Clin Transl Oncol (2018) 20:508–516
https://doi.org/10.1007/s12094-017-1743-x

RESEARCH ARTICLE

Acquired temozolomide resistance in human glioblastoma cell line U251 is caused by mismatch repair deficiency and can be overcome by lomustine

J. Stritzelberger[1] · L. Distel[1] · R. Buslei[1,2] · R. Fietkau[1] · F. Putz[1]

Received: 28 June 2017 / Accepted: 17 August 2017 / Published online: 20 August 2017

Abstract

Purpose Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults. While the alkylating agent temozolomide (TMZ) has prolonged overall survival, resistance evolution represents an important clinical problem. Therefore, we studied the effectiveness of radiotherapy and CCNU in an in vitro model of acquired TMZ resistance.

Methods We studied the *MGMT*-methylated GBM cell line U251 and its in vitro derived TMZ-resistant subline, U251/TMZ-R. Cytotoxicity of TMZ, CCNU, and radiation was tested. Both cell lines were analyzed for *MGMT* promotor status and expression of mismatch repair genes (MMR). The influence of MMR inhibition by cadmium chloride ($CdCl_2$) on the effects of both drugs was evaluated.

Results During the resistance evolution process in vitro, U251/TMZ-R developed MMR deficiency, but *MGMT* status did not change. U251/TMZ-R cells were more resistant to TMZ than parental U251 cells (cell viability: 92.0% in U251/TMZ-R/69.2% in U251; $p = 0.032$) yet more sensitive to CCNU (56.4%/80.8%; $p = 0.023$). The effectiveness of radiotherapy was not reduced in the TMZ-resistant cell line. Combination of CCNU and TMZ showed promising results for both cell lines and overcame resistance. $CdCl_2$-induced MMR deficiency increased cytotoxicity of CCNU.

Conclusion Our results confirm MMR deficiency as a crucial process for resistance evolution to TMZ. MMR-deficient TMZ-resistant GBM cells were particularly sensitive to CCNU and to combined CCNU/TMZ. Effectiveness of radiotherapy was preserved in TMZ-resistant cells. Consequently, CCNU might be preferentially considered as a treatment option for recurrent *MGMT*-methylated GBM and may even be suitable for prevention of resistance evolution in primary treatment.

Keywords Glioblastoma · Antineoplastic drug resistance · Temozolomide · Lomustine · Mismatch repair

Abbreviations

7AAD	7-Amino-actinomycin D
AxV	Annexin V
CCNU	Lomustine
$CdCl_2$	Cadmiumchloride
DMEM	Dulbecco's Modified Eagle Medium (Medium für Zellkultur)
GBM	Glioblastoma multiforme
Gy	Gray
MD	Mean difference
MGMT	O^6-Methylguanin-DNA-methyltransferase
MMR	Mismatch-repair
SF	Survival fraction
TMZ	Temozolomide

✉ F. Putz
florian.putz@uk-erlangen.de

J. Stritzelberger
jenny.stritzelberger@fau.de

L. Distel
luipold.distel@uk-erlangen.de

R. Buslei
rolf.buslei@uk-erlangen.de

R. Fietkau
rainer.fietkau@uk-erlangen.de

[1] Department of Radiation Oncology, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Universitaetsstraße 27, 91054 Erlangen, Germany

[2] Department of Neuropathology, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), 91054 Erlangen, Germany

Introduction

Glioblastoma (GBM) is the most common malignant primary tumor of the brain in adults and associated with a particular poor prognosis [1]. The current standard of care includes surgery, radiotherapy, and the monofunctional alkylating agent temozolomide (TMZ). Although TMZ has improved overall survival, most patients still develop tumor recurrence within a period of 7 months [2]. Therapy failure is often due to resistance evolution processes against TMZ, one of the major obstacles in GBM treatment [3].

Amongst DNA adducts created by monofunctional agents like TMZ, O^6-methylguanine assumedly is the most important lesion mediating TMZ toxicity. These adducts are repaired by O^6-methylguanin-DNA-methyltransferase (*MGMT*); if *MGMT* is absent, base mispairing triggers repetitive but unsuccessful mismatch repair (MMR) leading to subsequent DNA strand breaks, cell cycle arrest, and apoptosis [4, 5]. Resistance to TMZ at the time of diagnosis is mostly due to high levels of *MGMT* [6, 7]. In contrast, tumors with methylated *MGMT* promotor are very sensitive to TMZ, but resistance almost inevitably develops during treatment. Several authors suggested MMR deficiency being responsible for acquired TMZ resistance in *MGMT*-methylated tumors [6, 8–13] and strategies to restore the effect of MMR system have been postulated to improve the effect of TMZ [14].

Aside from TMZ, lomustine (CCNU) is another alkylating agent with proven efficacy in GBM therapy [15, 16]. While a proficient MMR is vital for the expression of TMZ cytotoxicity, it is supposedly inversely related to CCNU toxicity as interstrand links caused by bifunctional agents such as CCNU are repaired by MMR. MMR deficiency indeed was shown to increase sensitivity to bifunctional agents in different MMR-deficient non-glioma cell lines [17–20].

Clinical trials showed promising results for the combination of CCNU and TMZ in patients with newly diagnosed GBM, with the largest clinical benefit found for *MGMT*-methylated patients [21, 22]. In addition, CCNU monotherapy demonstrated good efficacy for recurrent cases [15]. However, the underlying mechanisms have not been elucidated to date.

We hypothesized that acquired resistance to TMZ in *MGMT*-methylated GBM cells is mediated by MMR deficiency and, therefore, might be accompanied by increased sensitivity to CCNU. We, therefore, sought to investigate the combination and the differential effects of TMZ and CCNU in the human GBM cell line U251 and a TMZ-resistant line U251/TMZ-R with regard to the role of MMR and *MGMT*.

Materials and methods

Cell lines and primary culture

The human glioblastoma cell line U251 was obtained from Cell Line Service (CLS; Eppelheim, Germany). Cells were maintained and cultured in Dulbecco's Modified Eagle's Medium (DMEM; PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Darmstadt, Germany) and cultured at 37 °C in a 5% CO_2 incubator.

