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Human Glioma Cells Acquire Temozolomide Resistance After Repeated Drug Exposure *Via* DNA Mismatch Repair Dysfunction

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Abstract. *Background/Aim: Temozolomide (TMZ) induces prolonged arrest of human glioma cells in the G_2/M phase and inhibition of the G_2 checkpoint intensifies the effect of TMZ. These findings suggest that the G_2 checkpoint is linked to DNA repair mechanisms. Materials and Methods: To clarify the mechanism of TMZ resistance, we established TMZ-resistant (TR) clones by serial treatment of U87MG cells with TMZ. We evaluated TMZ-induced cell cycle arrest and the effect of various G_2 checkpoint inhibitors. Results: We observed that longer exposure (over 6 months) to TMZ enriched the proportion of TR clones that underwent only minimal G_2 arrest following TMZ treatment compared to short exposure (4 months) to TMZ. Expression of MSH6 was reduced in these clones. None of the G_2 checkpoint inhibitors could resensitize TR clones to TMZ. Conclusion: Longer drug treatment may induce resistance of cells to DNA damaging agent(s) by means of mismatch repair modification.*

Temozolomide, a DNA alkylating agent, is the main chemotherapeutic agent in the management of glioblastoma. TMZ creates a methyl adduct at the O^6 position of guanine in DNA (1, 2). Although O^6-methylguanine itself does not cause serious DNA damage, the presence of O^6-methylguanine in cells with insufficient activity of O^6-methylguanine-DNA methyltransferase (MGMT) causes guanine/thymine (GT) mismatch during DNA replication (3). GT mismatches are recognized by the DNA mismatch repair system (MMR), which removes thymine. However, as long as O^6-methylguanine exists, thymine is continuously incorporated into the pairing side, the GT mismatches are not eliminated, and thymine removal is repeated. TMZ administration causes the barren cycle to repeat, which leads to eventual ATP depletion and DNA double strand breaks, which in turn lead to cytotoxicity (4).

Previous studies have shown that the most prominent event in glioma cells exposed to TMZ is prolonged G_2 phase cell cycle arrest (5-7), and we have previously reported that the G_2 checkpoint inhibitors, including the chk1 inhibitor and the cyclin-dependent kinase 1 (cdk1; cdc2) inhibitor, blocked TMZ-induced G_2 arrest leading to an increase in cell death (8, 9). These results suggest the linkage between TMZ-induced cell cycle regulation and DNA repair, although little is known about how DNA repair is mediated through G_2 checkpoint activation. Furthermore, we have also previously published that Akt, which promotes cell survival and is commonly activated in many neoplasms including glioblastoma, inhibits both TMZ-induced G_2 arrest and cell death (10). These results suggest a complex interaction of the G_2 checkpoint and other pathways.

MGMT is a well-known resistance mechanism because it removes methyl adduct from O^6-methylguanine, the main cause of TMZ-induced cytotoxicity (11). However, a number of glioblastomas show methylation in the promoter region of MGMT, which leads to a decrease in MGMT expression (12-15). Considering that even glioblastomas with decreased MGMT expression come to acquire resistance to TMZ (16), it becomes necessary to clarify the mechanism of TMZ resistance caused by factors other than MGMT.

In this study, we aimed to clarify the mechanism of TMZ resistance in glioblastomas with low MGMT expression to address this problem. We established TMZ- resistant cell clones from human glioblastoma U87MG cells, and analyzed TMZ-induced cell cycle arrest, expression of the proteins related with drug sensitivity, and the effect of various G_2 checkpoint inhibitors.

Materials and Methods

Cell culture and drug administration. The human glioblastoma cell line U87MG was cultured in Dulbecco's Modified Eagle Medium

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with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) at 37˚C and 5% CO_2. The cultures were seeded for over 2 days before drug treatment.

Drugs and treatment. TMZ (FUJIFILM Wako Chemicals, Osaka, Japan), rabusertib (RS), and MK-8776 (MK) (Sellek Chemicals, Houston, TX, USA) were dissolved in dimethyl sulfoxide (DMSO) (FUJIFILM Wako Chemicals, Osaka, Japan). Flavopiridol (FP) was supplied by the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, and was dissolved in DMSO.

