Temozolomide suppresses MYC via activation of TAp63 to inhibit progression of human glioblastoma

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Glioblastoma multiforme (GBM) is a highly invasive and chemoradioresistant brain malignancy. Temozolomide (TMZ), a DNA-alkylating agent, is effective against GBM and has become the standard first-line drug. However, the mechanism by which TMZ regulates the progression of GBM remains elusive. Here, we demonstrate that TMZ targets TAp63, a p53 family member, inducing its expression to suppress the progression of human GBM. High levels of TAp63 expression in GBM tissues after TMZ treatment was an indicator of favourable prognosis. In human GBM cells, TMZ-induced TAp63 directly repressed MYC transcription. Activation of this TAp63-MYC pathway by TMZ inhibited human GBM progression both in vitro and in vivo. Furthermore, downregulation of MYC mRNA levels in recurrent GBMs after TMZ treatment correlated with better patient survival. Therefore, our results suggest that the TAp63-mediated transcriptional repression of MYC is a novel pathway regulating TMZ efficacy in GBM.

Results
High TAp63 expression correlates with favorable prognosis in human GBM. To investigate the expression levels of p63 and p73 in human GBM, 69 newly diagnosed malignant gliomas (59 GBMs; 7 anaplastic astrocytomas; 3 anaplastic oligoastrocytomas) were selected for RNA and DNA analyses (Supplementary Table S1). Forty-nine cases had received procarbazine, ACNU and vincristine (PAV) therapy and twenty had

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received TMZ therapy. First, our clinical data showed that TMZ treatment significantly prolonged the survival period of patients with malignant glioma ($P = 0.0394$; Supplementary Fig. S1a), and that high levels of $MGMT$ expression were significantly associated with unfavourable prognoses ($P = 0.0015$; Supplementary Fig. S1b). Following quantitative RT-PCR for the analysis of $TAp63$ and $TAp73$ expression, the samples were divided into groups with high or low levels of $TAp63$ (Fig. 1a) and $TAp73$ (Supplementary Fig. S2) based on their respective expression levels in a normal adult brain. Kaplan–Meier cumulative survival curves showed that high $TAp63$ expression was significantly associated with prolonged survival ($P = 0.0373$; Fig. 1b). In contrast, the levels of $TAp73$ expression showed no prognostic potential (Supplementary Fig. S2). Therefore, from here on we focused on $TAp63$ and selected the 20 TMZ-treated GBM samples to perform immunohistochemical staining for p63. We found that p63 immunopositivity (Fig. 1c) was significantly associated with better recurrence-free survival ($P = 0.0066$, Fig. 1d), and as $ANp63$ mRNA was not detectable in these samples (data not shown), the positive p63 immunohistochemical staining may largely reflect the expression levels of $TAp63$ protein. Therefore, a high expression level of $TAp63$ is a favourable prognostic indicator of human GBM for cases that undergo TMZ treatment.