Drugs

TMZ, CCNU, and $CdCl_2$ were obtained from Sigma-Aldrich (St Louis, USA). TMZ was dissolved in dimethyl sulfoxide (DMSO), CCNU in ethanol, and $CdCl_2$ in sterilized water. Stock solutions were stored at −20 °C.

Generation of a TMZ-resistant cell line

U251 cells were cultured in 75 cm^2 cell flasks (Cellstar; Greiner BioOne, Nürtlingen, Germany) and allowed to adhere overnight. Cells were treated with 100 µM TMZ. Cell treatment was repeated every 24 h for 5 consecutive days. After those 5 days, exposure to the fresh TMZ was repeated every 3 days to a total of 3 weeks. This procedure has been previously described for U251 [13].

Treatment with drugs and irradiation

Cells were seeded in 25 cm^2 flasks at a density of 2.0×10^5 cells. The chemotherapeutics were added 48 h later. $CdCl_2$ was added 2 h before chemotherapy treatment for pre-incubation according to a protocol introduced by Yamauchi et al. [23]. 1 h after chemotherapy treatment, cells were irradiated with 2 Gy, corresponding to the daily dose employed in clinical practice [24]. Irradiation was performed with an X-ray generator (120 kV, 22.7 mA, variable time; GE Inspection Technologies, Ahrensburg, Germany).

Cell death detection

We used APC Annexin V (AxV)/7-amino-actinomycin D (7AAD) staining and flow cytometry to investigate TMZ and CCNU-induced cell death. After harvesting the cells 72 h after treatment by trypsinization, cell suspension (100 µl, 1×10^5 cells) and 5 µl of AxV and 7AAD (both BD Biosciences, Franklin Lakes, USA), respectively, were combined with 400 µl Ringer solution (B. Braun

Melsungen AG, Melsungen, Germany) and incubated at 4 °C for 30 min. Cell death was determined using flow cytometry (Gallios, Beckman Coulter, Brea, USA) and its associated Kaluza 1.3 Software (Beckman Coulter, Krefeld, Germany). For each sample, a minimum of 2×10^4 events was assayed. Experiments were performed at least thrice with two replicates per run. AxV/7AAD-double-negative cells were considered to be viable cells, AxV-positive/7AAD-negative cells early apoptotic cells, and AxV/7AAD-double-positive cells late apoptotic/necrotic cells [25–27].

Cell cycle analysis

Cell cycle distribution was analyzed by Hoechst 33342 staining (BD Biosciences, Franklin Lakes, USA) and flow cytometry. 72 h after treatment, cells were harvested. 2 ml of cell suspension ($\triangleq 2 \times 10^6$ cells) were combined with 10 ml of 70% ethanol (Carl Roth, Karlsruhe, Germany) at 4 °C for at least 2 h to fix the cells. Following incubation, cells were resuspended in 1 ml Ringer solution, combined with 3 µl of Hoechst, and incubated at 4 °C for 20 min. In each sample, 2×10^5 cells were assayed using flow cytometry. The cell cycle phase distribution was determined with the Kaluza 1.3 software.

Clonogenic assay

The effects of irradiation were determined with clonogenic assays. Cells were plated in 60-mm dishes (Nunc Thermo Fisher, Waltham, USA) with 300–1600 cells per dish. After 6–12 h, cells were treated with drugs and irradiated with increasing doses of 2, 4, 6, 8, and 10 Gy (see above). After incubation for 10–14 days in drug-free fresh, medium cells were fixed with methylene blue for 30 min. Subsequently, colonies containing >50 cells were counted. The survival fraction (SF) was calculated as follows: SF at a given condition = colonies counted of the given condition/(cells seeded of the given condition × plating efficiency/100). Plating efficiency = percentage of untreated cells seeded that grow into colonies.

Immunostaining

To assess MMR protein expression, immunostaining and subsequent image analyses were performed following a standard protocol [28] with slight modifications. The following mouse monoclonal antibodies were used: anti-MLH1, anti-MSH6, anti-PMS2 (all BD Biosciences), and anti-MSH2 (Merck Millipore, Darmstadt, Germany). Primary antibodies were applied at a dilution of 1:100 and Alexa labelled secondary antibodies (Invitrogen; Life Technologies GmbH, Darmstadt, Germany) at 1:400. Images were captured by fluorescence microscopy (Leica DM 6000). Overlays were built using an image-processing software (Biomas 3.3 10/2004 MSAB). MLH1 foci were counted as previously described [28, 29].

Pyrosequencing for promotor status determination

In both cell lines, quantitative methylation analyses of the *MGMT* promotor were performed by pyrosequencing (PyroMark Q24 MGMT-Kit [Qiagen]) in the Institute of Neuropathology, Erlangen.

Statistical analysis

If not indicated otherwise, results are expressed as the mean ± standard deviation (SD) of three independent experiments. Statistics were performed with IBM SPSS Statistics 22.0 for Windows (IBM Corporation, New York, USA) using the two-sided *t* test and the non-parametric Mann–Whitney *U* test. Significant differences with a p value of ≤ 0.05 are marked as *, very significant differences ($p \leq 0.01$) as **, and highly significant differences ($p \leq 0.001$) as ***.

Results

U251/TMZ-R was more resistant to TMZ than U251

Cytotoxicity of 500 µM TMZ for 72 h was determined by analyzing cell viability, apoptotic, and necrotic cell death using AxV/7AAD staining (Fig. 1a). Following exposure to TMZ, the percentage of viable U251/TMZ-R cells (AxV−/7AAD−) was significantly elevated compared to parental U251 cells (92.0 ± 2.0% for U251/TMZ-R and 69.2 ± 8.8% for U251, $p = 0.03$). Both early apoptotic cells (AxV+/7AAD−) and late apoptotic/necrotic cells (AxV+/7AAD+) were notably decreased in U251/TMZ-R compared to U251 (mean difference (MD) in apoptosis: 0.8 ± 0.3 vs. 2.7 ± 0.5%; $p = 0.02$; MD in necrosis: 5.9 ± 1.5 vs. 22.3 ± 6.63%; $p = 0.04$) (Fig. 1a, b).