Unsynchronized cells were treated with TMZ (100 μM) for 3 h, washed with culture medium, and collected subsequently in a subconfluent state. In the colony formation efficiency assay, cells were treated with TMZ [50 μM or 100 μM, 3 h], FP [50 nM, 3 days (d)], MK (500 nM, 3 d), or RS (250 nM, 3 d). FP, RS, and MK were also dosed in combination with TMZ.

Cell cycle phase evaluation. Cells attached to culture dishes were trypsinized at each time point and collected together with the cells floating in the media. Then, cells were washed with phosphate-buffered saline (PBS), fixed with 70% (v/v) ethanol, and if needed, stored at −20˚C for a maximum of 2 weeks. Cells were then washed with PBS once and incubated with PBS-containing 40 μg/ml propidium iodide (Sigma-Aldrich, St Louis, MO, USA) and 200 μg/ml RNase A (Sigma-Aldrich) at 20°C in the dark for one hour. The stained nuclei were analyzed using the Becton Dickinson FACScan (San Jose, CA, USA) or Beckman Caulter Gallios (Brea, CA, USA).

Western blot. Preparation of protein extracts and western blots was performed as previously described (5). The membranes onto which proteins were transferred and blocked were labeled with MGMT (Kamiya Biomedical Co., Tukwila, WA, USA), α tubulin, cdc2, MSH2, and MSH6 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), β actin, chk1, chk2, phosphorylated chk1 (p-chk1), phosphorylated chk2 (p-chk2), phosphorylated cdc2 (p-cdc2) (Cell Signaling Technology, Danvers, MA, USA) antibodies, which were identified using an enhanced chemiluminescence detection system.

Colony formation efficiency. Cells were seeded at 500 cells/well in 6-well culture plates. After overnight culture, cells were treated with each drug (TMZ, FP, RS, or MK) in the conditions stated above and allowed to form colonies in culture medium with no drug. Fifteen days after drug exposure, cells were stained with methylene blue and colonies with more than 50 cells were counted. We performed at least three independent experiments.

Statistical analysis. We used the Mann-Whitney *U*-test to assess colony formation efficiency.

Results

G_2 cell cycle checkpoint was not activated by TMZ treatment in human glioma clones selected after long-term repetitive exposure to the drug. To investigate the mechanism through which TMZ-treated cells acquire resistance to TMZ, U87MG cell were treated with TMZ for 3 h at gradually increasing concentrations (10 μM → 25 μM → 50 μM → 100 μM → 200 μM) every 2 weeks, and survived cells were maintained with repetitive TMZ administration at 200 μM/2 weeks. After 4 months of treatment, the surviving colonies were selected as TMZ-resistant (TR) clones (TR1-9). These clones were confirmed for their proliferation activity in the presence of TMZ by colony formation efficiency (Figure 1a). FACS analysis showed that TMZ induced G_2 arrest transiently in some clones, whereas no cell cycle phase arrest was observed in others, which suggested that the response to TMZ varied among the resistant cell clones selected though the same treatment (Figure 1b). Based on this observation, we hypothesized that longer exposure to TMZ may result in a more uniform response of TRs to TMZ. We harvested TRs from another U87MG culture after repeated TMZ exposure for a long period of time (over 6 months). All of them (TR11, TR14, TR17, and TR20) showed no cell cycle arrest in response to TMZ (Figure 1c). The colony formation efficiency assay confirmed that all TMZ–induced G_2-arrest-resistant clones acquired high TMZ resistance (Figure 1d). Phosphorylation of G_2 checkpoint proteins chk1 and chk2 in response to DNA damage increases the phosphorylated form of cdc2 and inhibits exit of cells from the G_2 phase (17-27). Treatment of U87MG cells with 100 μM TMZ resulted in increased expression of p-chk1 (Ser345), p-chk2 (Thr 68), and p-cdc2 (Tyr15). However, treatment of TRs without TMZ-induced G_2 arrest (TR11, TR14, TR17, and TR20), with 100 μM TMZ had no effect on the expression of p-chk1 (Ser345), p-chk2 (Thr 68), and p-cdc2 (Tyr15), which have previously been shown to be key events in TMZ-induced G_2 arrest (Figure 2a).The total levels of expression of these proteins was not affected (Figure 2b). All these clones did not show detectable levels of the MGMT expression, suggesting acquired resistance was not a consequence of increased MGMT activity (Figure 3, upper panel). As a control for MGMT expression, we used the human glioblastoma cell line SF767, which expresses MGMT (11). However, expression of MSH2 and MSH6, major proteins in MMR activation, after the formation of G:T genomic DNA mismatch, was decreased in TR11, TR14, TR17, and TR20 compared with their parental U87MG cells suggesting that MMR dysfunction led the cells to acquire TMZ resistance (Figure 3, lower panel).