**$TAp63$ directly represses MYC transcription in response to TMZ.** Since other DNA damaging agents activate $TAp63$ by inducing its mRNA expression\textsuperscript{13}, we next investigated the role of TMZ in $TAp63$ expression. The steady-state mRNA levels of $TAp63$ in four human GBM cell lines were analysed (Supplementary Fig. S3a), and $TAp63$ isoforms was the major $TAp63$ isoform expressed (Supplementary Fig. S3b). TMZ stimulated $TAp63$ expression in both $TP53$ mutant (YH-13) and wild-type (U87MG) cells, and induced the expression of $TAp63$ target genes, including $BAX$, $CDKN1A$ ($p21$) and $MDM2$ (Fig. 2a; Supplementary Fig. S4). In recent reports on functional network modelling analyses of GBM, a significant association between the $p53$ and MYC pathways has been suggested\textsuperscript{14,15}. In addition, deletions of $TP53$ and $PTEN$ in murine brains caused the spontaneous development of GBM with a simultaneous induction of MYC\textsuperscript{16}. Therefore, we examined the expression of MYC in TMZ-treated GBM. Interestingly, TMZ induced $TAp63$ expression and suppresses MYC expression in a dose-dependent manner in both U87MG and YH-13 cells (Fig. 2a). Immunoblotting also showed an increase in $TAp63$ with suppression of MYC (Fig. 2b). Since $p53$ is reported to directly repress MYC transcription\textsuperscript{17,18}, we investigated whether $TAp63$ could regulate the transcription of MYC. Regardless of the status of $TP53$, siRNA knockdown of $TAp63$ induced MYC expression at both the mRNA (Fig. 2c; Supplementary Fig. S5a) and protein (Fig. 2d) levels, whereas $TAp63$ overexpression suppressed MYC (Fig. 2e; Supplementary Fig. S5b) and induced $BAX$, $CDKN1A$ and $MDM2$ (Supplementary Fig. S6a,b). MYCN levels remained unaffected in GBM cell lines (Supplementary Fig. S7a,b). To further confirm if $TAp63$-mediated suppression of MYC occurred in primary GBM cells, we introduced...
the use of cancer tissue-originated spheroids (CTOS) composed of pure tumor cells derived directly from the GBM tissue. As shown in Supplementary Fig. S8, the TAp63- MYC regulatory pathway was intact in the CTOS. To examine whether TAp63 directly represses MYC transcription, we performed a chromatin immunoprecipitation (ChIP) assay. We designed four primer sets (R1, R2, R3 and R4) to amplify the indicated genomic regions of the putative TP53 binding sequence (Fig. 2f). Our results showed that endogenous TAp63 was recruited to the upstream promoter and the intron 1 region of the MYC gene in YH-13 cells, but not intron 2, which has been previously shown as a p53-binding site (Supplementary Fig. S9).

TAp63 overexpression in YH-13 and U87MG cells inhibited MYC promoter activity (Fig. 2j; Supplementary Fig. S10), suggesting that TMZ stimulates the TAp63-mediated repression of MYC transcription in GBM cells.

The TAp63-MYC pathway regulates sphere formation and invasion in GBM. MYC is a regulator of stemness in glioma, and its expression is required for glioma cell neurosphere formation. Consistent with these notions, TAp63 knockdown increased both the number and size of YH-13 neurospheres, while knockdown of MYC suppressed the sphere-forming ability (Fig. 3a,b). Moreover, the number of spheres formed by YH-13 cells co-transfected with both siTAp63 and siMYC was less than that of cells transfected with siTAp63 alone, suggesting that MYC induction by TAp63 knockdown contributes to the sphere-forming ability of tumour cells.

TAp63 also plays a critical role in the regulation of cancer invasion. To assess whether the TAp63-MYC pathway also regulates invasion, TAp63 and MYC were knocked down in YH-13 cells with siRNA for 24 and 48 h. Although knockdown of endogenous TAp63 and MYC had no effect on cell proliferation by day 2 of culture (Fig. 3c), knockdown of TAp63 alone significantly promoted the cellular invasion of YH-13 cells. Additionally, co-transfection of siTAp63 and siMYC decreased the percentage of invading cells compared to siControl-transfected cells (Fig. 3d), suggesting that TAp63 downregulates GBM invasion via MYC suppression.

Temozolomide suppresses tumour cell growth and invasion via TAp63 induction. We assessed whether TMZ affects the invasive property of GBM cells via the TAp63-MYC pathway. The treatment of U87MG cells with 75 μM TMZ inhibited cell proliferation and significantly inhibited cellular invasion on day 5 of exposure (Fig. 4a,b). Since apoptotic cell death was not observed under these experimental conditions (Supplementary Fig. S11), TMZ may have anti-invasive properties. Moreover, U87MG cells were transfected with the indicated siRNAs for TAp63 and MYC and subjected to Boyden chamber invasion assays. TAp63 knockdown in U87MG cells induced MYC mRNA expression and rescued the TMZ-inhibited cellular invasion, compared to the control (Fig. 4c). Next, to assess the antitumour effects of TAp63 in vivo, we knocked down...
TAp63 in the tumors of U87MG xenograft mouse models. Our results showed that TAp63 knockdown promotes tumor cell proliferation (Supplementary Fig. S12a,b). To investigate the role of TAp63 in TMZ efficacy, we implanted U87MG cells into the hind legs of mice (n = 4) and, 7 days later, injected siControl and siTAp63 into the palpable tumors, which were then treated with TMZ (15 mg/kg) intraperitoneally on the same day. At day 14, tumors subjected to siTAp63 knockdown with TMZ treatment were considerably larger compared to the control tumors, indicating a drug-resistant phenotype (Fig. 4d). Finally, we assessed whether the TMZ-induced activation of the TAp63-MYC pathway is associated with the clinical outcome of patients. To this end, we analysed the RNA from 20 paired (initially diagnosed tumour and recurrent tumour from the same patient) malignant glioma samples (17 GBMs, three anaplastic oligoastrocytomas) from TMZ-treated patients. The expression levels of MYC mRNA were significantly decreased after the treatment (Fig. 4e). Furthermore, the subgroup with decreased MYC expression after TMZ treatment showed significantly better overall survival (Fig. 4f). We performed a similar analysis using only the GBM mRNA data and both TAp63 expression and MYC suppression indicated good prognoses (Fig S13a–f). Taken together, these findings suggest that the TMZ-mediated suppression of MYC via TAp63 activation is a key pathway for the drug's efficacy against GBM.

