Biochemical and Biophysical Research Communications 445 (2014) 263–268

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

RAD18 mediates resistance to ionizing radiation in human glioma cells

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ARTICLE INFO

Article history:
Received 27 January 2014
Available online 8 February 2014

Keywords:
Glioblastoma multiforme
RAD18
Translesion DNA synthesis
Radioresistance

ABSTRACT

Radioresistance remains a major challenge in the treatment of glioblastoma multiforme (GBM). RAD18 a central regulator of translesion DNA synthesis (TLS), has been shown to play an important role in regulating genomic stability and DNA damage response. In the present study, we investigate the relationship between RAD18 and resistance to ionizing radiation (IR) and examined the expression levels of RAD18 in primary and recurrent GBM specimens. Our results showed that RAD18 is an important mediator of the IR-induced resistance in GBM. The expression level of RAD18 in glioma cells correlates with their resistance to IR. Ectopic expression of RAD18 in RAD18-low A172 glioma cells confers significant resistance to IR treatment. Conversely, depletion of endogenous RAD18 in RAD18-high glioma cells sensitized these cells to IR treatment. Moreover, RAD18 overexpression confers resistance to IR-mediated apoptosis in RAD18-low A172 glioma cells, whereas cells deficient in RAD18 exhibit increased apoptosis induced by IR. Furthermore, knockdown of RAD18 in RAD18-high glioma cells disrupts HR-mediated repair, resulting in increased accumulation of DSB. In addition, clinical data indicated that RAD18 was significantly higher in recurrent GBM samples that were exposed to IR compared with the corresponding primary GBM samples. Collectively, our findings reveal that RAD18 may serve as a key mediator of the IR response and may function as a potential target for circumventing IR resistance in human GBM.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and the deadliest of the malignant primary brain tumors in adults. Despite advances in current multi-modal treatment options, the overall prognosis of patients with GBM remains dismal [1]. Ionizing radiation (IR) plays a major role in the treatment of patients with GBM. However, the efficacy of this therapeutic modality is often limited by the occurrence of radioresistance [2].

Translesion DNA synthesis (TLS) is a DNA damage tolerance mechanism that allows cells to avoid the detrimental consequences of persistent stalled replication forks. TLS involves a "polymerase switching" in which the stalled replicative polymerase is replaced by a specialized TLS polymerase. TLS polymerases can bypass the damage by incorporating a non-complementary nucleotide opposite the lesion [3]. Over the past decade, a set of specialized TLS polymerases has been identified, including polymerase κ (Pol κ), Pol ι, Pol η and REV1 in the Y-family and polymerase ζ (Pol ζ) in the B-family. However, because of the low fidelity and processivity of TLS polymerases, the ensuing lesion bypass is often error-prone and preserves replication forks at the cost of mutagenesis [4,5]. Accumulating evidence has suggested that these low-fidelity TLS polymerases may contribute to spontaneous and DNA damage-induced mutations during DNA replication, thereby leading to genomic instability and increased cancer susceptibility [6–9]. Indeed, aberrant expression of TLS polymerases is frequently observed in a variety of cancers, including non-small cell lung cancer [10], breast cancer [11], colorectal cancer [12], esophageal cancer [13], and glioma [14,15]. More recently, several studies have reported that elevated expression of TLS polymerases confers resistance to multiple chemotherapeutic agents [15–18].

RAD18 is an integral protein that is involved in the TLS and is essential for the ubiquitination of PCNA, which results in the recruitment of TLS polymerases to the sites of DNA damage [19]. RAD18 has ubiquitin-ligase (E3) activity and interacts with the human homologs of the RAD6 protein (HHR6A and HHR6B), which participate in post-replication repair (PPR) and contribute to the maintenance of genomic stability [20,21]. It has been shown that inactivation of RAD18 in mouse embryonic stem cells or chicken DT40 cells resulted in hypersensitivity to various DNA-damaging agents and the presence of defective PRR [22–24]. Several molecular epidemiological studies evaluated the functional consequences