Cell cycle distribution after exposure to 500 µM TMZ was analyzed by Hoechst 33342 staining (Fig. 2a). TMZ induced a G2/M block in parental U251 cells (G2/M fraction; Controls: 11.0 ± 1.8%, TMZ: 45.3 ± 15.4%; $p = 0.05$) and a distinct reduction of cells in G1 phase (76.5 ± 1.6 vs. 34.7 ± 7.6%; $p = 0.03$). U251/TMZ-R cells, however, did not accumulate in G2/M after TMZ treatment, and the cell cycle distribution remained largely unchanged (Fig. 2b).

Growth inhibition was assessed using colony formation assay. TMZ suppressed colony formation in U251, but not

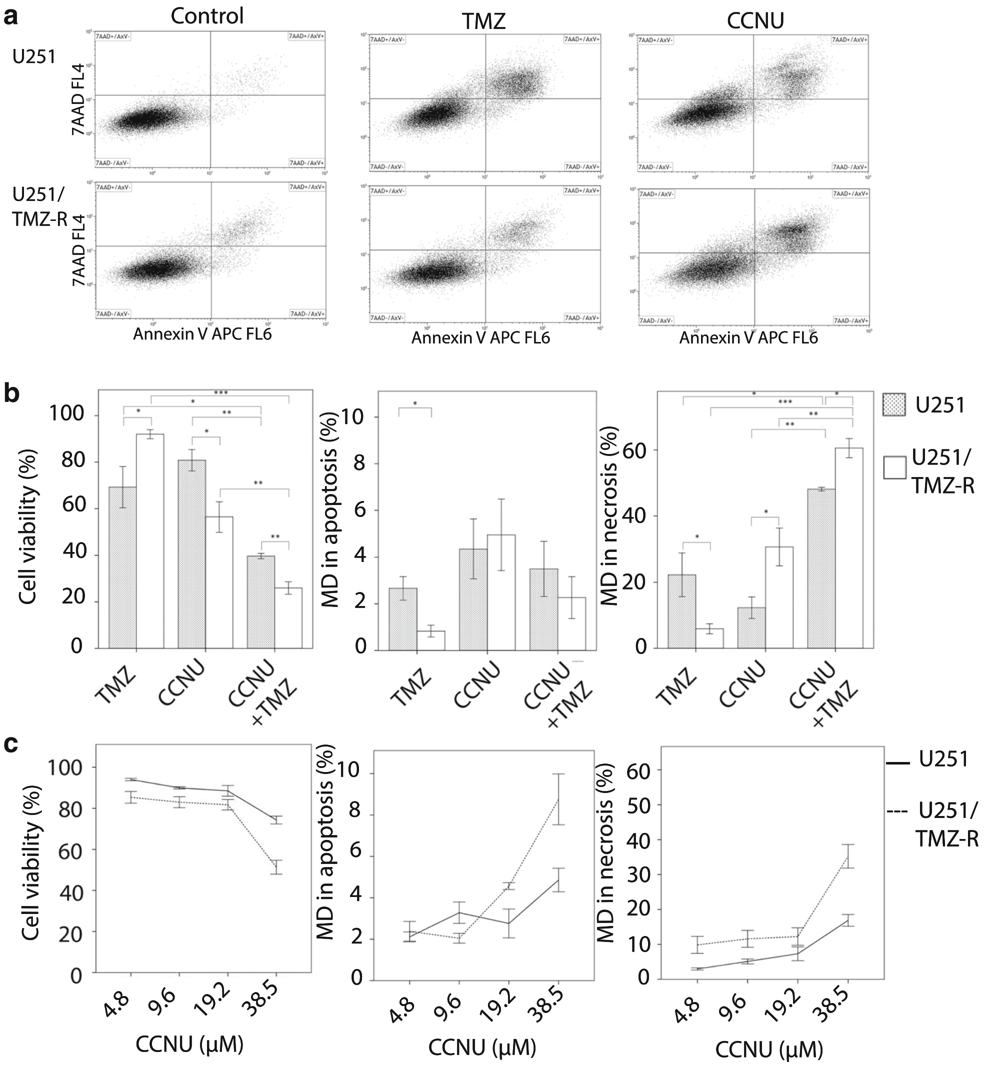

Fig. 1 Flow cytometric analysis of cytotoxicity of TMZ, CCNU, and combination treatment. Cells were treated with 500 µM of TMZ, 38.5 µM of CCNU, or a combination of both. Cell viability, apoptosis, and necrosis were detected by 7AAD/AxV staining. When U251 was compared to U251/TMZ-R, cell viability was calculated as cell viability (treatment condition)/cell viability (control). To compare apoptosis and necrosis rates between both cell lines, mean differences (MD) were calculated as the differences between apoptosis/necrosis rates of treatment condition and matching control of the relevant cell line. **a** Representative flow-cytometric histograms; **b** cell viability, apoptosis and necrosis after mock treatment, TMZ, CCNU or CCNU+TMZ for U251 and U251/TMZ-R. **c** Cell viability, apoptosis, and necrosis after treatment with increasing doses of CCNU. Note: U251/TMZ-R showed increased cell viability after treatment with TMZ but reduced cell viability after CCNU compared to U251. Combination treatment with CCNU and TMZ resulted in highly significantly reduced cell viability in U251 and U251/TMZ-R compared to CCNU or TMZ alone. Accordingly, apoptosis and necrosis rates were decreased after TMZ therapy and increased after CCNU/CCNU+TMZ (**a**, **b**). The differential effects of CCNU on parental and resistant cells were apparent for various concentrations of CCNU (**c**)

in U251/TMZ-R (SF: 41.3 ± 11.6 vs. 100.0 ± 2.8%; $p = 0.003$).

U251/TMZ-R cells did not develop cross resistance to CCNU and cytotoxicity of CCNU was enhanced in U251/TMZ-R

Cytotoxicity of CCNU was analyzed using AxV/7AAD staining and flow cytometry as described for TMZ (Fig. 1a). After exposure to 38.5 µM CCNU for 72 h, cell viability (AxV−/7AAD−) was decreased in U251/TMZ-R compared to U251 (56.4 ± 6.5 vs. 80.8 ± 4.6%; $p = 0.02$). Late apoptosis/necrosis (AxV+/7AAD+) was notably increased in U251/TMZ-R (30.7 ± 5.7 vs. 12.3 ± 3.3%; $p = 0.04$). Thus, cells were very sensitive to CCNU-induced cell death with an even more cytotoxic effect of CCNU in U251/TMZ-R cells than in U251 cells (Fig. 1b, c). CCNU induced G2/M arrest (Fig. 2b) and suppressed colony formation in both cell lines (data not shown), strengthening the conclusion that TMZ-R cells were not cross-resistant to CCNU.