**Discussion**

In this study, we report that TAp63 regulates MYC transcription and tumor progression in TMZ-treated GBM. The study highlighted four points. First, TAp63 expression is a favourable prognostic factor in TMZ-treated GBM. Second, TMZ induces TAp63 to suppress growth and invasion. Third, TAp63 directly represses MYC expression in response to TMZ treatment, and fourth, MYC downregulation correlates with TMZ efficacy.

A possible mechanism of TMZ action could be that TMZ induces TAp63 expression in GBM cells, and in turn, activated TAp63 inhibits cellular invasion via suppression of MYC expression. These findings are consistent with the previous reports in which TAp63 suppresses invasion through coordinated transcriptional regulation of its downstream target genes such as SERPINB5, CCNG2, BHLHE41 and DICER1^{21–24}. A prior study on the regulatory mechanisms of p53 upon its downstream targets showed that in response to hypoxic stress, p53 is recruited onto intron 2 of the MYC gene to directly inhibit MYC transcription^{19}. Our work has identified a critical role for TAp63 as
Figure 4 | TMZ inhibits GBM progression via the TAp63-MYC pathway. (a) Cell viability assay showing suppression of proliferation in cells treated with TMZ, 75 μM. (b) Cellular invasion was suppressed by day 5, at which point the treated and control cultures were adjusted to 5 × 10^4 cells/500 μl and subjected to Boyden chamber invasion assays. *P < 0.05 (two-tailed t-test). (c) Percentage of U87MG cells invading the Matrigel relative to control migration, following TAp63 knockdown and TMZ treatment. Corresponding mRNA analysis. *P < 0.05 (two-tailed t-test). (d) Effect of TAp63 knockdown on the U87MG cells treated with TMZ treatment at 14 days after subcutaneous transplantation in nude mice. The end volumes were compared to the volumes at implantation. Tumour growth was measured with callipers and calculated by the formula: volume = length (A) × width (B) × width (B) × 0.5, where A and B are the long and short axes respectively. TMZ in PBS was administered intraperitoneally at 15 mg/kg once a week. Data are representative of five independent experiments (n = 5). *P value by two-tailed t-test. Photographs in (d) are representative of n = 5 mice. Bar, 10 mm. (e) MYC downregulation in recurrent tumours after TMZ plus radiotherapy (n = 20; solid line: mean difference; dashed lines: 95% confidence interval. P value by paired t-test. (f) Overall survival according to MYC downregulation (≥1.5-fold decrease in primary/recurrent (p/r) MYC mRNA), post-TMZ treatment. MYC decrease: n = 11, mean p/r = 2.65 ± 0.31 s.d.; no change: n = 9, mean p/r = 0.9 ± 0.13 s.d. P value by log-rank test.
an alternative regulator of MYC in human GBM. Distinct from the p53-MYC regulatory mechanism, Tap63 is activated in response to TMZ-induced stress, and does not bind to intron 2 but is recruited onto the upstream promoter region of MYC to directly repress transcription. In addition, the interaction between the p53 and MYC signaling networks are important for cellular proliferation and differentiation in the prevention of GBM pathogenesis. However, p53 is functionally inactivated in many aggressive GBMs. Therefore, the newly identified Tap63-MYC regulatory pathway may serve as an alternative activator of the p53-regulated tumour suppressive pathways.