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http://dx.doi.org/10.1016/j.bbrc.2014.02.003
0006-291X/

of non-synonymous SNPs in RAD18, which have been associated with colorectal cancer and non-small cell lung cancer risk [25,26] (Kanzaki et al., 2007; Nakamura et al., 2009). More recently, Wong et al. reported that RAD18 was significantly overexpressed in primary and metastatic melanoma and was involved in the regulation of melanoma cell proliferation [27]. Furthermore, increased expression of RAD18 was observed in esophageal squamous cell carcinoma [13]. However, the relationship between RAD18 and GBM remains unknown.

In the present study, we investigated the relationship between RAD18 and resistance to IR and examined the expression levels of RAD18 in primary and recurrent GBM samples.

2. Materials and methods

2.1. Cell culture

A172, U87, U251 and T98 glioma cell lines were obtained from the American Type Culture Collection and were grown in DMEM supplemented with 10% FBS. Normal human astrocytes (NHAs) were purchased from Lonza (Walkersville, MD, USA) and maintained in astrocyte growth medium supplemented with rhEGF, insulin, ascorbic acid, GA-1000, L-glutamine and 5% FBS.

2.2. Human tissue samples

Forty primary GBM tissues were obtained postoperatively between 2004 and 2007 from the Department of Neurosurgery, The Fourth Affiliated Hospital of Harbin Medical University. Ten normal brain tissue (NBT) samples were obtained by collecting donations (with consent) from individuals who died of traumatic brain injury. Our study also included six randomly selected pairs of primary and recurrent GBM tumor samples (each pair was from the same patient) from our department, from 2007 to 2011. All six patients had received radiotherapy after surgery. The histological features of all of the specimens were confirmed by pathologists according to the WHO criteria. This study was reviewed and approved by the Ethical Committee of Harbin Medical University, and written informed consent was obtained from all patients.

2.3. Plasmids, Lentivirus production and transduction

The entire coding sequences of RAD18 were obtained from HUVEC mRNA by RT-PCR. RAD18 was purified and cloned into a pcDNA3.1 vector. Pre-made lentiviral RAD18 short hairpin RNA (shRNA) constructs and a shNC construct were purchased from Open Biosystems (Huntsville, AL). pLKO-shBRCA1 and lentiviral helper plasmids (pCMV-dR8.2 dvpr and pCMV-VSV-G) were obtained from Addgene (Cambridge, MA). Lentivirus stocks were prepared following the manufacturer's protocol. To select for cells that were stably expressing shRNA constructs, the cells were plated at sub-confluent densities and infected with 1 mL of virus-containing medium containing 10 μg/mL polybrene. Selection with 0.375–1 μg/mL of puromycin was started 48 h after infection. After 4 weeks of selection, monolayers of stably infected pooled clones were harvested for use and cryopreserved.

2.4. Colony formation assay

Stable shRAD18 and shCtrl cells (1×10^3) were cultured in 6-well plates and allowed to attach overnight. The cells were then treated with different doses (1, 2, 3 and 4 Gy) of γ-irradiation (Gammacell 1000 Elite; Best Theratronics, Ottawa, Ontario, Canada). The medium was replaced every 3 days, and the cells were allowed to grow for 10–14 days in regular culture medium with 300 μg/mL G418. The colonies were then fixed with ice-cold methanol and stained with crystal violet. The survival fraction was determined by dividing the number of colonies (more than 50 cells) formed in the radiation-treated group by the number of colonies formed in the untreated control group. Each dose was replicated in triplicate, and the experiments were repeated at least three times.

