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XRCC3 contributes to temozolomide resistance of glioblastoma cells by promoting DNA double-strand break repair

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ABSTRACT

Glioblastoma is the most frequent and aggressive form of high-grade malignant glioma. Due to the dismal prognosis faced by patients suffering from this disease, there is a need for identifying new targets that might improve therapy. The aim of this study was to determine the contribution of the DNA double-strand break (DSB) repair protein X-ray repair cross-complementing 3 (XRCC3) to the resistance of glioma cells to the chemotherapeutic drug temozolomide. Analysis of a publicly available database, E-GEOD-4290, showed that gliomas overexpress XRCC3 (NM_005432) compared to normal brain tissue. Using an isogenic glioma cell system, in which XRCC3 was downregulated by interference RNA, we demonstrate that XRCC3 protects glioma cells against temozolomide-induced reproductive cell death, apoptosis and cell cycle inhibition. Furthermore, XRCC3 knockdown significantly reduced the rate of repair of DSBs following TMZ treatment, which results in increased drug sensitivity. This study confirms the importance of homologous recombination in the resistance of glioma cells to the methylating drug temozolomide and adds XRCC3 to the list of homology-directed DNA repair proteins as possible targets for therapeutic intervention.

1. Introduction

Glioblastoma accounts for up to 70% of high-grade malignant gliomas [37]. The prognosis for patients suffering from this type of brain cancer is bleak, as the median length of survival is only 10 mounts [12]. During therapy, the tumour is removed surgically with maximal safe margins and the patients receive radiotherapy and concomitant and adjuvant chemotherapy with temozolomide (TMZ) [33]. Taken the abysmal outlook for patients suffering from this disease, a need exists for identifying targets that may potentially improve current therapy, until a more permanent therapeutic solution can be found for this cancer.

TMZ is an S_N1 methylating chemotherapeutic agent that can pass the blood-brain barrier [19]. At physiological pH, it hydrolyzes spontaneously to form 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC), while MTIC subsequently forms 4-amino-5-imidazole carboxamide and the methyl diazonium (MDI) ion. Although MDI methylates DNA at multiple sites, the critical DNA lesion for therapy is O^6-methylguanine (O^6MeG) [2,5,36]. O^6MeG is repaired by O^6-methylguanine-DNA methyltransferase (MGMT) in a stoichiometric damage reversal reaction that restores guanine and, at the same time, inactivates MGMT [14]. The expression of MGMT and consequently the resistance of gliomas to TMZ based therapy, is governed by epigenetic modification of CpG islands in the MGMT promoter [7]. If these CpG islands are methylated, *MGMT* is silenced and O^6MeG persists in the DNA. Promoter methylation of *MGMT*, as well as MGMT activity, has been shown to be prognostic for glioma therapy outcome, which supports the importance of O^6MeG in glioma therapy [5,6,10].

O^6MeG does not trigger cell death in gliomas directly, it requires DNA replication and mismatch repair to facilitate the formation of DNA double-strand breaks (DSBs) [9,21,27]. These DSBs are thought to be the ultimate killing lesions. Glioma cells tolerate TMZ-induced DSBs in a process termed recombination bypass that requires RAD51 and BRCA2, two components of the homologous recombination repair pathway [24]. Other RAD51 paralogues, XRCC2, XRCC3, RAD51B, RAD51C and RAD51D may also play a role in the tolerance of chemotherapeutics like TMZ that induce O^6MeG. Here we focused on the role of the RAD51 paralogue X-ray repair cross-complementing 3 (XRCC3) in the protection of glioma cells against TMZ.

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XRCC3 has been shown to promote the repair of DSBs by homologous recombination and to be essential for the maintenance of genomic integrity and cell survival upon exposure to ionizing radiation, mitomycin C and cisplatin [22,35]. XRCC3 is phosphorylated in an ataxia telangiectasia and Rad3-related protein (ATR) dependent manner at stalled replication forks in S-phase, which is thought to lead to its activation [32]. It interacts directly with RAD51 [17] and is required for the loading of RAD51 onto DNA [3,32,38].

A functional single-nucleotide polymorphism, Thr241Met, has been identified in XRCC3 [31]. It was thought that any decrease in DNA repair activity caused by this polymorphism may increase the risk of developing gliomas. Various studies have been performed, some showing an increased risk [16] and some showing no increase in risk [8]. The differences in these findings could be due to differences in the genetic background of the populations or differences in their environment. The clarity of the results will also be influenced by the fact that XRCC3 containing the Thr241Met polymorphism is still able to perform its homologous recombination repair function [1].

In this study, we addressed whether XRCC3 contributes to the resistance of glioma cells to the chemotherapeutic drug TMZ, which is used first line in glioblastoma therapy. Analysis of a publicly available database revealed that glioblastomas overexpress XRCC3, which might contribute to their intrinsic drug resistance. Here we show that knockdown of XRCC3 sensitizes glioma cells to TMZ-induced cell death, apoptosis and inhibition of cell cycle progression. These effects are attributed to the decreased repair of DSBs generated following TMZ treatment in the XRCC3 knockdown cells. Our finding that XRCC3 causes resistance of glioma cells to TMZ corroborates the importance of homologous recombination in the tolerance of TMZ-induced DSBs [24,29] and supports a strategy for targeting this repair pathway during TMZ-based glioma therapy.

2. Materials and methods

2.1. Cell line and culture conditions

The human glioblastoma cell lines LN229 and T98G were purchased from American Type Culture Collection (ATCC, LGC Standards, GmbH, Wesel, Germany) while the human glioblastoma cell line A172 was purchased from Cell Lines Service (CLS, Eppelheim, Germany). The human fibroblasts VH10tert was a generous gift from Prof. L. Mullenders (Leiden) while the smooth muscle cells (HUASMC), human pericytes from placenta (hPC-PL) and the primary humans umbilical cord endothelial cells (HUVEC) were purchased from PromoCell (Heidelberg, Germany) and maintained according to the provider's protocol. Upon receipt, the cells were amplified for cryopreservation in liquid-N_2 and freshly thawed cell stocks were used for every battery of tests. LN229, T98G, A172, VH10tert and the LN229 transfectant cells were cultured in DMEM (Gibco, Life Technologies Corporation, Paisley, UK) supplemented with 10% FBS and penicillin/streptomycin (PAA Laboratories GmbH, Cölbe, Germany). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Microarray dataset

The microarray dataset E-GEOD-4290, reported on in Ref. [34], was downloaded from ArrayExpress (https:/www.ebi.ac.uk/arrayexpress). The dataset comprised microarray data obtained from 180 tissue samples, 23 from patients suffering from epilepsy, 42 from patients suffering from grade II oligodendroglioma and astrocytoma, 31 from patients suffering from grade III oligodendroglioma and astrocytoma and 81 from patients suffering from grade IV glioblastoma. The intensity values for *XRCC3* (NM_005432) were extracted from the dataset and plotted in the graph.