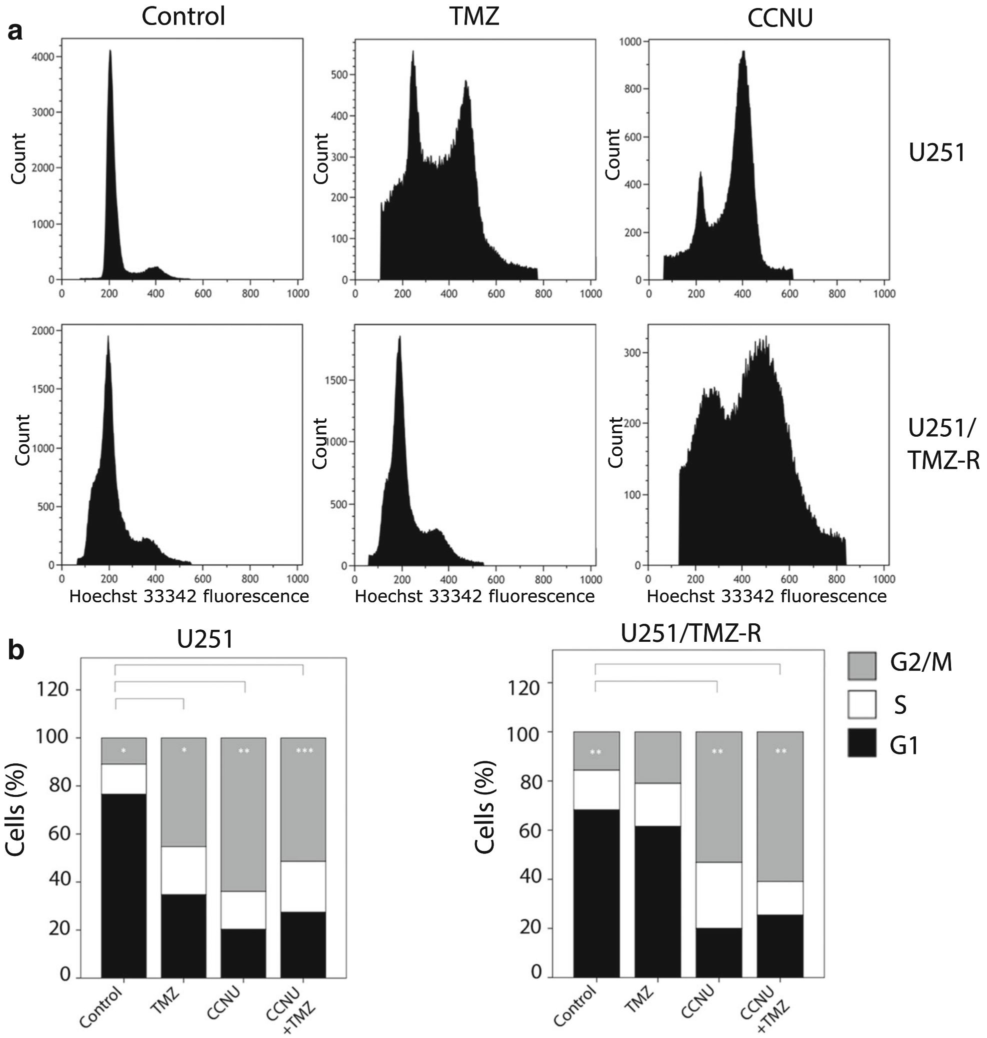

Fig. 2 Cell cycle effects of TMZ, CCNU, or combination treatment. Cells were treated with 500 µM of TMZ, 38.5 µM of CCNU, or a combination of both. Cell cycle distribution was analyzed using Hoechst staining and flow cytometry. **a** Representative flow-cytometric histograms; **b** cell cycle distributions after mock treatment, TMZ, CCNU or CCNU+TMZ for U251 and U251/TMZ-R. Note: TMZ did not induce a G2/M block in U251/TMZ-R, whereas CCNU and combination treatment induced a highly significant G2/M block. TMZ, CCNU, and CCNU+TMZ induced G2/M block in U251

Combination of CCNU and TMZ led to increased cytotoxicity in both cell lines and overcame resistance

The combination of CCNU (38.5 µM) and TMZ (500 µM) had stronger effects on cell viability and late apoptosis/necrosis than each single drug for U251 and U251/TMZ-R (Fig. 1b). The effects of CCNU+TMZ were even stronger in resistant cells (cell viability: 26.0 ± 2.7 vs. 39.7 ± 1.1%; $p = 0.01$, MD in necrosis: 60.6 ± 2.9 vs. 48.1 ± 0.6%; $p = 0.02$). Similarly, the combination of TMZ and CCNU led to an increase in G2/M arrest (Fig. 2b).

Inhibition of MMR increased sensitivity to CCNU in U251

Cells were pre-incubated with $CdCl_2$, an inhibitor of MMR, at a concentration of 2.5 µM, chosen for its minimally toxic effects (Fig. 3a). 72 h after exposure to 4.8 µM CCNU, cells pre-incubated with $CdCl_2$ showed significantly increased rates in late apoptosis/necrosis (3.3 ± 0.9 vs. 8.2 ± 1.0% for CCNU vs. CCNU + $CdCl_2$; $p = 0.02$) and decreased rates in cell viability (93.6 ± 1.8 vs. 86.5 ± 1.8%; $p = 0.05$, Fig. 3d).

Correspondingly, G2/M arrest after exposure to CCNU was significantly more distinct in cells pre-incubated with $CdCl_2$ (G2/M fraction: 15.7 ± 2.2 vs. 23.5 ± 1.4% for CCNU vs. CCNU+$CdCl_2$; $p = 0.04$, Fig. 3b). Although TMZ toxicity was slightly decreased in cells pre-incubated with $CdCl_2$ with regard to cell viability (60.7 ± 8.0 vs. 65.1 ± 1.0%; $p = 0.64$), late apoptosis/necrosis rate (30.1 ± 4.7 vs. 25.8 ± 2.6%; $p = 0.53$), and G2/M arrest (61.1 ± 2.3 vs. 58.1 ± 4.8%; $p = 0.61$), no significant effects were observed (Fig. 3c). Our results indicate that MMR inhibition sensitized cells to CCNU but not to TMZ.