2.5. Quantitative real-time PCR

Total RNA was extracted from surgically resected tissues using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time PCR was performed using the Applied Biosystems 7900 Sequence Detection system. The following primers were used for the amplification: RAD18, forward primer 5′-TTCACAAAAGGAAGCCGCTG-3′ and reverse primer 5′-TTACTGAGGTCATATTATCTTC-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified in the same PCR as an internal control using primers: 5′-GAAGGTG AAGGTCGGAGTC-3′ (forward) and 5′-GAAGATGGTGATGGGA TTTC-3′ (reverse). The reaction conditions included a denaturation program (95 °C for 3 min) and an amplification and quantification program repeated 40 times (95 °C for 10 s and 55 °C for 45 s). Each sample was tested in triplicate. The expression of RAD18 was defined based on the threshold cycle (Ct), and relative expression levels were calculated as $2^{-[(\text{Ct of RAD18}) - (\text{Ct of GAPDH})]}$ after normalization with reference to GAPDH expression.

2.6. Western blotting

The supernatants were clarified by centrifugation and stored at −80 °C. Whole cell lysates were prepared by suspending the cell pellets in lysis buffer. Protein concentrations were determined using the Bradford method. The proteins were isolated with SDS–polyacrylamide gel, and immunoblotting was performed as previously described [28]. Antibodies against RAD18 (Sigma–Aldrich), cleaved caspase-3 (cell Signaling), γ-H2AX (Millipore, Bedford, MA, USA) and β-actin (Bethyl Laboratories, Montgomery, TX, USA) were used for Western blotting analysis.

2.7. Cellular apoptosis analysis

Apoptosis assays were performed as previously described [28]. In brief, cells were treated with different doses of IR. After treatment, both floating and attached cells were collected for analysis. Cells were washed with PBS and resuspended in PBS containing 25 mg/mL Annexin V and 50 mg/mL propidium iodide before being analyzed by flow cytometry (BD Pharmingen).

2.8. Immunohistochemistry assays

The immunohistochemistry assays were conducted on human primary and recurrent GBM tissues to detect and score RAD18 expression using methods as described previously [28].

2.9. Immunofluorescent staining

Cells grown on coverslips were fixed with 4% paraformaldehyde at room temperature for 15 min and then permeabilized with PBS containing 0.25% Triton X-100 for 10 min. The cells were blocked with 1% bovine serum albumin for 20 min before incubation with primary antibodies at room temperature for 1 h. After washing with PBS, the cells were incubated with Alexa Fluor-conjugated goat anti-mouse IgG secondary antibodies at room temperature for 1 h. After a final wash with PBS, the coverslips were mounted

with anti-fading mounting medium containing 6-diamidino-2-phenylindole (DAPI). The cells on coverslips were washed twice in PBS, incubated in cytoskeleton buffer (PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% Triton X-100) for 5 min on ice and then incubated in stripping buffer (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1% Tween 20, and 0.25% sodium deoxycholate) for 3 min on ice. The cells were washed three times in ice-cold PBS and were then fixed and processed as described above. The images were captured with a Nikon ECLIPSE E800 fluorescence microscope.

2.10. Homologous recombination DNA repair assay

A synthetic HR repair substrate DR-GFP system was developed and kindly provided by Dr. Huibo Wang (Nanjing Medical University, Nanjing, China). This DR-GFP HR repair substrate consists of two differentially mutated GFP genes oriented as direct repeats. The expression of I-SceI endonuclease results in a site-specific DSB within one of the mutated GFP genes, which, when repaired by gene conversion, will result in a functional GFP gene. We transfected the DR-GFP plasmid into U87 cells, and a positive clone integrated with a single copy of the reporter was identified. The GBM cells were then selected with hygromycin to generate the stable DR-GFP cell lines. To evaluate homologous recombination repair of the DSBs, DR-GFP cells were sequentially transfected with shRAD18 or shBRCA1 and the pCBA-SceI plasmid. Flow cytometric analysis was then performed to determine the percentages of the GFP-positive cells that resulted from HR repair induced by the DSBs.

