Oncology Research, Vol. 26, pp. 1365–1373
Printed in the USA.

0965-0407/18 $90.00 + .00
DOI: https://doi.org/10.3727/096504018X15154085345907
E-ISSN 1555-3906
www.cognizantcommunication.com

Silencing of E3 Ubiquitin Ligase RNF8 Enhances Ionizing Radiation Sensitivity of Medulloblastoma Cells by Promoting the Deubiquitination of PCNA

Fei Li,*[1] Bin Liu,†[1] Xiaolan Zhou,* and Quan Xu‡

*College of Nursing, Xi'an Medical University, Xi'an, Shaanxi, P.R. China
†Department of Plastic Surgery, Xi'an Central Hospital, Xi'an, Shaanxi, P.R. China
‡Department of Pediatric Surgery, Northwest Women and Children's Hospital, Xi'an, Shaanxi, P.R. China

DNA damage response induced by ionizing radiation (IR) is an important event involved in the sensitivity and efficiency of radiotherapy in human medulloblastoma. RNF8 is an E3 ubiquitin ligase and has key roles in the process of DNA damage and repair. Our study aimed to evaluate the effect of RNF8 in the DNA damage repair induced by IR exposure in medulloblastoma cells. We found that the levels of RNF8 were significantly upregulated by γ-ray irradiation in a dose-dependent manner in medulloblastoma cells and colocalized with γ-H2AX, a sensitive marker of DNA double-strand breaks induced by γ-ray radiation. RNF8 knockdown was observed to enhance the sensitivity of IR in medulloblastoma cells, as evaluated by reduced cell survival. The apoptosis and cell cycle arrest of medulloblastoma cells were dramatically increased by RNF8 suppression after IR treatment. Furthermore, RNF8 inhibition did not affect the protein levels of BRCA1, a crucial protein involved in IR-induced DNA damage repair, but significantly decreased the recruitment of BRCA1 and increased the level of γ-H2AX at DNA damage sites compared to the control. A significant increase in OTM was observed in medulloblastoma cells treated by RNF8 shRNA after exposure to IR, indicating the effect of RNF8 on DNA damage and repair. Additionally, PCNA, a major target for ubiquitin modification during DNA damage response, was found to be monoubiquitinated by E3 ligase RNF8 and might contribute to the low radiosensitivity in medulloblastoma cells. Altogether, our findings may provide RNF8 as a novel target for the improvement of radiotherapy in medulloblastoma.

Key words: RNF8; Ionizing radiation (IR); Sensitivity; PCNA ubiquitination; DNA damage; Medulloblastoma

INTRODUCTION

Medulloblastoma is a malignant brain tumor and commonly occurs in children, which remains one of the most challenging neuroepithelial tumors in central nervous system malignancy, accounting for about 25% of all pediatric brain tumors[1]. Currently, the standard treatment strategies for medulloblastoma patients mainly include surgical resection and postoperative chemotherapy or radiotherapy[2]. Although these therapeutic approaches evolve and greatly benefit for clinical practice, no significant improvement in the prognosis of these patients has been recorded in the last two decades[3]. Therefore, identification of genes and biological pathways contributing to therapeutic inefficiency will greatly benefit for improving the prognosis of medulloblastoma.

The human hereditary substance DNA is persistently threatened by exogenous detrimental factors such as chemicals, mechanical stress, ionizing radiation (IR), and ultraviolet, as well as endogenous damaging factors including metabolic products and reactive oxygen species (ROS). DNA double-strand breaks (DSBs) are one of the most harmful DNA damages because unrepaired or misrepaired DSBs can lead to genome rearrangement, apoptosis, tumorigenesis, and immune deficiency. Eukaryotic cells maintain genome integrity by the DNA damage response (DDR) network, which initiates a series of signal transduction cascades to lead to subsequent DNA repair, apoptosis, and chromatin remodeling[4]. Upon activation of DSBs, the DDR initiates DNA damage repair by regulating the cell cycle or apoptosis of stimulated cells, which may cause limited sensitivity of medulloblastoma cells to radiotherapy.

Ring finger protein 8 (RNF8) is a E3 ubiquitin (Ub) ligase with RING finger and has key roles in the process

[1]These authors provided equal contribution to this work.
Address correspondence to Xiaolan Zhou, College of Nursing, Xi'an Medical University, 1 Xinwang Road, Xi'an, Shaanxi, 710021, P.R. China. Tel: +86-2986168967; E-mail: xiaolanzxian@163.com

of DNA damage and repair. RNF8 could promote the recruitment of several proteins, such as tumor protein p53-binding protein 1 (53BP1), Rad51, and breast cancer 1, early type (BRCA1), at the sites of DNA damage by regulating the ubiquitination of histone H2A and histone H2A family, member X (H2AX) to facilitate DNA repair. Previous studies reported that aberrantly expressed RNF8 may disrupt the transduction of DDR and the DNA damage repair[5,6]. However, the exact mechanism by which RNF8 affects the process of DDR during IR exposure is not completely known.