2.3. siRNA, plasmids, and generation of a stable knockdown

A pSuper (OligoEngine, Seattle, USA) construct was generated to express shRNA targeting XRCC3 mRNA using the sequence 5′-AACUGAAAUCGGUAAAGGA-3’. The plasmids pSuper.basic.XRCC3sh and pSV2-neo were co-transfected into cells using Effectene (Qiagen GmbH, Hilden, Germany). XRCC3 knockdown and neomycin resistant cells were selected for using 0.75 mg/ml G418 (Invitrogen, ThermoFisher Scientific, Darmstadt, Germany) until colonies formed. Single colonies were picked, expanded and tested for XRCC3 knockdown by Western blot. LN229 knockdown clones were routinely cultured in medium containing 0.75 mg/ml G418, which was omitted during the experiments.

siRNA targeting XRCC3 and non-targeting siRNA were purchased from Life Technologies GmbH (Darmstadt, Germany). For transient knockdown of XRCC3 in LN229 cells the siRNA were transfected using Lipofectamine RNAiMAX (ThermoFisher Scientific, Darmstadt, Germany) according to the manufactures protocol. For transient knockdown of XRCC3 in T98G and A172 cells the pSuper.basic.XRCC3sh or Empty plasmid was transfected using Effectene (Qiagen GmbH, Hilden, Germany) according to the manufactures protocol.

2.4. Drugs and drug treatment

Temozolomide (TMZ, generously provided by Dr Geoff Margison, The University of Manchester, Centre for Occupational and Environmental Health, United Kingdom, Manchester) stocks were prepared by dissolving in dimethyl sulfoxide (DMSO, Carl Roth GmbH, Karlsruhe, Germany) and then diluting it in two parts sterile dH_2O to a concentration of 35 mM. These TMZ stocks were aliquoted and stored at −80 °C until use. Methyl methanesulfonate (MMS, Sigma, Steinheim, Germany) was diluted in sterile dH_2O to a stock solution of 100 mM before exposing cells to the indicated concentrations. For MMS, cells were exposed to the methylating agent for 1 h before MMS was removed by changing the medium. O^6-benzylguanine (O^6BG, Sigma, Steinheim, Germany) was dissolved in DMSO to a stock concentration of 10 mM, aliquoted and stored at −20 °C.

2.5. Colony survival assay

LN229 pS-empty, LN229XRCC3kd.c2, LN229XRCC3kd.c8, T98G and A172 glioma cells in logarithmic growth were seeded at appropriate numbers on 60 mm Petri dishes. Cells were allowed to attach for 6 h before TMZ or MMS exposure with the indicated concentrations. After approximately two weeks, colonies were fixed with acetic acid:methanol:H_2O (1:1:8), and stained with a staining solution containing 1.25% Giemsa and 0.125% violet crystal.

2.6. Apoptosis determination by flow cytometry

LN229 pS-empty, LN229XRCC3kd.c2, LN229XRCC3kd.c8, LN229 transfected with siRNA targeting XRCC3 and A172 glioma cells were exposed to the indicated concentration of TMZ and 144 h after TMZ exposure the samples were prepared for analysis. Unfixed cells were labelled with Annexin V-FITC (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions and stained with 1 μg/ml propidium iodide (PI) (Sigma, Steinheim, Germany) before the analysis by flow cytometry. Annexin V positive cells were classified as apoptotic while Annexin V and PI double-positive cells were classified as necrotic/late-apoptotic. The flow cytometric analysis was carried out using a FACS Canto II flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany). The data were analysed using the BD FACSDiva software.

2.7. Cell cycle analysis

Exponentially growing LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 glioma cells were exposed to 50 μM TMZ and prepared for cell cycle analysis at indicated time points. Cells were harvested and fixed in 70% ethanol at −20 °C. Following removal of the ethanol, RNA was digested (30 μg/ml RNase for 1 h at room temperature) and the DNA of the cells were stained with 16.5 μg/ml PI before flow cytometric data acquisition using a FACS Canto II flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany). Cell cycle distribution of the cells was determined using ModFit LT version 3.3 software (Verity Software House, Topsham, ME, USA). Apoptotic cells were excluded from analysis.

2.8. Immunofluorescence microscopy

LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 glioma cells were plated onto microscope coverslips. The next day the cells were exposed to 50 μM TMZ and the samples were prepared at the indicated time points after TMZ exposure. Cells were fixed in ice-cold (−20 °C) methanol:acetone (7:3) for 9 min at −20 °C. The samples were washed three times with PBS, blocked with 5% BSA in 0.25% Triton-X100/PBS for 1 h at room temperature. Samples were exposed to the primary antibody (anti-phospho-S139-H2AX, 1:1000, Cell Signalling) overnight at 4 °C. Following removal of the primary antibody by washing the samples in PBS, the samples were exposed to the secondary antibody (anti rabbit-Alexa Fluor 488, 1:500, Invitrogen, ThermoFisher Scientific, Darmstadt, Germany) for 2 h at room temperature. DNA was counterstained with 1 mM TO-PRO-3 (Invitrogen, ThermoFisher Scientific, Darmstadt, Germany). Slides were mounted in Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA). Microphotographs were acquired by laser scanning microscopy (LSM710, Carl Zeiss MicroImaging).

2.9. Statistical analysis

For statistical analysis, the unpaired two-tailed *t*-test or the one-way ANOVA test was performed using GraphPad Prism version 3 software. p-Values $\leq$ 0.05 were considered to be significant and were marked with asterisks in the graphs.

3. Results

3.1. Glioblastoma tumour tissue exhibits a high level of XRCC3

In an effort to determine whether glioblastomas deregulate the expression of XRCC3 and thereby potentially become more resistant to genotoxic based therapies, we made use of a publically available microarray database and compared the differences in *XRCC3* mRNA expression between normal brain tissue obtained from patients suffering from epilepsy and malignant tissue obtained from patients suffering from brain tumours. The expression of *XRCC3* increased with tumour grade and malignancy and glioblastomas showed the strongest and most significant increase in expression compared to normal tissue (Fig. 1A). We should note that the expression of *XRCC3* in the glioblastoma samples varied greatly, ranging from tumours that express comparable levels of *XRCC3* to normal tissue to tumours that express up to 400% more *XRCC3*. This points to a possible sub-group of gliomas that will potentially be more resistant to genotoxic based therapies.