2.11. Statistical analysis

All values are expressed as means ± SD. Statistical analyses were conducted using Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Expression of RAD18 in glioma cells correlates with their resistance to IR

Previous studies have suggested that the overexpression of TLS DNA polymerases confers resistance to DNA damaging agents and/or radiotherapy [15–18]. We speculate that RAD18 might play a part in acquired IR resistance in glioma cells. To test this hypothesis, we initially examined the expression of RAD18 in four glioma cell lines (A172, T98, U87 and U251) and in normal human astrocytes (NHAs) by Western blot analysis. As shown in Fig. 1A, we found that RAD18 expression was significantly higher in U87, U251 and T98 glioma cells than in A172 and NHA cells (Fig. 1A). Next, we overexpressed RAD18 in RAD18-low A172 cells. The Western blot analysis confirmed that the expression of RAD18 increased in A172 cells transfected with pcDNA3.1/RAD18 compared with those transfected with a control plasmid (Fig. 1B). The clonogenic analysis showed that the overexpression of RAD18 rendered A172 cells more resistant to IR treatment (Fig. 1C and Supplementary Fig. 1). For comparison, we infected two glioma cells (U87 and U251) with lentivirus-based shRNA to knockdown the expression of RAD18. Western blotting analysis showed that the expression of RAD18 was significantly inhibited (Fig. 1D). Depletion of RAD18 by shRNAs in two glioma cells sensitized their response to IR treatment as demonstrated by reduced colony-forming ability ($p < 0.01$, Fig. 1E and F). Taken together, these results suggest that overexpression of RAD18 confers resistance to IR treatment in glioma cells.

3.2. RAD18 overexpression inhibits IR-induced apoptosis

To determine the mechanisms by which RAD18 enhances the resistance of glioma cells to IR, we analyzed the effect of RAD18 overexpression on IR-induced apoptosis. A172 cells stably expressing RAD18 were exposed to 2 Gy IR for 15 min, and then apoptosis was determined by the Annexin V/PI flow cytometric assay after 48 h. The results showed that A172 cells with stable RAD18 expression exhibited a significantly reduced level of IR-induced apoptosis compared to the control cells, leading to a reduction from 67.51% to 10.63% ($p < 0.05$, Fig. 2A). These findings were further confirmed by subsequent changes in the expression levels of cleaved caspase-3. As shown in Fig. 2B, the expression of cleaved caspase-3 was increased in A172 cells, and significantly decreased after forced overexpression of RAD18 in response to IR. In contrast, the RAD18-depleted U87 and U251 glioma cells showed a marked increase in IR-induced apoptosis compared to the control cells ($p < 0.01$, Fig. 2C). An increase in the amount of cleaved caspase-3 was also consistently observed in RAD18-depleted U87 and U251 cells compared to the control cells in response to IR (Fig. 2D). These data suggest that RAD18 overexpression confers resistance to IR-mediated apoptosis in glioma cells.

3.3. Knockdown of RAD18 disrupts HR-mediated repair and results in increased accumulation of DSB

Recent studies have reported that RAD18 may participate in homologous recombination (HR)-mediated DSB repair in MEF cells [19,29]. Therefore, we tested whether RAD18 could potentially impact HR-mediated DSB repair in GBM cells. We measured HR activity using a GFP-based DSB repair system in human U87 GBM cells, which contain an integrated single-copy HR reporter with an I-SceI recognition site (U87 DR-GFP cells). The expression of RAD18 was knocked down in U87 DR-GFP cells by RNA interference. It is known that BRCA1 knockdown leads to decreased HR repair. Similar to the role of BRCA1 downregulation in HR-mediated DSB repair, we found that knocking down RAD18 expression dramatically impaired HR efficiency as demonstrated by the reduced numbers of GFP-positive U87 cells ($p < 0.01$, Fig. 3A), indicating that RAD18 is indeed involved in HR repair in glioma cells.