G_2 checkpoint inhibitors did not resensitize TMZ resistance clones to TMZ. We have previously reported that the cdk inhibitor FP enhanced TMZ-induced cell death through the inhibition of cdc2 (cdk1) in the U87MG cells (9). FP also restored TMZ sensitivity in U87MG-derived TMZ-resistant clones that showed cdc2 phosphorylation in response to TMZ treatment and could overcome TMZ-resistance induced by Akt hyperactivity (9). However, TRs established in this study (TR11, TR14, TR17, and TR20) did not show increase in cdc2 phosphorylation as mentioned. In these clones, FP

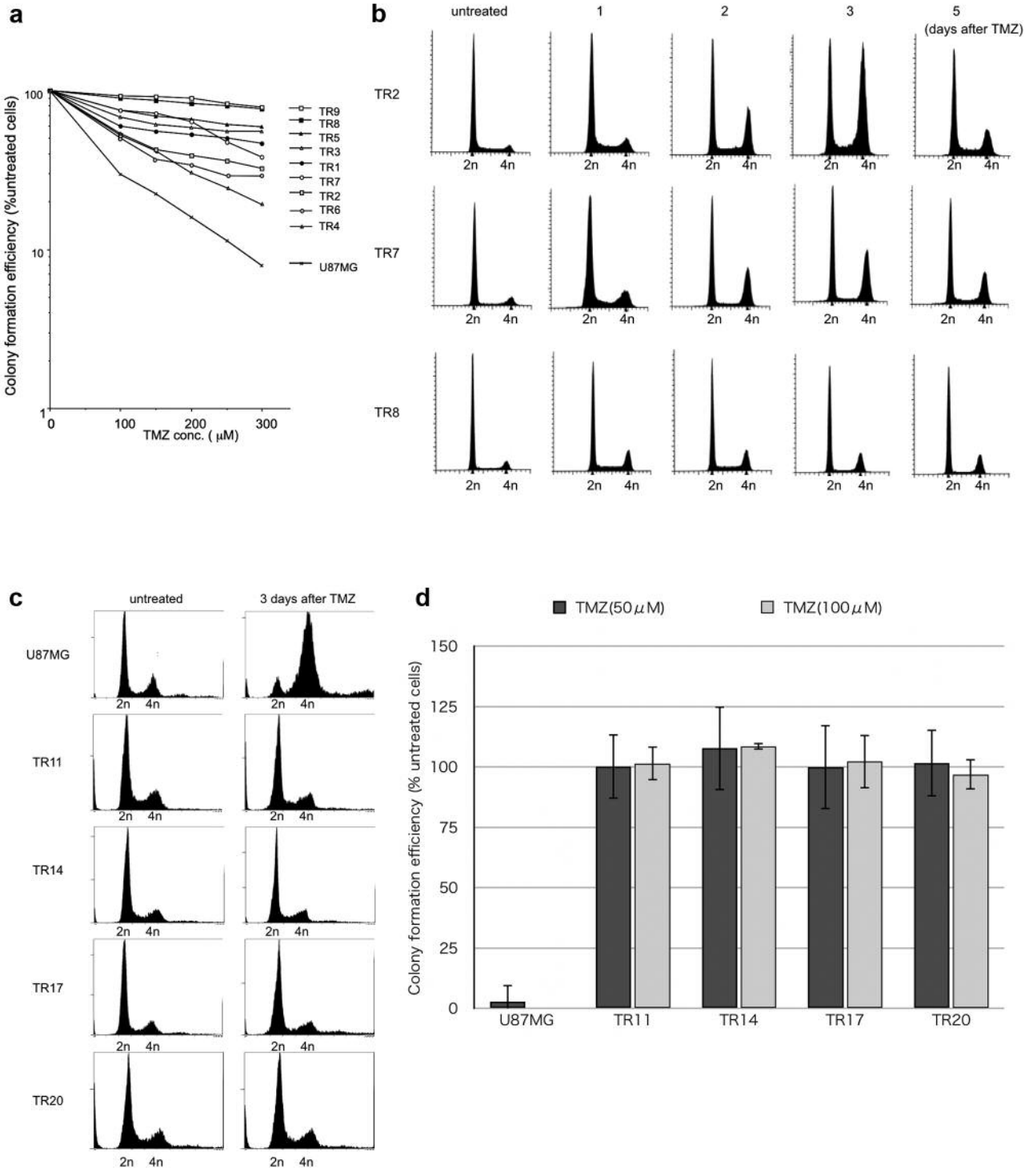

Figure 1. *Confirmation of TMZ resistance in TR-clones, and cell cycle analyzation of U87MG and TR clones after TMZ treatment. a. Colony formation efficiency of U87MG and TMZ-resistant cell lines (TR1-9). Cells were treated with TMZ at 100-300 μM for 3 h. b. The resistant cell lines confirmed in Figure 1a were treated with TMZ (100 μM, 3 h), and cells were collected on days 1, 2, 3, and 5 after TMZ treatment, to analyze the cell cycle. c. Four TMZ-resistant cell lines (TR11, TR14, TR17, and TR20) that were generated by repetitive TMZ administration over a long period of time (over 6 months) were treated with TMZ (100 μM, 3 h), and cells were collected 3 days after TMZ treatment to analyze cell cycle. d. Colony formation efficiency of U87MG and TMZ-resistant cell lines (TR11, TR14, TR17, and TR20). Cells were treated with TMZ at 50 μM or 100 μM for 3 h. The data from the groups treated with 50 μM TMZ were obtained from 12 independent experiments, and those with 100 μM TMZ were obtained from 3 independent experiments. The values represent the mean±standard deviation obtained from three independent experiments.*