In order to determine whether the glioma cell lines used in this study express XRCC3 at levels higher than untransformed cells, the

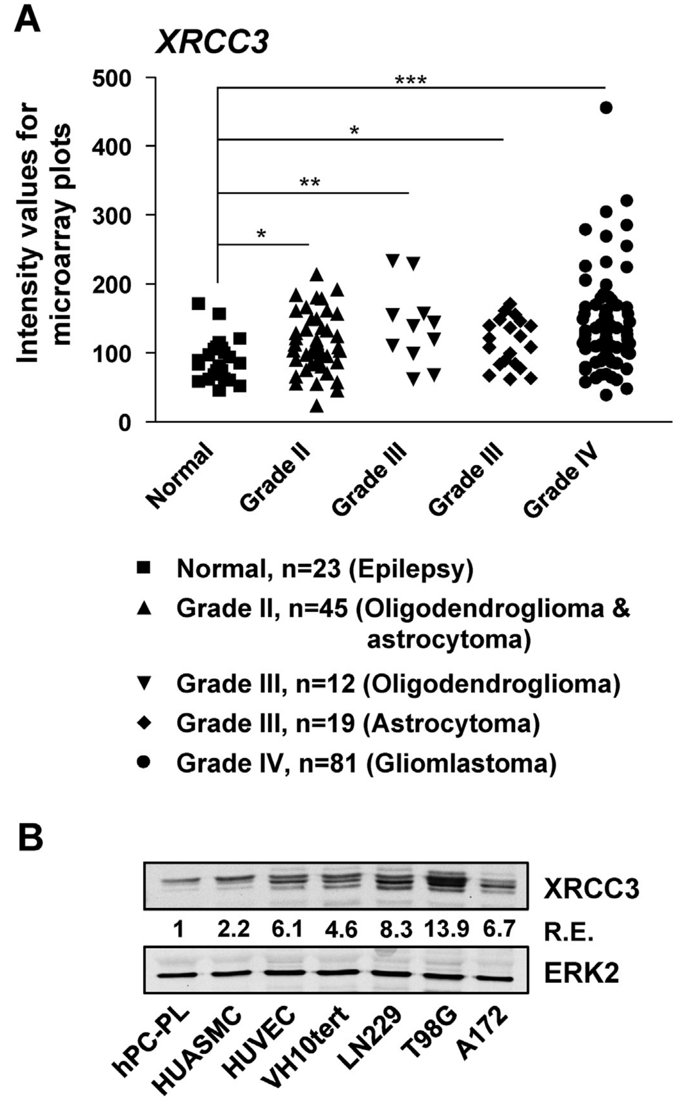

Fig. 1. Glioblastoma tumours overexpress *XRCC3*. (A) Using a public available microarray database the expression of *XRCC3* (NM_005432) was analysed in brain tissue from 23 epilepsy patients as well as tumour tissue from 45 grade II oligodendroglioma and astrocytoma patients, 12 grade III Oligodendroglioma patients, 19 grade III astrocytoma and 81 glioblastoma patients. p-Values of <0.05 are marked as *, <0.005 as ** and <0.001 as ***. (B) Western blot analysis of the differences in XRCC3 expression in the untransformed cells hPC-PL, HUASMC, HUVEC and VH10tert compared to the glioma cells LN229, T98G and A172. ERK2 was used as loading control.

XRCC3 protein level in the glioblastma cells LN229, T98G and A172 were compared to the XRCC3 protein level in primary smooth muscle cells (HUASMC), primary human pericytes from placenta (hPC-PL), primary humans umbilical cord endothelial cells (HUVEC) and diploid human fibroblasts using Western blot. Quantitative analysis shows that LN229, T98G and A172 glioma cells clearly express a higher level of XRCC3 than hPC-PL and HUASMC and a similar to higher level than VH10tert and HUVEC (Fig. 1B). Although we were not able to compare the expression of XRCC3 in the glioblastoma cells with glial cells due to the lack of availability, the high expression of XRCC3 in glioblastoma cell lines is in line with what was observed with the microarray data.

3.2. XRCC3 protects glioma cells against TMZ-induced cell death

Having found that glioblastoma overexpresses *XRCC3 in situ*, we next addressed the question of whether this DNA repair protein protects glioblastoma cells against the chemotherapeutic TMZ. To this end, XRCC3 was stably knocked-down in the glioblastoma cell line LN229 (Fig. 2A). LN229 transfected with the empty vector, LN229 pS-empty, was used as a non-knockdown control. The XRCC3 knockdown lines LN229XRCC3kd.c2 and LN229XRCC3kd.c8 expressed 63% and 38% XRCC3 protein compared to the empty vector control (Fig. 2A). As it was reported that XRCC3 protects rodent cells from MMS induced cell death [20], we determined whether the glioblastoma knockdown cells exhibit the same sensitivity. LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 cells were exposed to increasing doses of MMS and the cell's resistance was determined using the colony survival assay. Knockdown of XRCC3 significantly sensitized glioblastoma cells to MMS (Fig. 2B), confirming what was found in rodent cells. Having established an isogenic glioblastoma cell system to determine the contribution of XRCC3 to the resistance of this cancer type to the chemotherapeutic TMZ, LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 were exposed to TMZ and overall survival was tested for by colony survival assay. XRCC3 knockdown