We next investigated if RAD18 depletion-induced hypersensitivity to IR was attributed to impaired DSB repair. We performed immunofluorescent staining of γ-H2AX, an early marker for DSB, to monitor the presence of DSB and the associated repairing activity. Depletion of RAD18 resulted in an increase in cells containing >10 γ-H2AX foci compared with control cells in the absence or presence of IR ($p < 0.01$, Fig. 3B). To confirm these results, Western blotting analysis was performed to compare the relative levels of γ-H2AX after IR. We consistently found that γ-H2AX levels were significantly increased in RAD18-depleted cells in response to 2 Gy of IR at the 24-h time point (Fig. 3C). Collectively, these results indicate that loss of RAD18 led to the disruption of HR-mediated repair and to the accumulation of DSB.

3.4. The association of RAD18 expression with IR resistance is clinically relevant

To determine whether the association of RAD18 expression with IR resistance is clinically relevant, we examined the expression levels of RAD18 in a cohort of 40 GBMs and 10 NBTs using qRT-PCR assays. Rad18 was found to be significantly upregulated in these GBM specimens compared with NBTs ($p < 0.01$, Fig. 4A). Furthermore, we examined the mRNA expression of RAD18 in tumor tissues from patients who received IR treatment. RAD18 was measured in six pairs of frozen GBM specimens, including

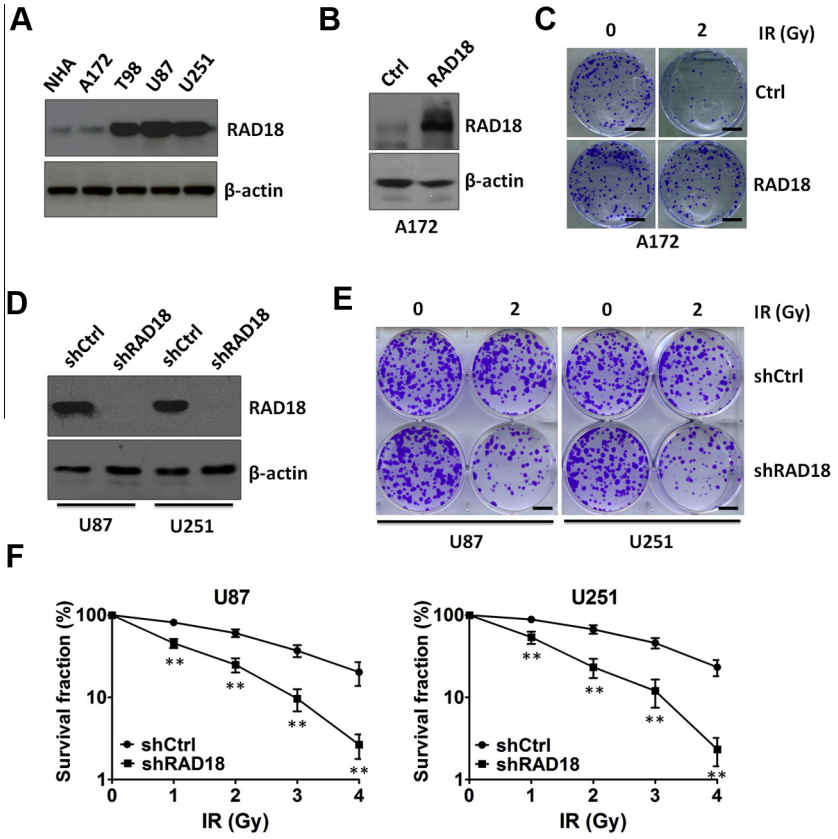

Fig. 1. Expression of RAD18 in glioma cells correlates with their resistance to IR. (A) Western blot analysis showed the expression of RAD18 in four glioma cell lines and NHAs. β-Actin served as a loading control. (B) Western blot analysis of RAD18 in A172 cells transfected with pcDNA3.1-RAD18 or vector control. (C) Representative images of A172 cells transfected with pcDNA3.1-RAD18 or vector control in the absence or presence of IR treatment (2 Gy). (D) Western blot analysis of RAD18 protein expression in U87 and U251 cells transfected with ctrl shRNA or RAD18 shRNA. β-Actin was used as the loading control. (E) Representative images of U87 and U251 cells transfected with shCtrl or shRAD18 in the absence or presence of IR treatment (2 Gy). (F) The colony-forming experiments were performed three times using triplicate samples, and average scores are indicated with error bars on the histogram. $^{**}p < 0.01$.