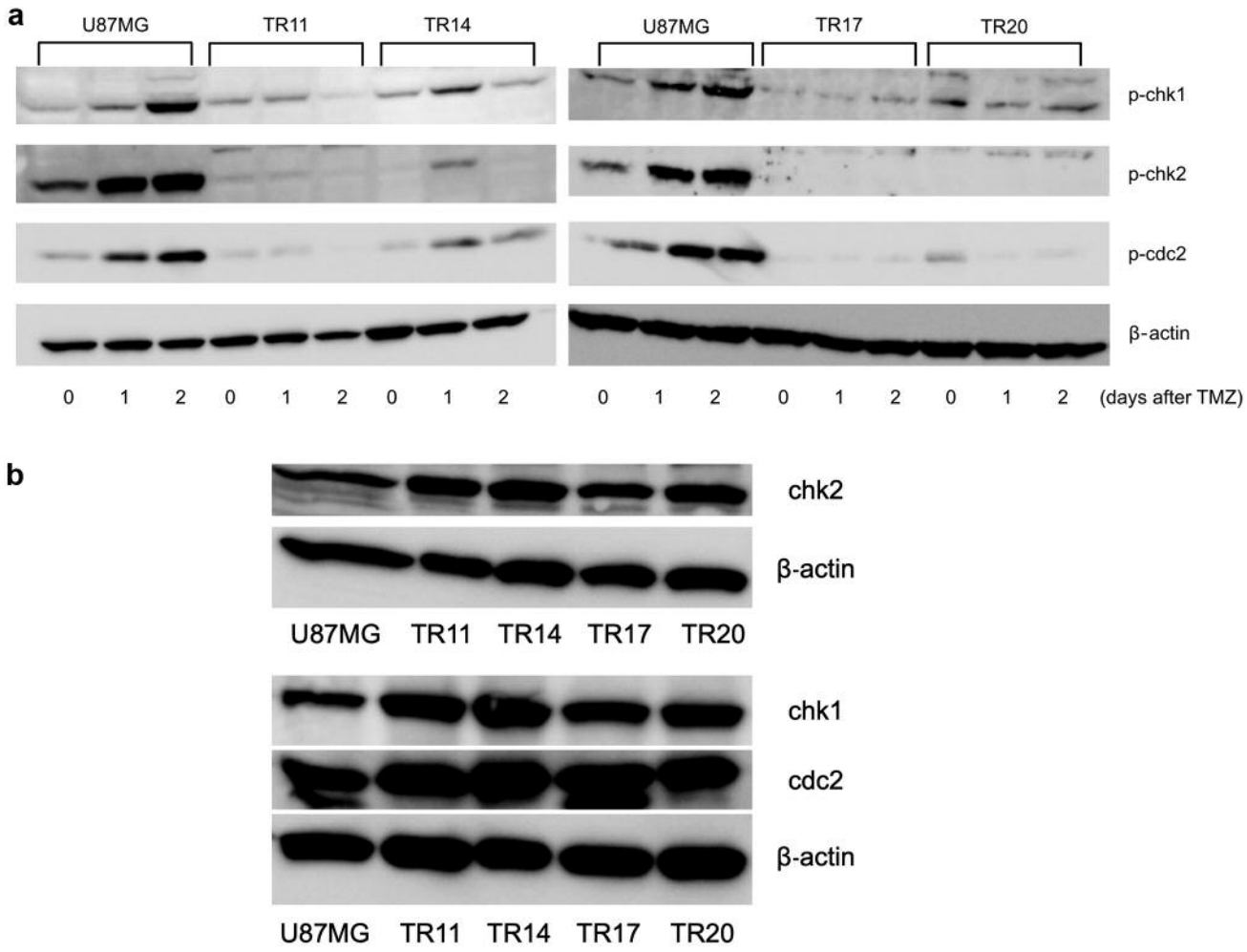

Figure 2. *Western blot analysis of G_2 checkpoint proteins. a. Western blot analysis of p-chk1, p-chk2, and p-cdc2 levels in U87MG and TMZ–induced G_2/M-arrest-resistant cell lines (TR11, TR14, TR17, and TR20). For analysis of p-chk1, p-chk2, and p-cdc2, cells were collected on days 0, 1, and 2 after TMZ treatment. b. Western blot analysis of chk1, chk2, cdc2 levels in U87MG and TMZ–induced G_2/M-arrest-resistant cell lines (TR11, TR14, TR17, and TR20).*

did not potentiate TMZ toxicity. Thus, the effect of the cdk inhibitor depended on the activity of cdc2 (Figure 4).

Next, we examined the effect of two different chk1 inhibitors, MK and RS, because a staurosporine derivative (UCN-01), which inhibits chk1, enhanced TMZ toxicity in human glioma cells (8). To exclude that their activity was due to effects other than those of the chk1 inhibitor, we first treated U87MG cells with MK or RS at different concentrations, up to over 100-fold of their IC_{50} (MK 3 nM, RS 7 nM) in combination with TMZ, and the lowest concentration at which an antitumor effect was observed by colony formation efficiency assay was chosen in the following experiments. Because the cells exposed to MK or RS, which could induce cell cycle arrest, required long term observation, the colony-formation efficiency assay was an appropriate method to study cell survival and suitable drug concentration. Our results showed that the chk1 inhibitors did not enhance the suppressive effect of TMZ in TR11, TR14, TR17, and TR20 clones (Figure 5). Combined with the FP experiments, the G_2 checkpoint inhibitor could not resensitize TR clones obtained after repeated long-term TMZ treatment.