Radio-sensitivity

To investigate effects of irradiation, clonogenic assays were performed. No significant differences between

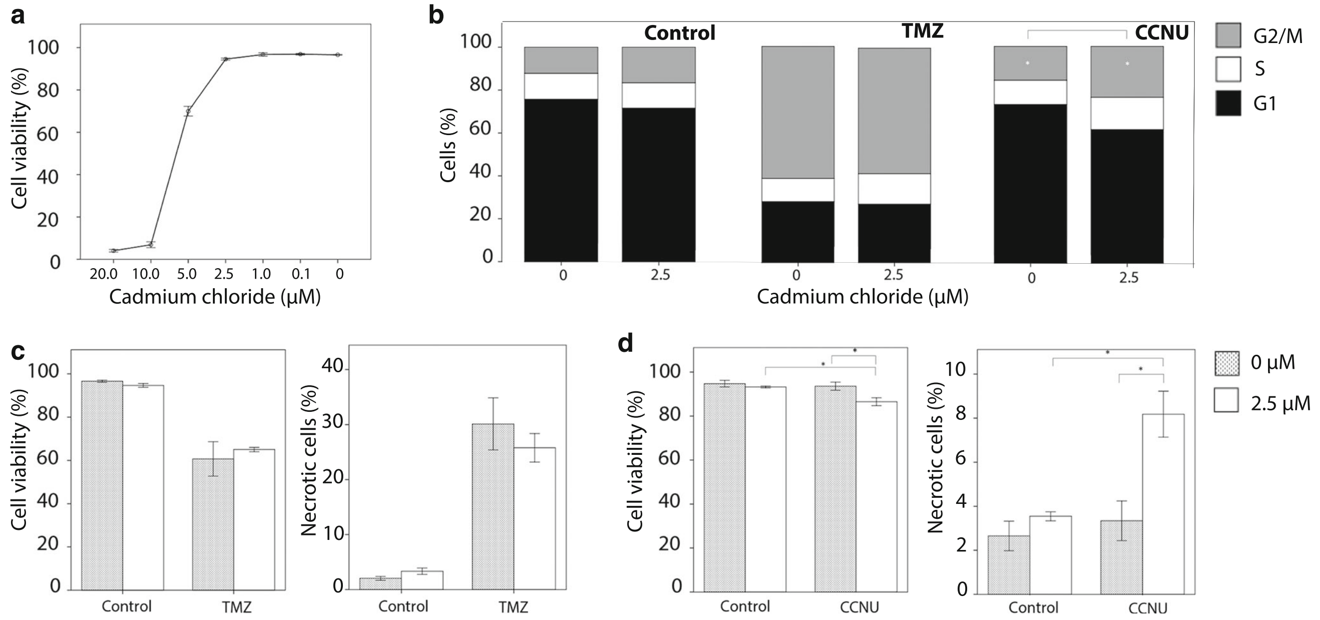

Fig. 3 Inhibition of DNA MMR by $CdCl_2$. 2 h before drug treatment, cells were pre-incubated with $CdCl_2$, an inhibitor of MMR, at a concentration of 2.5 μM considered to have no effects on cell viability (**a**) and cell cycle distribution (**b**). CCNU was applied at a minimally toxic concentration of 4.8 μM to avoid additive cytotoxic effects, TMZ was applied at 500 μM. Cell cycle phases (**b**), cell viability, and late apoptosis/necrosis rates (**c**, **d**) were compared. Cells treated with CCNU and TMZ but without $CdCl_2$ served as controls. *Note* CCNU-induced G2/M-block was more distinct in cells pre-incubated with $CdCl_2$ than in controls. Rate of necrosis was significantly increased in CCNU treated cells after pretreatment with $CdCl_2$ compared to CCNU or $CdCl_2$ alone and single treatment with either CCNU or $CdCl_2$ did not result in significantly elevated necrosis rate compared to control levels

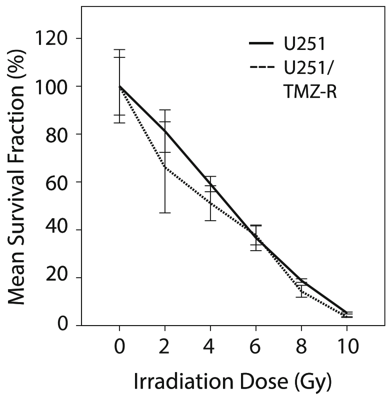

Fig. 4 Effects of radiation on U251 and U251/TMZ-R. Radiation with 2–10 Gy suppressed colony formation at an effective level in both parental U251 and resistant U251/TMZ-R with dose-dependent toxicity. SF for U251 vs. U251/TMZ-R: 2 Gy 66.1 ± 8.64 vs. 81.25 ± 4.03%, 4 Gy 51.13 ± 3.32 vs. 59.13 ± 1.46%, 6 Gy 37.69 ± 1.54 vs. 36.66 ± 2.08%, 8 Gy 14.32 ± 0.96 vs. 18.79 ± 0.29%, 10 Gy 3.53 ± 0.05 vs. 5.25 ± 0.15%

parental and resistant cells were detected for doses of 2 and 6 Gy with regard to growth restriction. However, significantly reduced colony formation was observed in TMZ-resistant cells for radiation doses of 4, 8, and 10 Gy, although the observed effects were minimal (Fig. 4). Furthermore, we did not observe differences in radio-sensitivity between both cell lines with regard to cell viability, apoptosis, and cell cycle distribution (data not shown).

Molecular characterization of U251/TMZ-R

U251 and U251/TMZ-R were tested for *MGMT* promotor methylation by pyrosequencing and promotor methylation was detected in both cell lines (Fig. 5). When tested for expression of MMR proteins, MLH1 was only activated in U251 (17.7 ± 1.1 vs. 0.4 ± 0.0 foci/cell; $p = 0.04$). For MSH2, MSH6, and PML1, no countable foci were detected in both cell lines. Our results suggest that TMZ resistance acquired in U251/TMZ-R was not related to changes in *MGMT* status but to deficiency of the MMR protein MLH1 (Fig. 6).