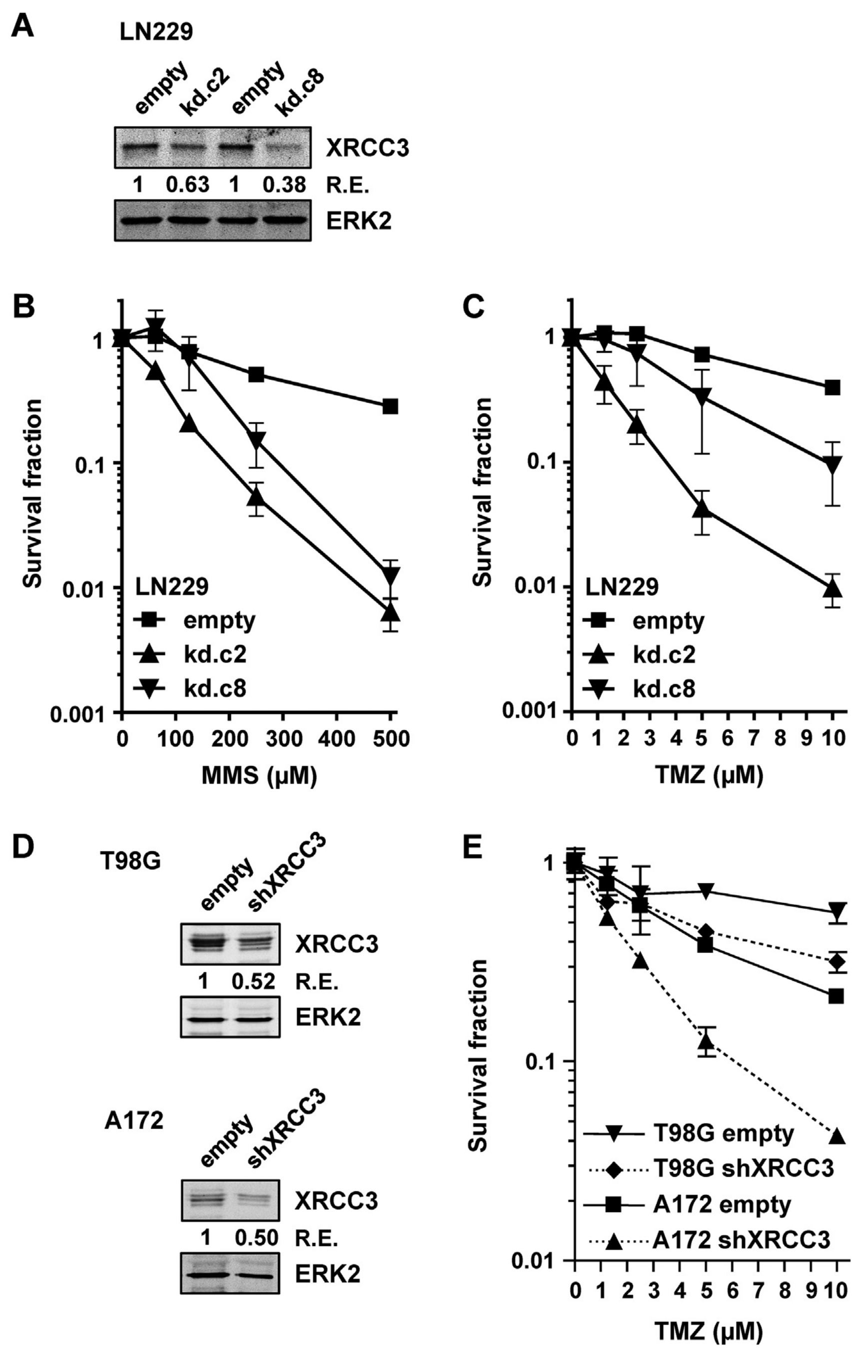

Fig. 2. XRCC3 protects glioma cells against methylating agent-induced cell death. (A) Knockdown of XRCC3 in LN229 glioma cells. Western blot analysis of XRCC3 protein level in LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8. ERK2 was used as loading control. Colony survival of LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 cells exposed to the S_N2 methylating agent MMS (B) and the S_N1 methylating chemotherapeutic TMZ (C). (D) Western blot analysis of XRCC3 protein level in T98G and A172 upon transient knockdown of XRCC3. ERK2 was used as loading control. (E) Colony survival of T98G and A172 upon transient knockdown of XRCC3 exposed to TMZ. The designation “empty” means stable or transient transfection with the control vector not expressing XRCC3 shRNA.

sensitized LN229 cells to TMZ-induced cell death (Fig. 2C). To verify the role of XRCC3 in glioma cells exposed to TMZ, XRCC3 was transiently knocked down using the pSuper.basic.XRCC3sh plasmid and the LN229 pS-empty plasmid for control in two additional glioma cell lines, namely T98G and A172, for knockdown see Fig. 2D. Similar to LN229, knockdown of XRCC3 sensitized T98G and A172 glioma cells to TMZ-induced cell death (Fig. 2E), confirming that XRCC3 causes resistance of glioblastoma cells to the killing effect of the methylating drug TMZ.

3.3. XRCC3 defends against temozolomide-induced apoptosis in glioma cells

It has been shown that TMZ-induced cell killing in glioblastoma cells is executed by apoptosis [28]. Therefore, the question of whether the increased cell death upon TMZ exposure in XRCC3 knockdown cells is due to an increase in apoptosis was addressed. LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 glioblastoma cells were exposed to TMZ and the apoptosis and necrosis response were quantified using annexin V/propidium iodide labelling and flow cytometry. XRCC3 knockdown significantly sensitized glioma cells to TMZ-induced apoptosis (Fig. 3A) and necrosis (Fig. 3B) for all the concentrations of TMZ tested. The main mode of cell death induced by TMZ in XRCC3 knockdown cells was apoptosis as 65–80% of cell death fraction was annexin V and only 20% PI positive (Fig. 3C).

To substantiate the results obtained in LN229 cells, XRCC3 was transiently knocked down using the pSuper.basic.XRCC3sh plasmid and the LN229 pS-empty plasmid for control in A172 glioma cells. Similar to what was observed in LN229 cells upon XRCC3