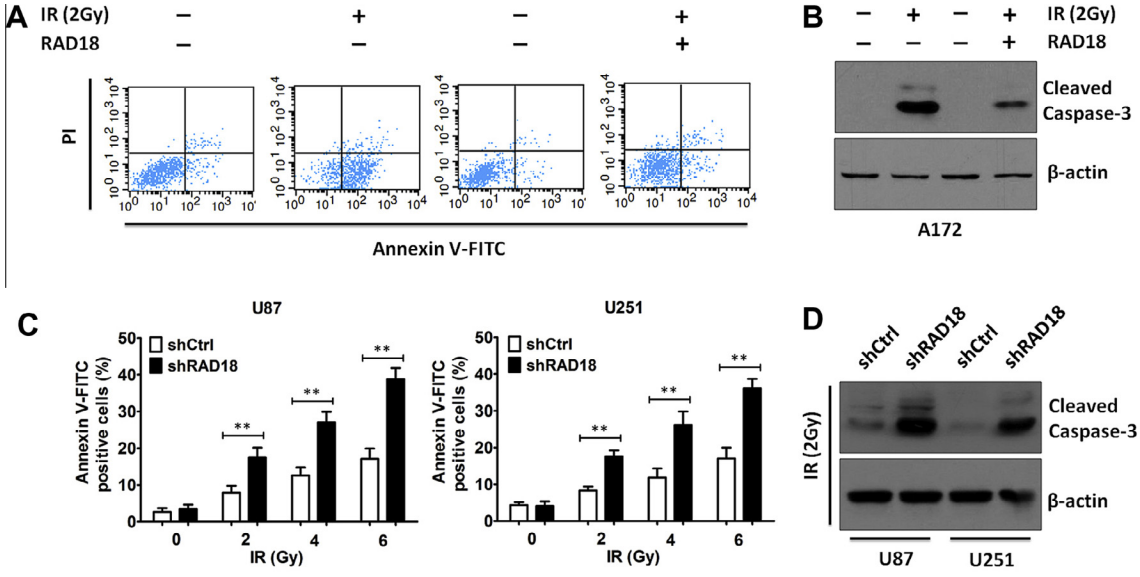

Fig. 2. RAD18 overexpression inhibits IR-induced apoptosis. (A) A172 cells stably transfected with pcDNA3.1-RAD18 or vector control were treated with IR (2 Gy) for 15 min, and then apoptosis was determined by Annexin V/PI flow cytometric assays after 48 h. (B) Western blot analysis of cleaved caspase-3 expression in A172 cells transfected with pcDNA3.1-RAD18 or vector control after exposure to IR (2 Gy). (C) Histograms summarizing three independent flow cytometry experiments of U87 and U251 cells transfected with shCtrl or shRAD18 after exposure to different doses of IR (2, 4 and 6 Gy). Data are the mean ± standard deviation. $^{**}p < 0.01$. (D) Western blot analysis of cleaved caspase-3 expression in U87 and U251 cells transfected with shCtrl or shRAD18 after exposure to IR (2 Gy).