Discussion

In our study, TMZ-resistant *MGMT*-methylated GBM cells showed increased resistance to TMZ-induced cell death, but intriguingly increased sensitivity to CCNU-induced cell death compared to parental U251 cells.

Beyond that, the combination of CCNU and TMZ was more effective than each single agent in both cell lines regarding drug-induced cell death. It is especially interesting that combination of CCNU und TMZ was even more effective in resistant cells than in non-resistant parental cells.

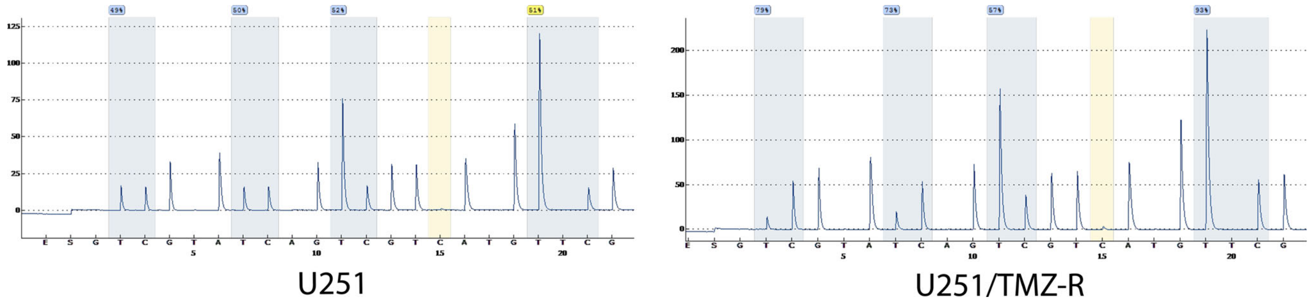

Fig. 5 MGMT promotor methylation status in U251 and U251/TMZ-R as assessed by pyrosequencing. U251 and U251/TMZ-R both showed methylation of the MGMT promotor

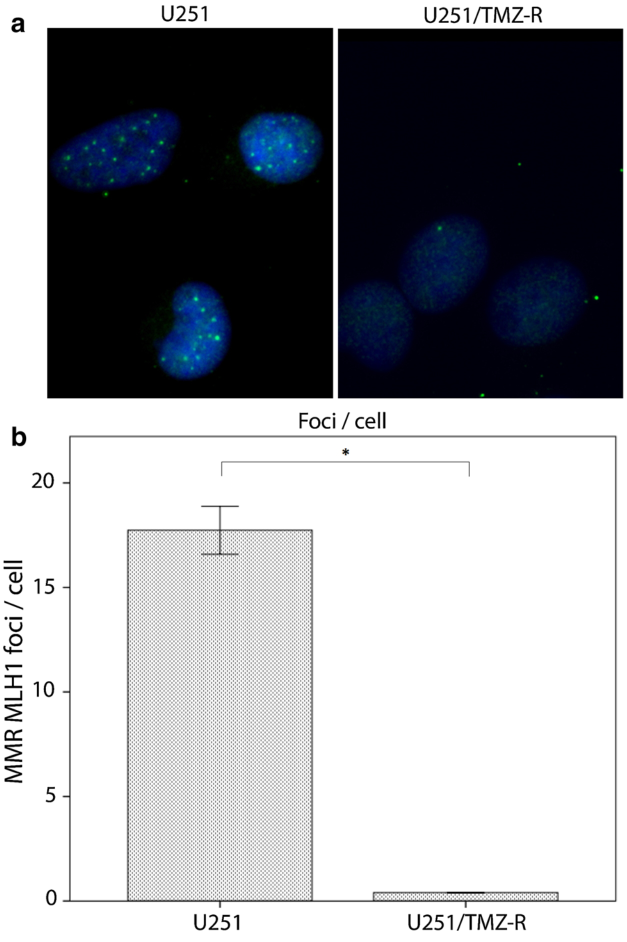

Fig. 6 Expression of MMR protein MLH1. U251 and U251/TMZ-R were stained by anti-MLH1 (*green*) and DAPI (*blue*) (**a**). MLH1 foci were counted and the number of foci per cell was calculated (**b**). MLH1 expression was significantly decreased in U251/TMZ-R compared to U251

Our observations are well supported by clinical trials. Herrlinger et al. investigated the combination of CCNU and TMZ in the single arm phase II UKT-03 trial. In an updated analysis after an extended follow-up, Glas et al. reported long-term survival especially in the subgroup that received intensified TMZ and CCNU dose [21, 22]. In the recurrent setting, the REGAL trial showed an impressive overall survival of 9.8 months in the arm that received CCNU alone [15].

Apart from repairing DNA adducts caused by monofunctional agents like temozolomide, *MGMT* also repairs the O^6-chloroethylguanine residues induced by bifunctional agents such as CCNU; thus, elevated *MGMT* levels lead to cross resistance between both drugs [4, 30, 31]. When *MGMT* capacity is saturated by an excess of O^6-methylguanine produced, TMZ treatment causes base-pair mismatches and replication errors, triggering repetitive but unsuccessful MMR. This leads to continuous DNA strand breaks, cell cycle arrest in G2 phase, and subsequent apoptosis in MMR-proficient cells [4, 5, 32]. In contrast, cells with MMR deficiency possess relative resistance to monofunctional agents such as TMZ and a correlation between MMR deficiency and GBM recurrence has been reported [10–12, 33]. In line with these previous studies, changes in *MGMT* promotor methylation were not involved in the acquisition of resistance in our study. Conversely, expression of the MMR protein MLH1 was strongly decreased in the resistant cell line. A decrease in MLH1 expression has been reported to occur early during acquisition of TMZ resistance in vitro and in vivo [12]. MLH1 expression was significantly decreased in recurrent GBM [33]. This proves the importance of MLH1 for a proficient MMR response in TMZ-sensitive GBM cells, supporting our results.

