm the protein concentration in the supernatants was assayed with BCA reagent® (PIERCE, Tokyo, Japan). After reduction in 60 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 100 mM DTT, and 0.002% bromophenol blue, 20 - 50 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes (trans-blot transfer medium®, BIO-RAD, Hercules, CA, USA). The membranes were immersed for 1 hr in blocking buffer [(5% non-fat dry milk or 2% BSA or 5% BSA in tris-buffered saline (TBS)] and then incubated with anti-human primary antibodies (Table S1). All primary antibodies were diluted in Can Get Signal Solution 1 (Toyobo). After washing in Tween-TBS (T-TBS), the membranes were incubated for 1 hr with horseradish peroxidase-conjugated secondary antibodies in Can Get Signal Solution 2 (dilution 1:3000). The protein-antibody complexes were detected with Amersham ECL plus® Western blotting detection reagents (GE Healthcare, UK) using a Lumino image analyzer (Image Quant LAS-4000,® GE Healthcare Japan, Tokyo, Japan). Each experiment was repeated four times. To calculate the protein levels obtained by Western blot analysis we used NIH ImageJ 1.46 software (http://rsb.info.nih.gov/ij/).

Quantitative real-time PCR

Total RNA isolated from cultured GBM cells was purified with the MagNA Pure Compact RNA Isolation Kit (Roche Diagnostics) according to the manufacturer's protocol and reverse-transcribed with Transcriptor Universal cDNA Master (Roche Diagnostics). Quantitative real-time PCR (qRT-PCR) was

performed under the conditions recommended by the manufacturer on a Light Cycler rapid thermal cycler (Roche Diagnostics). The forward and reverse primer sequences were 5'-AGGCCAACATACATGCCTTCATC-3' and 5'- GCTGACGTGGCTTCATCCAA-3' for MDR1, 5'-CTCTACCATCATCCGGTTTAGTC-3' and 5'- CTTTAGGTCTTCAGCTCCAATCT-3' for YB-1, and 5'-GGGTGTGAACCATGAGAAGTATGA-3' and 5'-TGCTAAGCAGTTGGTGGTGC-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers were optimized at Nihon Gene Research Laboratories Inc. (Sendai, Japan). The expression of DKK3 mRNA was normalized relative to the GAPDH mRNA content. Each experiment was repeated four times.