Recently, there have been several reports on the role of p53 in TMZ resistance in GBM. TMZ activates p53 to induce apoptosis in GBM cell lines, and functional inactivation of the p53 pathway by overexpression of the p53 family members increases TMZ sensitivity in GBM cell lines, and in vivo xenograft models, and that the status of p53 is not a molecular predictor of the response to chemotherapy with TMZ.

In conclusion, our results clearly indicate that MYC is a novel Tap63 target gene and that the Tap63-MYC pathway has a crucial role in mediating suppression of GBM progression. Pharmacological targeting of the Tap63-MYC pathway may therefore provide new rational therapeutic strategies against TMZ-resistant GBMs.

Methods

Glioma tumour samples and tissue dissection. A total of 89 malignant glioma samples comprising of newly-diagnosed GBM (World Health Organization grade 4, n = 59), newly-diagnosed anaplastic astrocytoma (WHO grade 3, n = 7), newly-diagnosed anaplastic oligoastrocytoma (WHO grade 3, n = 3) and 20 recurrent malignant gliomas (17 GBMs, 3 anaplastic oligoastrocytomas) were collected from 1994 and 2011 and obtained from the Chiba Cancer Center upon receiving informed consent under an institutional review board-approved protocol. The present project was approved by the Ethics Committee of the Faculty of Biology and Medicine at the Chiba Cancer Center (protocol 15–19). After surgery, the specimens (supplied by Chiba Cancer Center) were used to generate cancer tissue-p63-expressing sample as the threshold standard. A newly diagnosed GBM was used to sacrifice the tissue, and then snap-frozen in liquid nitrogen. The samples were then cotransfected with a horseradish peroxidase-conjugated secondary antibody (1:1000; IgG #7074, 1:2000; IgG #7076, 1:1000) and normal mouse IgG (015-000-003, Jackson ImmunoResearch Laboratories Inc.). The primary antibodies were visualized using a chemiluminescence-based detection kit (ECL and ECL pro kit; Amersham and PerkinElmer).

Chromatin immunoprecipitation (ChIP) assay. YH-13 cells transfected with siRNA or exposed to TMZ at increasing concentrations between 150–300 μM were harvested 24 h after the second transfection or after TMZ exposure. ChIP assays were performed with a ChIP assay kit (Millipore) according to the manufacturer’s instructions using an anti-p63 antibody (clone (4A4) sc-8431, Santa Cruz Biotechnology) and normal mouse IgG (015-000-003, Jackson Immunoresearch Laboratories Inc.). The primer sequences for the MYC promoter are described in the SI Methods.

Luciferase reporter assay. pBV-LUC Del1 (#16601) and Del2 (#16602) containing the MYC promoter regions at nucleotides –2268 to +525 and –1061 to +525 from the transcription start site, respectively, were obtained from Addgene. YH-13 and U87MG cells were seeded at 5 × 10⁴ cells/well in a 24-well plate and allowed to adhere overnight. The cells were subjected to lentiviral infection on the following day with control pLV vector or pLV-Tap63x vector. At 54 h after infection, the cells were seeded in triplicate on 12-well plates at 1 × 10⁵ cells/well and cultured for 24 h. They were then cotransfected with 400 ng of MYC luciferase reporter construct and 40 ng of Renilla TK with Lipofectamine 2000 (Invitrogen). At 18 h after the second transfection, the cells were harvested and the luciferase activity was determined using a dual-luciferase assay system (Promega) according to the manufacturer’s instructions.

Cell viability assay (MTT assay). Cell viability was quantified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cells were collected and seeded in 96-well plates at 1 × 10⁴ cells/well. After addition of 10 μl of MTT tetrazolium salt (Sigma) solution to each well, the plates were incubated in a CO₂ incubator. The absorbance of each well was measured using a Dynatech MR900 plate reader with a test wavelength of 450 nm and a reference wavelength of 690 nm.