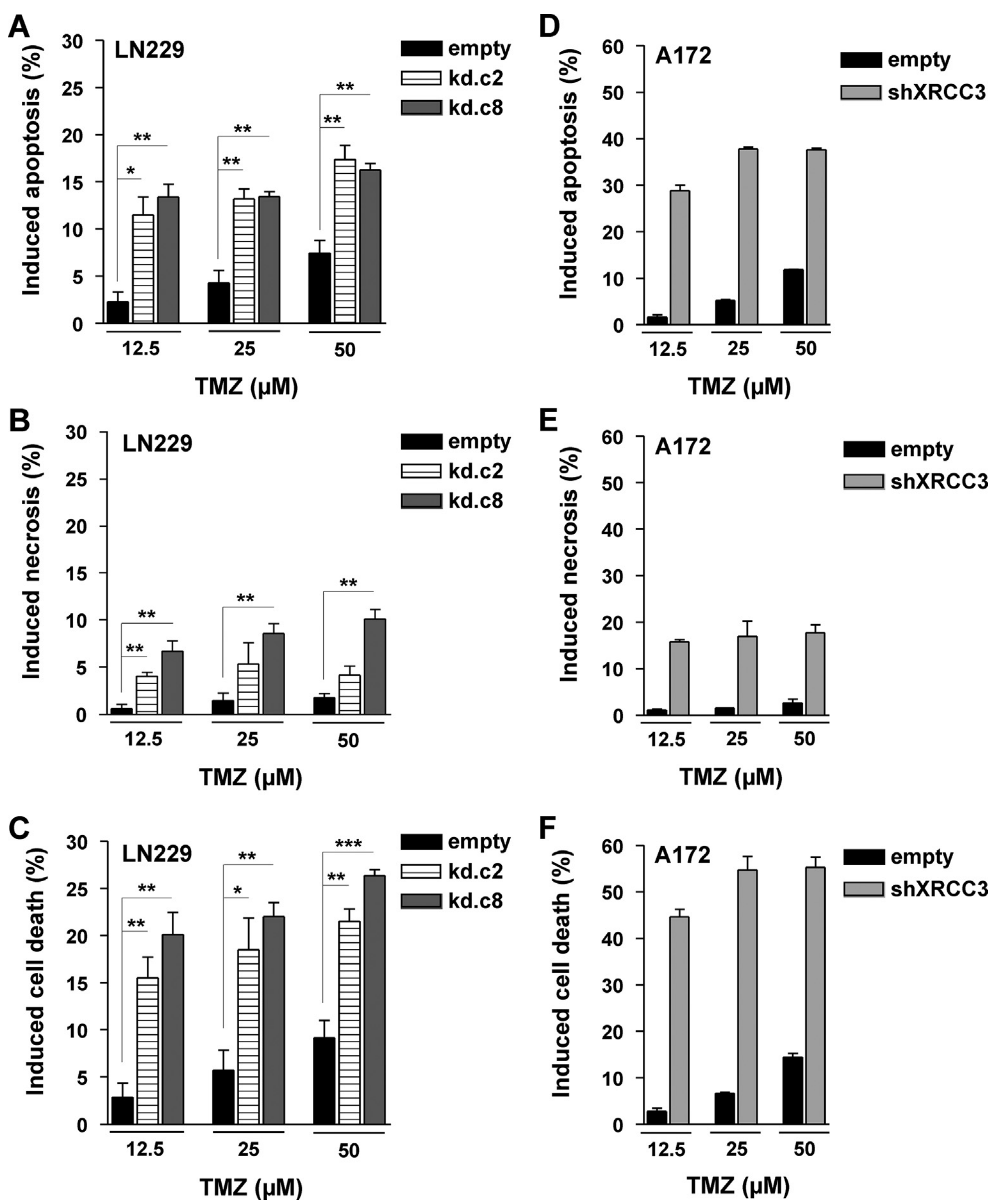

Fig. 3. XRCC3 protects glioma cells against TMZ-induced apoptosis. LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 cells were exposed to TMZ at the indicated concentrations and the (A) apoptosis and (B) necrosis response were determined by annexin V/PI double-staining and flow cytometry analysis 144 h later. (C) Cell death depicts the sum of apoptosis and necrosis. Error bars show standard error of the mean. p-Values of <0.05 are marked as *, <0.005 as ** and <0.001 as ***. XRCC3 was transiently knocked down in A172 cells and the cells were exposed to TMZ at the indicated concentrations and (D) apoptosis and (E) necrosis were determined by annexin V/PI double-staining and flow cytometry 144 h later. (F) Cell death depicts the sum of apoptosis and necrosis. The designation "empty" means transfections with the control vector not expressing shXRCC3.

knockdown, XRCC3 knockdown in A172 cells greatly increased the level of apoptosis (Fig. 3D), necrosis (Fig. 3E) and total cell death (Fig. 3F) in A172 cells. Further validation of the increased killing response was obtained by knocking down XRCC3 using siRNA targeting XRCC3 and non-targeting siRNA for control in LN229 cells. We should state that the sequence of the siRNA targeting XRCC3 differs from the sequence contained in the pSuper.basic.XRCC3sh plasmid. Upon transient knockdown of XRCC3 (Supplementary Fig. 1A), TMZ induced more apoptosis (Supplementary Fig. 1B), necrosis (Supplementary Fig. 1C) and total cell death (Supplementary Fig. 1D) in LN229 cells. From these data, we conclude that XRCC3 significantly protects glioblastoma cells from TMZ-induced apoptosis, and to a lesser extent, TMZ-induced necrosis.

3.4. XRCC3 contributes to the recovery from G2/M cell cycle arrest following TMZ exposure

TMZ exposure causes cells to accumulate in the G2/M phase of the cell cycle [11]. To determine whether XRCC3 plays a role in the recovery of glioma from a TMZ-induced G2/M cell cycle arrest, we exposed LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 cells to 50 μM TMZ and determined the cell cycle distribution 72, 96 and 120 h after exposure using PI-stained cells and flow cytometry analysis (Fig. 4). Exposure of cells to TMZ caused a G2/M arrest 72 h after exposure. This arrest was more pronounced in the XRCC3 knockdown cells. After 96 and 120 h of TMZ treatment, the control line started recovering from the G2/M arrest as the population of cells in G2/M decreased while the population in G1 increased. This recovering was not observed in the XRCC3 knockdown cells as the percentage of cells in G2/M and G1 of the cell cycle remained unchanged (Fig. 4). We interpret these data to mean that XRCC3 either alleviate the signalling that maintains the G2/M block or that XRCC3 contributes to the removal of the G2/M initializing signal, namely DSBs that form due to TMZ-induced replication stress.

3.5. XRCC3 stimulates the repair of temozolomide-induced DNA double-strand breaks

Unrepaired DNA adducts induced by TMZ cause DSBs to form in a replication-dependent manner [21,23]. Cells tolerate these breaks, which are formed transiently, in a process called recombination bypass [13] that require some components of homology-directed repair [24,25,29]. As the increased sensitivity observed upon XRCC3 knockdown could be due to less recombination bypass during S-phase, we quantified the amount of DSBs following TMZ exposure and XRCC3 knockdown. For this, we quantified the number of phosphorylated H2AX (γH2AX) foci, which is an accepted marker for DNA double-strand breaks [26]. LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 cells were exposed to 50 μM TMZ and 72 and 120 h later the average number of γH2AX foci per cell was quantified by fluorescence microscopy. TMZ induced significantly more γH2AX foci in the XRCC3 knockdown glioma cells than in the isogenic control (for representative micrographs see Fig. 5A). In addition, the γH2AX foci in the XRCC3