Proliferating cell nuclear antigen (PCNA) is a structurally and functionally conserved molecular coordinator in the core DNA synthesis machinery, which forms a homotrimeric ring encircling the DNA double helix and acts as a molecular platform to facilitate the protein–protein and protein–DNA interactions occurring at the replication fork[7,8]. Growing evidence has shown that PCNA is a major target for Ub modification during the DDR signaling pathways[9]. However, the role of PCNA ubiquitination in the repair of DNA damage in medulloblastoma cells remains unclear.

In the present study, we aimed to investigate the expression of RNF8 in human medulloblastoma cells treated by IR. Furthermore, we determined whether and how RNF8 affects the sensitivity of IR and DNA repair response in medulloblastoma cells. We also elucidated the interaction between RNF8 and PCNA ubiquitination to further clarify the potential role of RNF8 in the radiotherapy of medulloblastoma.

MATERIALS AND METHODS

Cell Culture and Treatment

Human medulloblastoma cell lines (BIU87, 5637, and D283 Med) and a urothelial cell line (SV-HUC-1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen), and maintained at 37°C in a humidified incubator of 5% CO_2. Cells were washed three times with phosphate-buffered saline (PBS) prior to radiation with γ-rays at 0, 2, 4, and 8 Gy. After radiation, cells were incubated in complete growth medium at 37°C. Then DNA DSBs were detected by neutral comet assay at 0.5, 1, and 1.5 h after γ-ray exposure.

The Transfection

The RNF8 short hairpin RNA (shRNA) or its negative control shRNA was, respectively, transfected into the D283 Med cells using Lipofectamine 2000 (Invitrogen) as described by the manufacturer's instructions. The short interfering RNA (siRNA) primer sequences were designed by Invitrogen Block-iT RNAi Designer (appendix). RNF8 shRNA_414: 5′-GGA CAA UUA UGG ACA ACA A dTdT-3′(F), 5′- UUG UUG UCC AUA AUU GUC C dTdT-3′(R). The pcDNA3.1/Ub-specific peptidase 7 (USP7) plasmid as well as control plasmid established by our colleague Dr. Fei Li (College of Nursing, Xi'an Medical University, Xi'an, P.R. China) were also transfected into the D283 Med cells using Lipofectamine 2000.

Neutral Comet Assay

The neutral comet assay was used to measure DNA DSB and performed as previously described[10]. Briefly, cells were placed in 0.25% trypsin digestion and then prepared for 5×10^4/ml single-cell suspension. The suspension (0.5 ml) was mixed with 1.5 ml of 1% low-melting-point agarose, which was quickly placed on precooling glass slides. The slides were dissociated for 3 h at room temperature and then were dipped into washing buffer overnight at 4°C. The treated slides were set for electrophoresis for 25 min at 0.55–0.66 V/cm. After electrophoresis, the gel was stained with 2.5 μg/ml propidium iodide (PI) for 30 min and then had comet image analysis within 48 h using CASP software (Wroclaw University, Poland). The degree of DNA damage was expressed as Olive tail moment (OTM), which was defined as the product of average distance of DNA migration and DNA content of the comet tail. The "comet" image of each cell was analyzed and recorded independently.

Western Blot

Total proteins or nuclear proteins were extracted using the Tissue or Cell Protein Extraction Kit (Amresco, USA) from cells. The CelLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA) was employed for extraction of nuclear proteins. The primary antibodies were purchased from Abcam (Cambridge, UK), except anti-PCNA and anti-ubiquityl-PCNA antibodies (CST, MA, USA). The proteins were separated by SDS-PAGE followed by electrotransfer to nitrocellulose (NC) membrane, and then the membranes were probed using antibodies against γ-H2AX (phospho S139) (1:1,000), β-actin (1:5,000), RNF8 (1:1,000), ubiquityl-PCNA (Lys164) (1:1,000), PCNA (1:2,000), followed by a horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam). Bands were revealed with an enhanced chemiluminescence (ECL) reagent (Millipore, Boston, MA, USA) and recorded on X-ray films (Kodak, P.R. China). The densitometry of each band was quantified by a gel imaging system and Quantity One 4.62 software (Bio-Rad, Hercules, CA, USA).