Sphere formation assay. We evaluated spheres derived from YH-13 cells transfected with the appropriate siRNA. After performing cell counts, we plated single cells in 60 mm non-coated dishes (2.5 × 10⁴ cells/dish; Iwaki) to check the sphere morphology, and used a 96-well ultra-low cluster plate (2.5 × 10⁵ cells/well; Costar) to count the spheres. The cells were allowed to proliferate in serum-free DMEM (Sigma) and F12 medium (Invitrogen) containing epidermal growth factor (Sigma) and 20 ng/ml basic fibroblast growth factor (Invitrogen) with 2% B27 supplement (Invitrogen). Half of the medium was replaced with fresh culture medium every 7 days.

Mouse xenograft models. Six- or seven-week-old male athymic BALB/c nu/nu nude mice were obtained from Japan SLC, Inc. The mice were anesthetized with intraperitoneal isoflurane and tracheotomy was performed at 20 mg/kg body weight. U87MG cells mixed with an equal volume of Matrigel were implanted into the right and left hind legs. One week after tumour cell implantation, we injected 50 μl of Aloe vera (Koken) with either control siRNA or siTap63 (10 μM) into the U87MG xenografts. A total of 15 mg/kg TMZ was administered intraperitoneally once per week. Tumour growth was
measured with calipers and calculated by the formula: volume \( V = \text{length} \times \text{width} \times \text{height} \). These studies were approved by the Committee for Animal Care at the Chiba Cancer Center Research Institute.

**Statistical analysis.** Data is presented as the mean ± the standard deviation. Statistical significances in the clinical data were calculated using Kaplan-Meier survival curves. Statistical analyses were performed with JMP® 10 (SAS institute Japan).

1. Chen, J., McKay, R. M. & Parada, L. F. Malignant glioma: lessons from genomics, mouse models, and stem cells. *Cell* **149**, 36–47 (2012).
2. Mrugala, M. M. & Chamberlain, M. C. Mechanisms of disease: temozolomide and glioblastoma - look to the future. *Nat. Clin. Pract. Oncol.* **5**, 476–486 (2008).
3. Stupp, R. et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N. Engl. J. Med.* **352**, 987–996 (2005).
4. Hegi, M. E. et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N. Engl. J. Med.* **352**, 997–1003 (2005).
5. Shah, N. et al. Comprehensive analysis of MGMT promoter methylation: correlation with MGMT expression and clinical response in GBM. *PLoS One* **6**, e16146 (2011).
6. Beier, D., Schulz, J. B. & Beier, C. P. Chemoresistance of glioblastoma cancer stem cells – much more complex than expected. *J. Cancer** **Research** **72**, 3463–3470 (2012).
7. Zhang, W. B. et al. Activation of AMP-activated protein kinase by temozolomide contributes to apoptosis in glioblastoma cells via p33 activation and mTORC1 inhibition. *J. Biol. Chem.* **285**, 40461–40471 (2010).
8. Blough, M. D. et al. Effect of aberrant p53 function on temozolomide sensitivity of glioblastoma cell lines and brain tumor initiating cells from glioblastoma. *J. Neurooncol.* **102**, 1–7 (2011).
9. Dinca, E. B. et al. p53 small-molecule inhibitor enhances temozolomide cytotoxic activity against intracranial glioblastoma xenografts. *Cell Death Differ.* **18**, 1003–1009 (2008).
10. Iuchi, T. et al. Hypofractionated high-dose irradiation for the treatment of malignant astrocytomas using simultaneous integrated boost technique by IMRT. *Int. J. Radiat. Oncol. Biol. Phys.* **64**, 1317–1324 (2006).
11. Ohira, M. et al. Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate risk neuroblastomas. *Cell Cancer Cell* **7**, 337–350 (2005).

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**Author contributions**

T.Y. and Y.S. designed, performed and analysed cellular and animal experiments and wrote the manuscript. T.I. collected clinical samples and analysed clinical data. M.O. assisted with discussions and experimental assistance with mice throughout the course of this study. This work was supported in part by a grant-in-aid from the Ministry of Health, Labour and Welfare for the Third Term Comprehensive Control Research for Cancer, Japan, and a grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

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