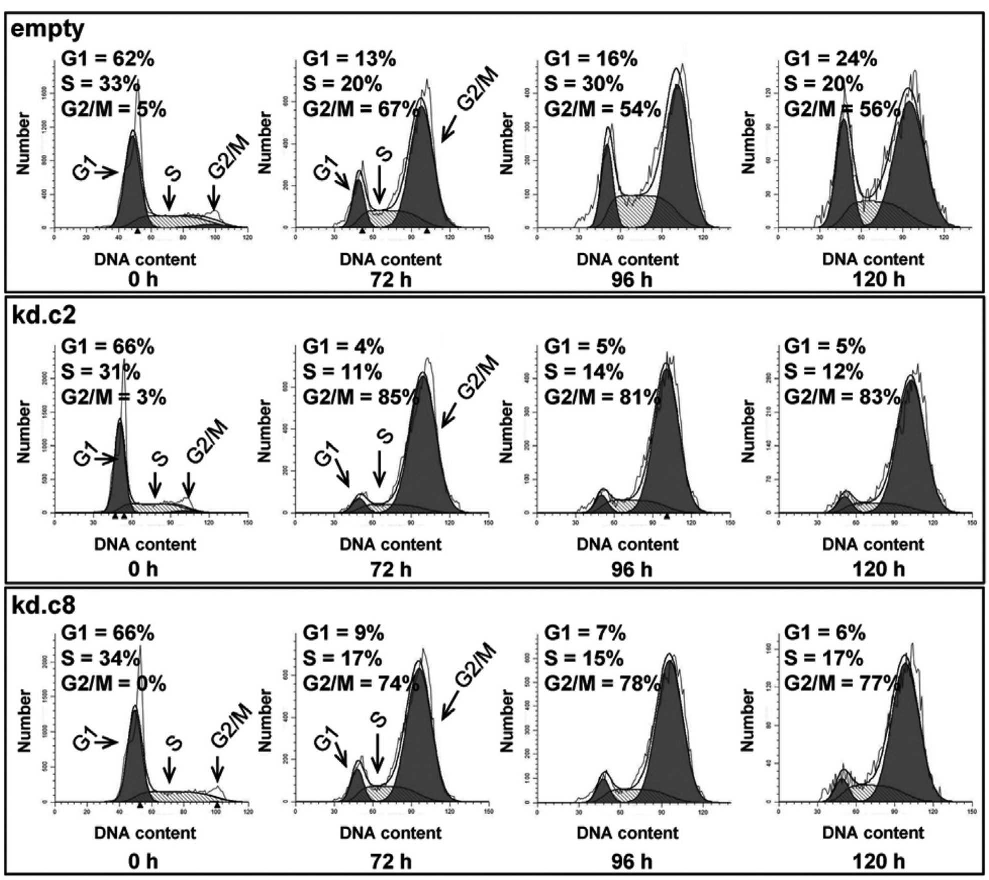

Fig. 4. XRCC3 stimulates the recovery of glioma cells from a TMZ-induced G2/M arrest. LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 cells were exposed to 50 μM TMZ and the cells were subjected to cell cycle analysis by flow cytometry at the indicated time points following exposure. Percentages of cells in G1, S and G2/M were determined by ModFit LT software.

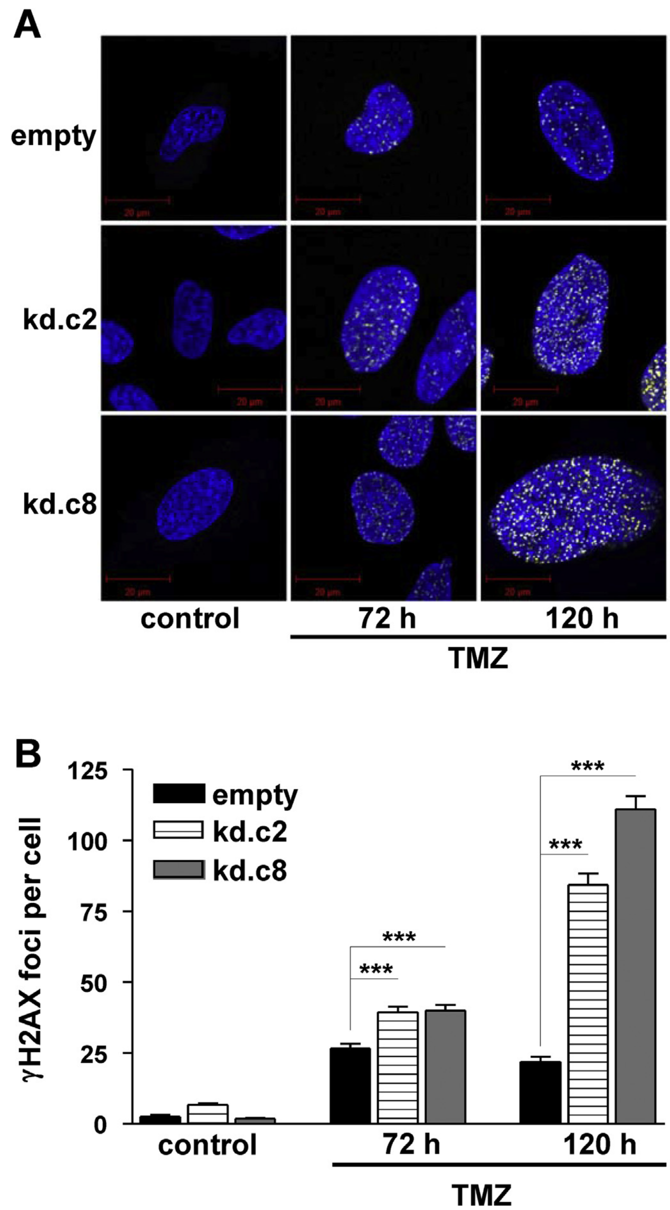

Fig. 5. XRCC3 contributes to the repair of TMZ-induced DNA double-strand breaks. LN229 pS-empty, LN229XRCC3kd.c2 and LN229XRCC3kd.c8 cells were exposed to 50 μM TMZ and samples were prepared for fluorescence microscopy analysis at the indicated time points. (A) Representative micrographs showing TMZ-induced γH2AX foci in yellow and nuclear staining in blue. (B) Quantification of TMZ-induced γH2AX foci. Error bars show standard error of the mean. p-Values of <0.001 are marked as ***. For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.

knockdown cells persisted longer than in the control line (Fig. 5B). These data obtained with isogenic cells clearly show that XRCC3 contributes to the repair of DSBs generated in response to TMZ, and lack of XRCC3 results in enhanced sensitivity of glioblastoma cells to TMZ.

4. Discussion

High-grade malignant glioma remains a therapeutic challenge. Arguably, the most encouraging advance for its therapy was the inclusion of the chemotherapeutic drug TMZ [30]. TMZ not only improved the standard of care at the time, but it is also well tolerated by the patients. The aim of this study was to identify targets for possibly improving the benefit seen with TMZ. We focused on a component of the homology-directed DSB repair pathway, namely XRCC3.