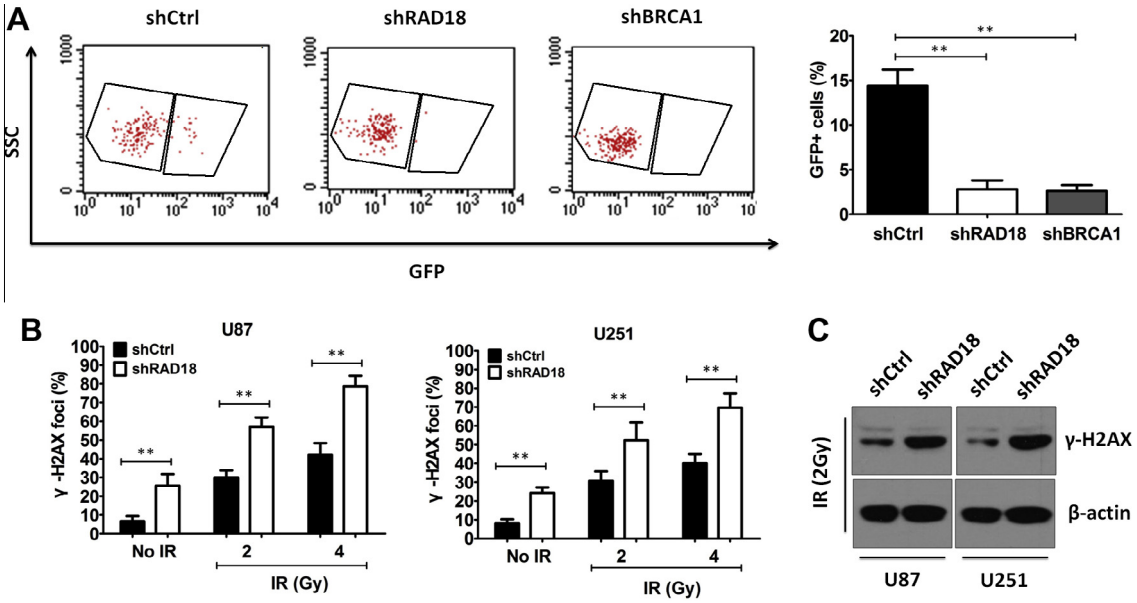

Fig. 3. Knockdown of RAD18 disrupts HR-mediated repair and results in increased accumulation of DSB. (A) U87 DR-GFP cells were sequentially transfected with shCtrl, shRAD18 or shBRCA1 and the pCBASceI plasmid. The GFP-positive cells were analyzed at 72 h by flow cytometry. Representative images of the FACS profiles are shown. (Left) Quantification of the FACS profiles is shown. (Right) $^{**}p < 0.01$. (B) The quantification of γ-H2AX foci was analyzed in U87 and U251 cells transfected with shCtrl or shRAD18 in the absence or presence of IR. The foci numbers of at least 10 cells were counted. The data are the means of three independent experiments ± SEM. $^{**}p < 0.01$. (C) Western blot analysis of γ-H2AX protein expression in U87 and U251 cells transfected with shCtrl or shRAD18 in response to IR (2 Gy). β-Actin was used as the loading control.