Discussion

We have reported that MMR activation caused by TMZ treatment leads to G_2 arrest glioma cells through chk1, and TMZ activity is enhanced by inhibiting the G_2 checkpoint and forcing cells to escape G_2 arrest (5-9). However, in the TR clones that acquired strong resistance to TMZ, TMZ-

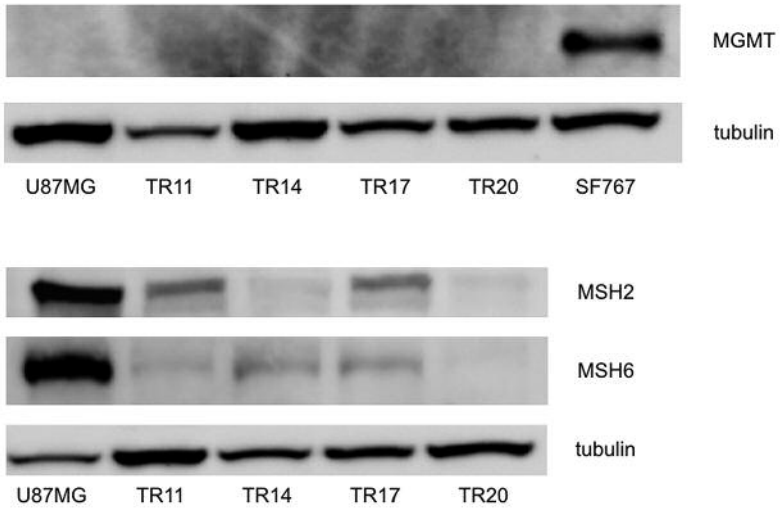

Figure 3. *Western blot analysis of MGMT in U87MG and TMZ–induced G_2/M-arrest-resistant cell lines (TR11, TR14, TR17, and TR20), using the SF767 cell line as a control (upper panel), and of MSH2 and MSH6 in U87MG and TMZ-resistant cell lines (TR11, TR14, TR17, and TR20) (lower panel).*

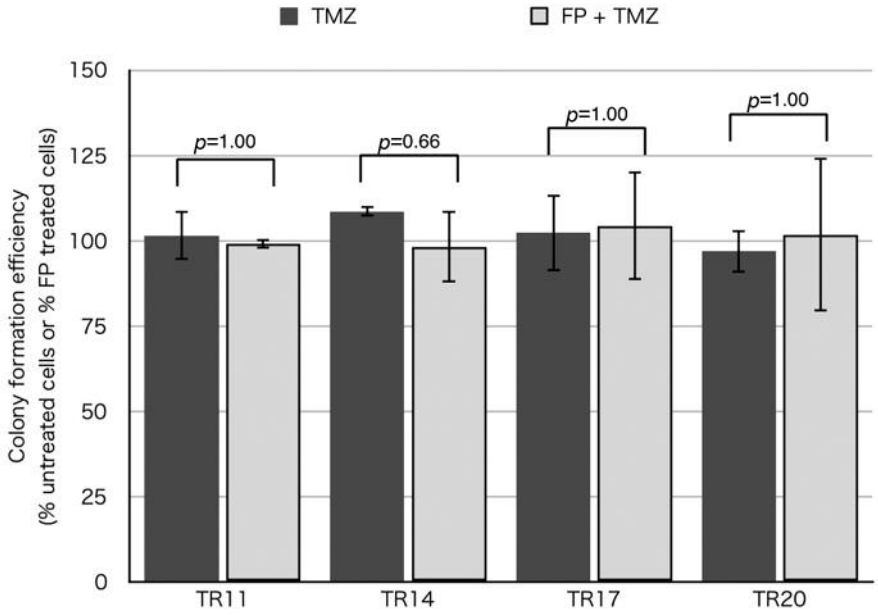

Figure 4. *Colony formation efficiency of TMZ–induced G_2/M-arrest-resistant cell lines (TR11, TR14, TR17, and TR20) treated with TMZ alone or in combination with FP. The resistant cell lines were treated with TMZ (100 μM, 3 h), FP (50 nM, 3 days), or TMZ (100 μM, 3 h) + FP (50 nM, 3 days). In the groups treated with TMZ alone, the ratios to untreated groups are shown; and in those treated with TMZ + FP, the ratios to groups treated with FP alone are shown. The data were obtained from three independent experiments and the values represent the mean±standard deviation obtained from the three experiments. There was no statistically significant difference between the groups treated with TMZ and those treated with TMZ + FP.*

induced G_2 arrest mediated through chk1 and chk2 was not observed, and the expression of MSH2 and MSH6 was significantly decreased in comparison with the parental U87MG cells, suggesting that the MMR dysfunction was a key factor in the acquisition of TMZ resistance. Our results agree with a previous study reporting that recurrent glioma after TMZ treatment commonly carries MSH6 abnormality (28-31). Taken together, a decrease in MSH6 expression due to continuous TMZ use in glioblastoma could be a common event in both cell cultures and tumors.