Our findings of increased CCNU toxicity in TMZ-resistant, MMR-deficient GBM cells are mechanistically supported by previous preclinical studies in non-glioma cell lines: Interstrand links caused by bifunctional agents like CCNU were shown to be repaired by MMR, leading to resistance to bifunctional agents [17–19]. To further examine the effects of MMR inhibition in U251, we used $CdCl_2$, a substance targeting several proteins involved in MMR, at a concentration of 2.5 μM. At this concentration, $CdCl_2$ effectively inhibits MMR [34, 35] and had minimally toxic effects (Fig. 3a). Sensitivity to CCNU was increased in cells pre-incubated with $CdCl_2$ which is in line with results from Yamauchi et al. who reported that BCNU-resistant leukemia cells were partially sensitized to CCNU as a result of $CdCl_2$-mediated MMR inhibition [23]. Although we would have expected decreased sensitivity to TMZ in cells pre-incubated with $CdCl_2$, this did not occur at a significant level in our experiments. This might be due to the low $CdCl_2$ concentration chosen on account of high toxicity levels; at a concentration of 100 µM, MMR efficiency is reduced by approximately 95%, but at 1 µM only by 2,7% [34]. A concentration of 2.5 µM might be just enough to cause MMR-deficiency-mediated sensitization to CCNU, yet not enough to induce TMZ resistance.

Aside from chemotherapy, the current standard of care includes radiotherapy [2]. Re-irradiation for recurrent GBM modestly increases overall survival [36, 37]. Accordingly, we did not detect meaningful differences in the effectiveness of radiotherapy between both cell lines. Both cell lines were sensitive to irradiation with dose-dependent growth restriction. The role of MMR in radiosensitization has been controversially discussed [11, 38, 39]. However, in our study, MMR deficiency did not affect radio-sensitivity in U251, confirming the importance of radiotherapy in recurrent GBM.

To the best of our knowledge, this preclinical investigation is the first to show that TMZ resistance mediated by MMR deficiency in *MGMT*-methylated GBM cells is accompanied by increased sensitivity to CCNU and to combined CCNU and TMZ. Radiosensitivity was preserved in resistant cells. These findings have important clinical implications as acquired TMZ resistance is one of the major obstacles in the treatment of GBM [3] and increased sensitivity to CCNU could be a work-around. As CCNU resistance is mediated by up-regulation and TMZ resistance by downregulation of MMR, we speculate that TMZ resistance evolution might even be preventable by concomitant CCNU administration. This may also explain the promising results for combined CCNU and TMZ in MGMT-methylated patients [21, 22].

Conclusion

This study showed promising results for CCNU in MMR-mediated TMZ resistance, indicating that further pre-clinical and clinical research is clearly warranted. Beyond that, our findings may provide the missing link between already well-described clinical and preclinical observations. Upcoming clinical trials like the German NOA-09 will provide further answers on the role of combined TMZ and CCNU.

Acknowledgements We would like to thank Mrs. Elisabeth Müller, Mrs. Doris Mehler, and Mrs. Renate Siebert for their technical expertise and help in conducting experiments. The present work was performed in fulfillment of the requirements for obtaining the degree "Dr. Med."

Author contributions JS and FP performed the experiments mentioned in the "Materials and methods", except for the pyrosequencing. RB performed pyrosequencing. JS, FP, LD were major contributors in writing the manuscript. RF provided materials and working space for the experiments we performed and revised the manuscript critically. All authors read and approved the final manuscript.

Compliance with ethical standards

Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest The authors declare that they have no competing interests.

Funding No funding was received.

References

1. Ostrom QT, Gittleman H, Liao P, Rouse C, Chen Y, Dowling J, et al. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2007–2011. Neuro-oncology. 2014;16(Suppl 4):iv1–63. doi:10.1093/neuonc/nou223.
2. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol. 2009;10(5):459–66.
3. Fan TY, Wang H, Xiang P, Liu YW, Li HZ, Lei BX, et al. Inhibition of EZH2 reverses chemotherapeutic drug TMZ chemosensitivity in glioblastoma. Int J Clin Exp Pathol. 2014;7(10):6662–70.
4. Drablos F, Feyzi E, Aas PA, Vaagbo CB, Kavli B, Bratlie MS, et al. Alkylation damage in DNA and RNA–repair mechanisms and medical significance. DNA Repair. 2004;3(11):1389–407. doi:10.1016/j.dnarep.2004.05.004.
5. Fink D, Aebi S, Howell SB. The role of DNA mismatch repair in drug resistance. Clin Cancer Res. 1998;4(1):1–6.
6. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. 2005;352(10):997–1003. doi:10.1056/NEJMoa043331.
7. Pegg AE. Repair of O(6)-alkylguanine by alkyltransferases. Mutat Res. 2000;462(2–3):83–100.
8. Sarkaria JN, Kitange GJ, James CD, Plummer R, Calvert H, Weller M, et al. Mechanisms of chemoresistance to alkylating agents in malignant glioma. Clin Cancer Res. 2008;14(10):2900–8. doi:10.1158/1078-0432.ccr-07-1719.
9. Liu L, Markowitz S, Gerson SL. Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis(2-chloroethyl)nitrosourea. Can Res. 1996;56(23):5375–9.
10. Felsberg J, Thon N, Eigenbrod S, Hentschel B, Sabel MC, Westphal M, et al. Promoter methylation and expression of MGMT and the DNA mismatch repair genes MLH1, MSH2, MSH6 and PMS2 in paired primary and recurrent glioblastomas. Int J Cancer Journal international du cancer. 2011;129(3):659–70. doi:10.1002/ijc.26083.
11. Cahill DP, Levine KK, Betensky RA, Codd PJ, Romany CA, Reavie LB, et al. Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. Clin Cancer Res. 2007;13(7):2038–45.
12. Shinsato Y, Furukawa T, Yunoue S, Yonezawa H, Minami K, Nishizawa Y, et al. Reduction of MLH1 and PMS2 confers temozolomide resistance and is associated with recurrence of glioblastoma. Oncotarget. 2013;4(12):2261–70.
13. Yip S, Miao J, Cahill DP, Iafrate AJ, Aldape K, Nutt CL, et al. MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance. Clin Cancer Res. 2009;15(14):4622–9.
14. Messaoudi K, Clavreul A, Lagarce F. Toward an effective strategy in glioblastoma treatment. Part I: resistance mechanisms and strategies to overcome resistance of glioblastoma to temozolomide. Drug Discov Today. 2015;20(7):899–905. doi:10.1016/j.drudis.2015.02.011.