By comparing the expression data from normal tissue and malignant brain tumours, the data showed that high-grade gliomas overexpress XRCC3. All the gliomas were pathologically graded according to the World Health Organization (WHO) standard for the time [34]. Although the WHO standard for grading gliomas has changed in 2016 [18], these findings are still of interest as the expression of XRCC3 showed a significant increase in all glioma tumour types. This notwithstanding, the data revealed a big variation of XRCC3 expression within the specific grade. This could be due to the dated classification system, as some tumours may have been misclassified, and it would be of interest to determine the XRCC3 expression using the new WHO standard as it may identify differences in XRCC3 expression between sub-types of gliomas more clearly. More likely, however, is that XRCC3 is naturally variably expressed, similiar to some other repair proteins such as MGMT [5].

The observed overexpression of XRCC3 in gliomas could cause this cancer to be resistant to TMZ-based therapies. To test this hypothesis, we created an isogenic glioma system where the expression of XRCC3 was downregulated by interference RNA. Upon XRCC3 knockdown, glioma cells showed a significant increase in cell killing upon TMZ exposure. This increase in cell killing in the XRCC3 knockdown glioma cells was paralleled by an increase in TMZ-induced apoptosis and an increase in cells blocked in the G2/M cell cycle phase. These results show that XRCC3 contributes to the resistance of glioma cells to TMZ-induced cell killing and points to a possible mechanism whereby this is achieved.

The importance of XRCC3 in the tolerance of TMZ-induced DNA lesions is clearly more obvious than its contribution to the protection against cancer formation. The contradictory results obtained when determining whether the Thr241Met polymorphism would increase the risk for developing gliomas [8,16] may be due to the low levels of DNA lesions subject to repair by XRCC3 that occur naturally. Any decrease in repair activity due to this polymorphism is negated by the low levels of naturally occurring DNA lesions dependent on their repair by XRCC3. During TMZ-based therapies, the aim is to overwhelm the cells capacity for repairing DNA damage. Under these conditions, the increased expression of XRCC3 in high-grade gliomas clearly contributes to their resistance.

TMZ methylates DNA at position 6 of guanine, and this methylation product, namely O^6MeG, leads to the formation of DSBs in a replication and mismatch repair-dependent manner [14]. Some components of the homologous recombination pathway, RAD51 [24], RAD51D [25], BRCC3 [4], XRCC2, and BRCA2 [29], and the non-homologous end-joining pathway, ligase IV [15] and DNA-PKcs [28], have been implicated in the tolerance of DSBs generated in response to TMZ. A study comparing the contribution of these two DNA repair pathway to the tolerance of O^6MeG-induced DSBs showed that homologous recombination playes a much more significant role than NHEJ [29]. As the relative contribution of homologous recombination is essential for protecting cells against methylating agents like TMZ, our finding that XRCC3 along with BRCC3 [4], RAD51 and BRCA2 [24], plays a major role in glioblastoma TMZ resistance strengthens the case that homologous recombination is an important target for clinical intervention in combination with TMZ. Collectively, the data support XRCC3 dependent homologous recombination as a key mechanism in the repair of TMZ-induced DNA lesions and suggests XRCC3 as a novel target for sensitizing high-grade gliomas in TMZ-based therapy.

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Conflicts of interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.03.025.

References

[1] F.D. Araujo, A.J. Pierce, J.M. Stark, M. Jasin, Variant XRCC3 implicated in cancer is functional in homology-directed repair of double-strand breaks, Oncogene 21 (2002) 4176–4180.

[2] J.C. Baer, A.A. Freeman, E.S. Newlands, A.J. Watson, J.A. Rafferty, G.P. Margison, Depletion of O6-alkylguanine-DNA alkyltransferase correlates with potentiation of temozolomide and CCNU toxicity in human tumour cells, Br. J. Canc. 67 (1993) 1299–1302.

[3] D.K. Bishop, U. Ear, A. Bhattacharyya, C. Calderone, M. Beckett, R.R. Weichselbaum, A. Shinohara, Xrcc3 is required for assembly of Rad51 complexes in vivo, J. Biol. Chem. 273 (1998) 21482–21488.

[4] K.M. Chai, C.Y. Wang, H.J. Liaw, K.M. Fang, C.S. Yang, S.F. Tzeng, Downregulation of BRCA1-BRCA2-containing complex subunit 3 sensitizes glioma cells to temozolomide, Oncotarget 5 (2014) 10901–10915.

[5] M. Christmann, G. Nagel, S. Horn, U. Krahn, D. Wiewrodt, C. Sommer, B. Kaina, MGMT activity, promoter methylation and immunohistochemistry of pretreatment and recurrent malignant gliomas: a comparative study on astrocytoma and glioblastoma, Int. J. Canc. 127 (2010) 2106–2118.

[6] M. Esteller, J. Garcia-Foncillas, E. Andion, S.N. Goodman, O.F. Hidalgo, V. Vanaclocha, S.B. Baylin, J.G. Herman, Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents, N. Engl. J. Med. 343 (2000) 1350–1354.

[7] M. Esteller, S.R. Hamilton, P.C. Burger, S.B. Baylin, J.G. Herman, Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia, Cancer Res. 59 (1999) 793–797.

[8] Y. Feng, M. Zeng, Q. Xu, Association between XRCC3 T241M polymorphism and glioma risk: a meta-analysis, Tumour Biol. 35 (2014) 5589–5592.

[9] C. Happold, P. Roth, W. Wick, N. Schmidt, A.M. Florea, M. Silginer, G. Reifenberger, M. Weller, Distinct molecular mechanisms of acquired resistance to temozolomide in glioblastoma cells, J. Neurochem. 122 (2012) 444–455.

[10] M.E. Hegi, A.C. Diserens, T. Gorlia, M.F. Hamou, N. de Tribolet, M. Weller, J.M. Kros, J.A. Hainfellner, W. Mason, L. Mariani, J.E. Bromberg, P. Hau, R.O. Mirimanoff, J.G. Cairncross, R.C. Janzer, R. Stupp, MGMT gene silencing and benefit from temozolomide in glioblastoma, N. Engl. J. Med. 352 (2005) 997–1003.

[11] Y. Hirose, M.S. Berger, R.O. Pieper, p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells, Cancer Res. 61 (2001) 1957–1963.

[12] D.R. Johnson, B.P. O'Neill, Glioblastoma survival in the United States before and during the temozolomide era, J. Neuro Oncol. 107 (2012) 359–364.

[13] B. Kaina, Mechanisms and consequences of methylating agent-induced SCEs and chromosomal aberrations: a long road traveled and still a far way to go, Cytogenet. Genome Res. 104 (2004) 77–86.