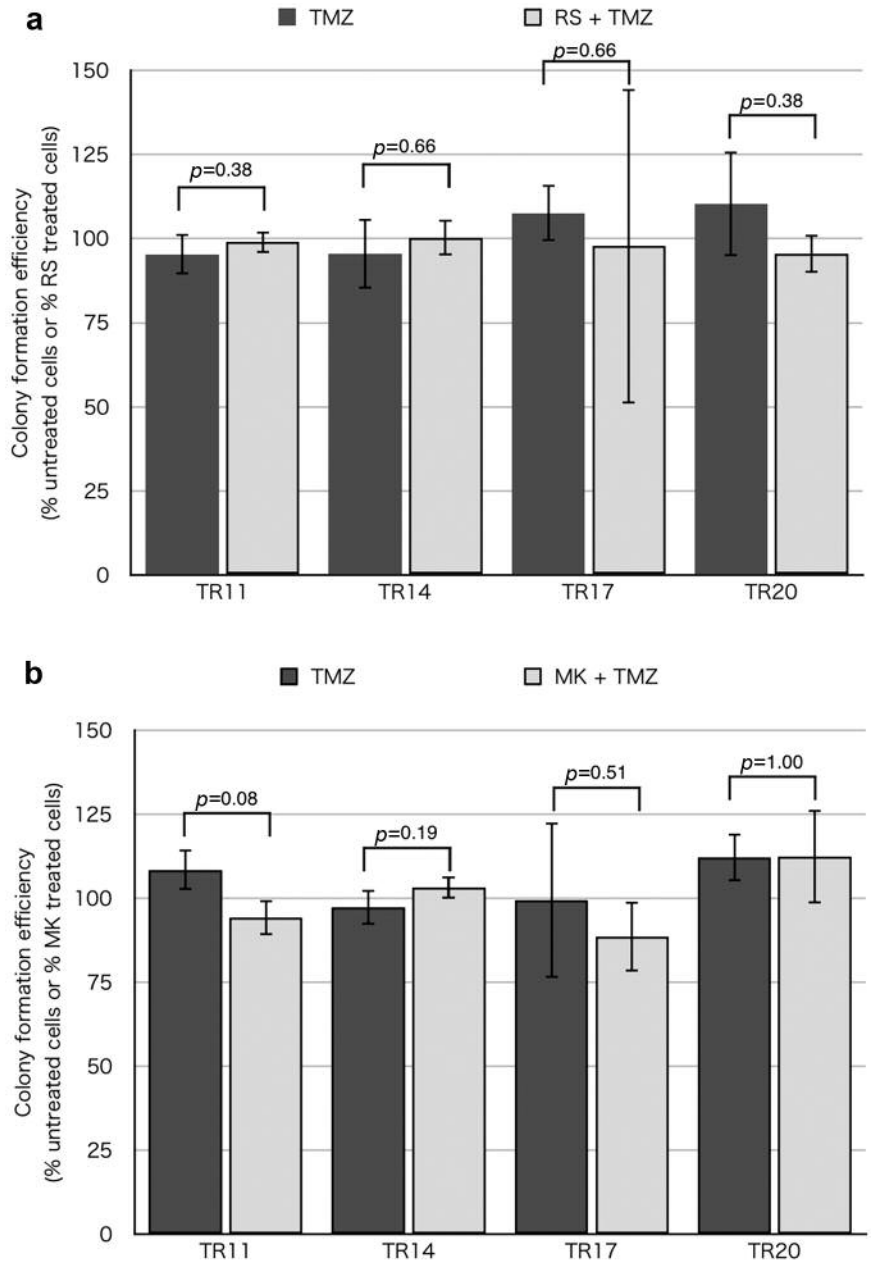

Figure 5. *Colony formation efficiency of TMZ–induced G_2/M-arrest-resistant cell lines (TR11, TR14, TR17, and TR20) treated with TMZ alone or in combination with chk1 inhibitor (RS or MK; a and b, respectively). The resistant cell lines were treated with TMZ (50 μM, 3 h), chk1 inhibitor (3 days), or TMZ (50 μM, 3 h) + chk1 inhibitor (3 days). In the groups treated with TMZ alone, the ratios to untreated groups are shown, and in those treated with TMZ + chk1 inhibitor, the ratios to groups treated with chk1 inhibitor alone are shown. The data were obtained from three independent experiments, and the values represent the mean±standard deviation from the three experiments. There were no statistically significant differences between the groups treated with TMZ and those treated with TMZ + chk1 inhibitor.*

We found there were two main types of cells which could survive after TMZ treatment; those with transient TMZ-induced G_2 arrest and those with no cell cycle phase arrest. Unlike the former type of TRs, as reported in our previous study (9), the latter was not resensitized to TMZ by the cdk inhibitor. Although both types were generated by the same method, using the same parental cell line (U87MG), different mechanisms of TMZ resistance were involved. TRs in which the cdk inhibitor affects TMZ resistance exhibited transient G_2 arrest and increased

phosphorylated-cdc2 to some degree in response to TMZ, whereas TRs without G_2 arrest did not exhibit an increase in phosphorylated-cdc2. This suggests that TMZ resistance is more advanced in clones obtained after prolonged TMZ treatment. Long-term exposure to TMZ may induce strong TMZ resistance without G_2 arrest, both in cell lines as well as in clinical cases. Since a number of TMZ-resistance mechanisms has been shown (28-39) in basic research studies and clinical trials, detailed studies are needed to investigate the multiple mechanisms of TMZ resistance with respect to differences in genetic tumor backgrounds and treatment regimens.