15. Batchelor TT, Mulholland P, Neyns B, Nabors LB, Campone M, Wick A, et al. Phase III randomized trial comparing the efficacy of cediranib as monotherapy, and in combination with lomustine, versus lomustine alone in patients with recurrent glioblastoma. J Clin Oncol. 2013;31(26):3212–8. doi:10.1200/jco.2012.47.2464.
16. Stupp R, Hegi ME, van den Bent MJ, Mason WP, Weller M, Mirimanoff RO, et al. Changing paradigms—an update on the multidisciplinary management of malignant glioma. Oncologist. 2006;11(2):165–80. doi:10.1634/theoncologist.11-2-165.
17. Aquilina G, Ceccotti S, Martinelli S, Hampson R, Bignami M. *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea sensitivity in mismatch repair-defective human cells. Can Res. 1998;58(1):135–41.
18. Fiumicino S, Martinelli S, Colussi C, Aquilina G, Leonetti C, Crescenzi M, et al. Sensitivity to DNA cross-linking chemotherapeutic agents in mismatch repair-defective cells in vitro and in xenografts. Int J Cancer J Int Cancer. 2000;85(4):590–6.
19. Pepponi R, Marra G, Fuggetta MP, Falcinelli S, Pagani E, Bonmassar E, et al. The effect of O^6-alkylguanine-DNA alkyltransferase and mismatch repair activities on the sensitivity of human melanoma cells to temozolomide, 1,3-bis(2-chloroethyl)1-nitrosourea, and cisplatin. J Pharmacol Exp Ther. 2003;304(2):661–8. doi:10.1124/jpet.102.043950.
20. Margison GP, Santibanez-Koref MF. O6-alkylguanine-DNA alkyltransferase: role in carcinogenesis and chemotherapy. BioEssays News Rev Mol Cell Dev Biol. 2002;24(3):255–66. doi:10.1002/bies.10063.
21. Glas M, Happold C, Rieger J, Wiewrodt D, Bahr O, Steinbach JP, et al. Long-term survival of patients with glioblastoma treated with radiotherapy and lomustine plus temozolomide. J Clin Oncol. 2009;27(8):1257–61. doi:10.1200/jco.2008.19.2195.
22. Herrlinger U, Rieger J, Koch D, Loeser S, Blaschke B, Kortmann RD, et al. Phase II trial of lomustine plus temozolomide chemotherapy in addition to radiotherapy in newly diagnosed glioblastoma: UKT-03. J Clin Oncol. 2006;24(27):4412–7. doi:10.1200/jco.2006.06.9104.
23. Yamauchi T, Ogawa M, Ueda T. Carmustine-resistant cancer cells are sensitized to temozolomide as a result of enhanced mismatch repair during the development of carmustine resistance. Mol Pharmacol. 2008;74(1):82–91.
24. Stupp R, Mason WP, Van Den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005;352(10):987–96.
25. Bedner E, Li X, Gorczyca W, Melamed MR, Darzynkiewicz Z. Analysis of apoptosis by laser scanning cytometry. Cytometry. 1999;35(3):181–95.
26. Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry. 1997;27(1):1–20.
27. Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV. Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry. 1992;13(2):204–8. doi:10.1002/cyto.990130216.
28. Endt H, Sprung CN, Keller U, Gaipl U, Fietkau R, Distel LV. Detailed analysis of DNA repair and senescence marker kinetics over the life span of a human fibroblast cell line. J Gerontol Ser A Biol Sci Med Sci. 2011;66(4):367–75. doi:10.1093/gerona/glq197.
29. Hecht M, Harrer T, Buttner M, Schwegler M, Erber S, Fietkau R, et al. Cytotoxic effect of efavirenz is selective against cancer cells and associated with the cannabinoid system. Aids. 2013;27(13):2031–40. doi:10.1097/QAD.0b013e3283625444.
30. Sankar A, Thomas DG, Darling JL. Sensitivity of short-term cultures derived from human malignant glioma to the anti-cancer drug temozolomide. Anti-cancer Drugs. 1999;10(2):179–85.
31. Faoro D, von Bueren AO, Shalaby T, Sciuscio D, Hurlimann ML, Arnold L, et al. Expression of O(6)-methylguanine-DNA methyltransferase in childhood medulloblastoma. J Neurooncol. 2011;103(1):59–69. doi:10.1007/s11060-010-0366-7.
32. Jiricny J. The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol. 2006;7(5):335–46. doi:10.1038/nrm1907.
33. Stark AM, Doukas A, Hugo H-H, Mehdorn HM. The expression of mismatch repair proteins MLH1, MSH2 and MSH6 correlates with the Ki67 proliferation index and survival in patients with recurrent glioblastoma. Neurol Res. 2010;32(8):816–20.
34. Jin YH, Clark AB, Slebos RJ, Al-Refai H, Taylor JA, Kunkel TA, et al. Cadmium is a mutagen that acts by inhibiting mismatch repair. Nat Genet. 2003;34(3):326–9.
35. Lützen A, Liberti SE, Rasmussen LJ. Cadmium inhibits human DNA mismatch repair in vivo. Biochem Biophys Res Commun. 2004;321(1):21–5.
36. Gallego O. Nonsurgical treatment of recurrent glioblastoma. Curr Oncol (Toronto, Ont). 2015;22(4):e273–81. doi:10.3747/co.22.2436.
37. Mizumoto M, Okumura T, Ishikawa E, Yamamoto T, Takano S, Matsumura A, et al. Reirradiation for recurrent malignant brain tumor with radiotherapy or proton beam therapy. Technical considerations based on experience at a single institution. Strahlentherapie und Onkologie: Organ der Deutschen Rontgengesellschaft [et al]. 2013;189(8):656–63. doi:10.1007/s00066-013-0390-6.
38. Berry SE, Kinsella TJ. Targeting DNA mismatch repair for radiosensitization. Semin Radiat Oncol. 2001;11(4):300–15.
39. Resnick KE, Frankel WL, Morrison CD, Fowler JM, Copeland LJ, Stephens J, et al. Mismatch repair status and outcomes after adjuvant therapy in patients with surgically staged endometrial cancer. Gynecol Oncol. 2010;117(2):234–8. doi:10.1016/j.ygyno.2009.12.028.