[14] B. Kaina, M. Christmann, S. Naumann, W.P. Roos, MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents, DNA Repair 6 (2007) 1079–1099.

[15] N. Kondo, A. Takahashi, E. Mori, K. Ohnishi, P.J. McKinnon, T. Sakaki, H. Nakase, T. Ohnishi, DNA ligase IV as a new molecular target for temozolomide, Biochem. Biophys. Res. Commun. 387 (2009) 656–660.

[16] H.J. Liang, Y.L. Yan, Z.M. Liu, X. Chen, Q.L. Peng, J. Wang, C.J. Mo, J.Z. Sui, J.R. Wu, L.M. Zhai, S. Yang, T.J. Li, R.L. Li, S. Li, X. Qin, Association of XRCC3 Thr241Met polymorphisms and gliomas risk: evidence from a meta-analysis, Asian Pac. J. Cancer Prev. APJCP Asian Pac. J. Cancer Prev. APJCP 14 (2013) 4243–4247.

[17] N. Liu, J.E. Lamerdin, R.S. Tebbs, D. Schild, J.D. Tucker, M.R. Shen, K.W. Brookman, M.J. Siciliano, C.A. Walter, W. Fan, L.S. Narayana, Z.Q. Zhou, A.W. Adamson, K.J. Sorensen, D.J. Chen, N.J. Jones, L.H. Thompson, XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages, Mol. Cell 1 (1998) 783–793.

[18] D.N. Louis, A. Perry, G. Reifenberger, A. von Deimling, D. Figarella-Branger, W.K. Cavenee, H. Ohgaki, O.D. Wiestler, P. Kleihues, D.W. Ellison, The 2016 World Health organization classification of tumors of the central nervous system: a summary, Acta Neuropathol. 131 (2016) 803–820.

[19] E.S. Newlands, M.F. Stevens, S.R. Wedge, R.T. Wheelhouse, C. Brock, Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials, Canc. Treat Rev. 23 (1997) 35–61.

[20] T. Nikolova, M. Ensminger, M. Lobrich, B. Kaina, Homologous recombination protects mammalian cells from replication-associated DNA double-strand breaks arising in response to methyl methanesulfonate, DNA Repair 9 (2010) 1050–1063.

[21] K. Ochs, B. Kaina, Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent, Cancer Res. 60 (2000) 5815–5824.

[22] A.J. Pierce, R.D. Johnson, L.H. Thompson, M. Jasin, XRCC3 promotes homology-directed repair of DNA damage in mammalian cells, Gene Dev. (1999) 2633–2638.

[23] S. Quiros, W.P. Roos, B. Kaina, Processing of O6-methylguanine into DNA double-strand breaks requires two rounds of replication whereas apoptosis is also induced in subsequent cell cycles, Cell Cycle 9 (2010) 168–178.

[24] S. Quiros, W.P. Roos, B. Kaina, Rad51 and BRCA2—New molecular targets for sensitizing glioma cells to alkylating anticancer drugs, PLoS One 6 (2011), e27183.

[25] P. Rajesh, C. Rajesh, M.D. Wyatt, D.L. Pittman, RAD51D protects against MLH1-dependent cytotoxic responses to O(6)-methylguanine, DNA Repair 9 (2010) 458–467.

[26] E.P. Rogakou, D.R. Pilch, A.H. Orr, V.S. Ivanova, W.M. Bonner, DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139, J. Biol. Chem. 273 (1998) 5858–5868.

[27] W. Roos, M. Baumgartner, B. Kaina, Apoptosis triggered by DNA damage O6-methylguanine in human lymphocytes requires DNA replication and is mediated by p53 and Fas/CD95/Apo-1, Oncogene 23 (2004) 359–367.

[28] W.P. Roos, L.F. Batista, S.C. Naumann, W. Wick, M. Weller, C.F. Menck, B. Kaina, Apoptosis in malignant glioma cells triggered by the temozolomide-induced DNA lesion O6-methylguanine, Oncogene 26 (2007) 186–197.

[29] W.P. Roos, T. Nikolova, S. Quiros, S.C. Naumann, O. Kiedron, M.Z. Zdzienicka, B. Kaina, Brca2/Xrcc2 dependent HR, but not NHEJ, is required for protection against O(6)-methylguanine triggered apoptosis, DSBs and chromosomal aberrations by a process leading to SCEs, DNA Repair 8 (2009) 72–86.

[30] K. Seystahl, W. Wick, M. Weller, Therapeutic options in recurrent glioblastoma—An update, Crit. Rev. Oncol. Hematol. 99 (2016) 389–408.

[31] M.R. Shen, I.M. Jones, H. Mohrenweiser, Nonconservative amino acid substitution variants exist at polymorphic frequency in DNA repair genes in healthy humans, Cancer Res. 58 (1998) 604–608.

[32] K. Somyajit, S. Basavaraju, R. Scully, G. Nagaraju, ATM- and ATR-mediated phosphorylation of XRCC3 regulates DNA double-strand break-induced checkpoint activation and repair, Mol. Cell Biol. 33 (2013) 1830–1844.

[33] R. Stupp, W.P. Mason, M.J. van den Bent, M. Weller, B. Fisher, M.J. Taphoorn, K. Belanger, A.A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R.C. Janzer, S.K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J.G. Cairncross, E. Eisenhauer, R.O. Mirimanoff, R., Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, N. Engl. J. Med. 352 (2005) 987–996.

[34] L. Sun, A.M. Hui, Q. Su, A. Vortmeyer, Y. Kotliarov, S. Pastorino, A. Passaniti, J. Menon, J. Walling, R. Bailey, M. Rosenblum, T. Mikkelsen, H.A. Fine, Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain, Canc. Cell 9 (2006) 287–300.

[35] M. Takata, M.S. Sasaki, S. Tachiiri, T. Fukushima, E. Sonoda, D. Schild, L.H. Thompson, S. Takeda, Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs, Mol. Cell Biol. 21 (2001) 2858–2866.

[36] M.J. Tisdale, Antitumor imidazotetrazines—XV. Role of guanine O6 alkylation in the mechanism of cytotoxicity of imidazotetrazinones, Biochem. Pharmacol. 36 (1987) 457–462.

[37] P.Y. Wen, S. Kesari, Malignant gliomas in adults, N. Engl. J. Med. 359 (2008) 492–507.

[38] T. Yoshihara, M. Ishida, A. Kinomura, M. Katsura, T. Tsuruga, S. Tashiro, T. Asahara, K. Miyagawa, XRCC3 deficiency results in a defect in recombination and increased endoreduplication in human cells, EMBO J. 23 (2004) 670–680.