Neither of the G_2 checkpoint inhibitors analyzed in this study could reverse TMZ resistance in MMR dysfunctional clones. In the TMZ-resistant cell lines in which MMR dysfunction emerged due to long-term TMZ exposure, GT mismatches following the formation of O^6-methylguanine are considered not cytotoxicity, and reversing resistance through MMR is likely to be difficult. Considering the possibility that the longer the period of TMZ exposure, the stronger the TMZ resistance becomes as the mechanism of resistance changes, the optimization of chemotherapy for MMR-functioning tumors (*i.e.* those at an earlier stage after initial therapy) may be required to improve the therapy for glioma, which could develop and lose the MMR function (and acquire resistance against DNA damaging agents).

Today, standard treatments for recurrent malignant glioma are not well defined and TMZ re-challenge is sometimes selected as a treatment for recurrence. A previous study has reported that the presence of an MGMT-promoter methylation is the main factor for the effectiveness of TMZ re-challenge (40). Based on this study, it is also important to consider the previous administration period of TMZ and the expression of MMR to predict the efficacy of TMZ re-challenge. However, although TMZ-resistant clones in this study acquired resistance by continuous exposure to TMZ, there are reports that TMZ re-challenge is more effective when the TMZ withdrawal period is long (40, 41). These reports suggest the possibility of changes in TMZ resistance by TMZ withdrawal and further research is warranted.

In conclusion, a longer drug treatment could induce the development of cells highly resistant to TMZ by means of MMR modification. For clinical management of glioblastomas using TMZ as main chemotherapeutic agent, it might be important to develop a new approach to intensify treatment before the tumor develops MMR deficiency. In other words, to improve treatment effectiveness, it may be useful to optimize chemotherapy for primary tumors, rather than to try to improve treatment effectiveness for tumors that have acquired strong TMZ resistance.

Conflicts of Interest

Yuichi Hirose has received a commercial research grant from Astellas, Boehringer Ingelheim, Eizai, Chugai Pharmaceuticals, Daiichi Sankyo, Nippon Kayaku, Otsuka Pharmaceuticals, Pfeizer and Takeda Pharmaceuticals, and received a speaker honorarium from Eizai, Chugai Pharmaceuticals and Kowa Company. The other Authors declare that they have no conflict of interest.

Authors' Contributions

KY and YH: Designed the study. KY and KN: Performed the experiments. KY and SO: Analyzed the data. KY and YH: Wrote the paper.

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