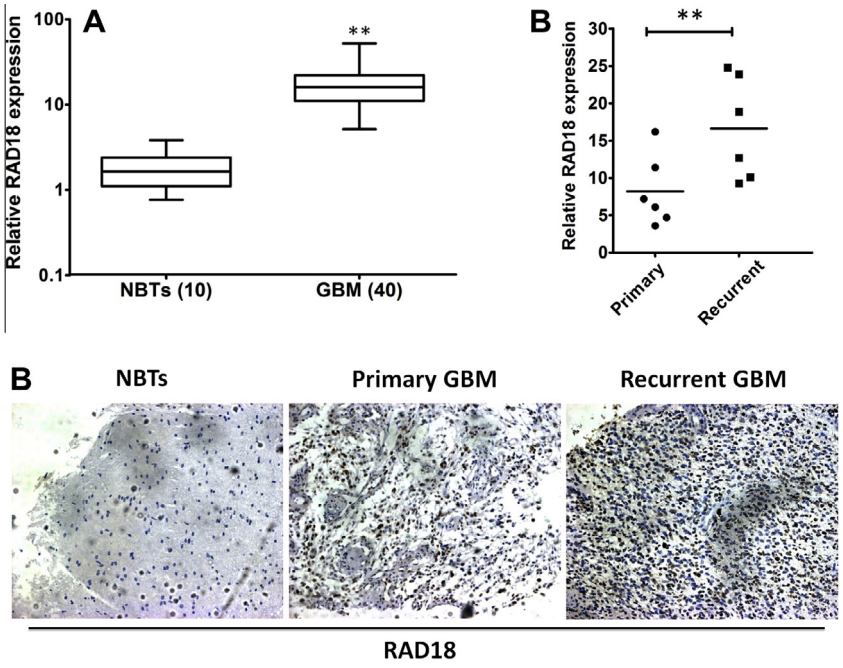

Fig. 4. The association of RAD18 expression with IR resistance is clinically relevant. (A) Quantitation of RAD18 expression in 40 glioblastoma multiforme (GBM) samples and 10 normal brain tissues (NBTs). GAPDH served as the loading control. Alteration of expression is shown as box plot presentations. $^{**}p < 0.01$. (B) Representative images of immunohistochemical analysis of RAD18 protein expression in NBTs and primary and recurrent GBM. (C) The relative mRNA expression level of RAD18 was determined by qRT-PCR in six paired samples of primary and recurrent GBM.

primary tumors and the corresponding recurrent tumors. The characteristics of the patients were shown in Supplementary Fig. 1. Histological analysis and qRT-PCR data showed that RAD18 expression was dramatically elevated in the recurrent tumors compared to the primary tumors after IR treatment ($p < 0.01$, Fig. 4B and C), indicating that the glioma cells with high expression of RAD18 were more resistant to the IR and more susceptible to death, whereas the glioma cells with low RAD18 levels were more sensitive to IR. Together, these results suggest that RAD18 is of clinical significance as a mediator of IR resistance.

4. Discussion

IR is a key component of standard therapy for GBM patients. However, acquired resistance limits the therapeutic efficacy of IR

[2]. Therefore, the identification of targets responsible for IR resistance is critical for successful GBM treatment. In this study, we show, for the first time, that RAD18 functions as a novel mediator of IR resistance in glioma cells.

The activation of TLS DNA polymerases confers resistance to various chemotherapeutic agents and radiotherapy [15–18]. However, whether RAD18 can modulate resistance to IR in gliomas is unknown. To explore the relationship between dysregulation of RAD18 and IR resistance in glioma cells, we first examined the level of RAD18 expression in several glioma cell lines and their responses to IR. Our data showed that the levels of RAD18 in glioma cells correlate with their resistance to IR. We have also shown that ectopic expression of RAD18 conferred the ability to acquire IR resistance. Conversely, depletion of endogenous RAD18 sensitized glioma cells to IR. In addition, we further explored the possible mechanisms involved in the RAD18-mediated resistance to IR by assessing the effect of RAD18 overexpression or knockdown on IR-induced apoptosis. Our data indicated that overexpression of RAD18 in RAD18-low A172 cells significantly inhibited IR-induced apoptosis, whereas depletion of RAD18 in RAD18-high glioma cells resulted in a marked increase in IR-induced apoptosis, indicating that RAD18 may regulate IR resistance by regulating the effects of IR on apoptosis in glioma cells. Furthermore, we showed that RAD18 is involved in HR-mediated repair and that knockdown of RAD18 led to the disruption of HR-mediated repair and to the accumulation of IR-induced genomic damage. In addition, we examined the expression levels of RAD18 in human GBM specimens and found that RAD18 was significantly upregulated in primary GBM tissues. More importantly, we found that elevated expression of RAD18 is associated with recurrent GBM patients who underwent IR therapy. To the best of our knowledge, the present study provides the first evidence that RAD18 may function as an important regulator during the process of IR-induced DNA damage and may play a critical role in GBM radioresistance. Thus, additional in vivo studies to evaluate the potential of RAD18 for use as a therapeutic target are warranted.

In summary, our findings reveal that RAD18 may serve as an important mediator of the response of glioma cells to IR. RAD18 may provide a novel therapeutic target for therapeutic intervention of gliomas, especially recurrent radioresistant tumors.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

This work was supported by a Grant from the National Natural Science Foundation of China (No. 81072079 to W. Yue).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.003.

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