RT-PCR

Total RNA was extracted using TRIzol reagents (Invitrogen) from D283 Med cells. Isolated RNA was electrophoresed on 1% agarose gel to examine the purity of total RNA. The first-strand cDNA was synthesized using 1.0 μg of total RNA and SuperScript® III Reverse Transcriptase (Invitrogen). PCR amplification was performed using a PCR amplification kit (Takara Biotechnology, Dalian, P.R. China). The specific primers were designed using the Primer Premier 6.0 software and synthesized by Sangon Biotech (Shanghai, P.R. China). The PCR production was electrophoresed on 1% agarose gel and visualized by the Gel Imaging System of Bio-Rad Corp.. Each band was analyzed by Quantity One 4.62 software (Bio-Rad).

Immunofluorescence Staining Assay

Cells were cultured on coverslips and permeabilized with PBS for 5 min. Then cells were incubated with primary antibodies [rabbit anti-RNF8 (1:200; Novus Biologicals), mouse anti-γ-H2AX (1:250; Millipore), rabbit anti-BRCA1 (1:200; Santa Cruz), and human centromere antibody HCT-0100 (1:100; Immunovision)] and secondary antibodies conjugated with Alexa Fluor 647 (Invitrogen) for immunofluorescence staining. Slides were measured using an Olympus BX-61 fluorescence microscope.

Cell Proliferation Assay

Transfected cells were cultured in 96-well plates (5×10^3 cells/ml) and treated with (2, 4, and 8 Gy) γ-ray irradiation for 1 h or (4 Gy) γ-ray irradiation for 0.5, 1, and 1.5 h. Then 10 μl of MTT solution was added to the cells for 4 h at 37°C, and the absorbance at 450 nm was tested by an ELISA plate reader (BioTek, Winooski, VT, USA).

Clone Formation Assay

Transfected cells were maintained in 24-well plates for 24 h and treated with (2, 4, and 8 Gy) γ-ray irradiation for 1 h. Then the clone formation assay was performed as described in a previous report[11].

Cell Apoptosis Assay

Transfected cells were maintained in serum-free DMEM for 12 h and harvested with PBS. Cells were then resuspended in binding buffer (5×10^6 cells) and incubated with PI and annexin V-fluorescein isothiocyanate (FITC) for 15 min. The FACSCalibur™ system (Becton Dickinson) was used to analyze the apoptosis.

Cell Cycle Analysis

Cell cycle progression was monitored by DAPI staining and analyzed by flow cytometry. In brief, D283 Med cells were harvested, washed twice with Ca^{2+}–Mg^{2+}-free PBS, and fixed in 95% ethanol. After being centrifuged at 2,200 rpm for 10 min, 2×10^5 of the cells were resuspended in 0.1% Triton X-100 solution supplemented with 300 nM DAPI. The cells were incubated for 30 min at 4°C and then were analyzed using fluorescence-activated cell sorting (FACS). The DNA profiles were analyzed using Multicycle AV software for cell cycle determination.

Statistical Analysis

Data are expressed as means ± standard deviation (SD) for at least three replicates per group. Data were analyzed by SPSS19.0 software (IBM, USA). Statistical differences between means were calculated using ANOVA, followed by LSD multiple comparison tests. Differences were considered significant at $p<0.05$.

RESULTS

RNF8 Is Upregulated in Medulloblastoma Cells

To determine the role of RNF8 in the DNA damage repair induced by IR exposure, we first performed expression assays to detect the expression of RNF8 in medulloblastoma cells. As shown in Figure 1A and B, the mRNA and protein levels of RNF8 were significantly upregulated in medulloblastoma BIU87, 5637, and D283 Med cells compared to the control urothelial cell SV-HUC-1. Besides, RNF8 was also observed to be highly expressed in the cell nucleus (Fig. 1B). Then we conducted the immunofluorescence staining assay to evaluate the localization in medulloblastoma cells treated by IR exposure. D283 Med cells were used in the follow-up experiments. After IR, RNF8 was clearly detected in medulloblastoma cells and colocalized with γ-H2AX, a sensitive marker of DSBs induced by γ-ray radiation[11], as evaluated by staining with anti-RNF8 and anti-γ-H2AX antibodies (Fig. 1C). Further, we found that the expression of RNF8 was significantly increased by γ-ray irradiation in a dose-dependent manner in medulloblastoma cells (Fig. 1D), indicating the involvement of RNF8 in DNA damage repair after radiotherapy.

RNF8 Silencing Enhances Sensitivity of IR in Cells

We then employed the specific shRNA of RNF8 to determine its effect on the sensitivity of IR in medulloblastoma cells. As seen in Figure 2A, the expression of RNF8 was obviously decreased by shRNA in D283 Med cells, suggesting the efficiency of RNF8 shRNA. Then the MTT assay and colony formation assay were performed to evaluate the change in sensitivity to IR in medulloblastoma cells. Results showed that the proliferation capacity of medulloblastoma cells was significantly decreased after RNF8 shRNA treatment, while there was

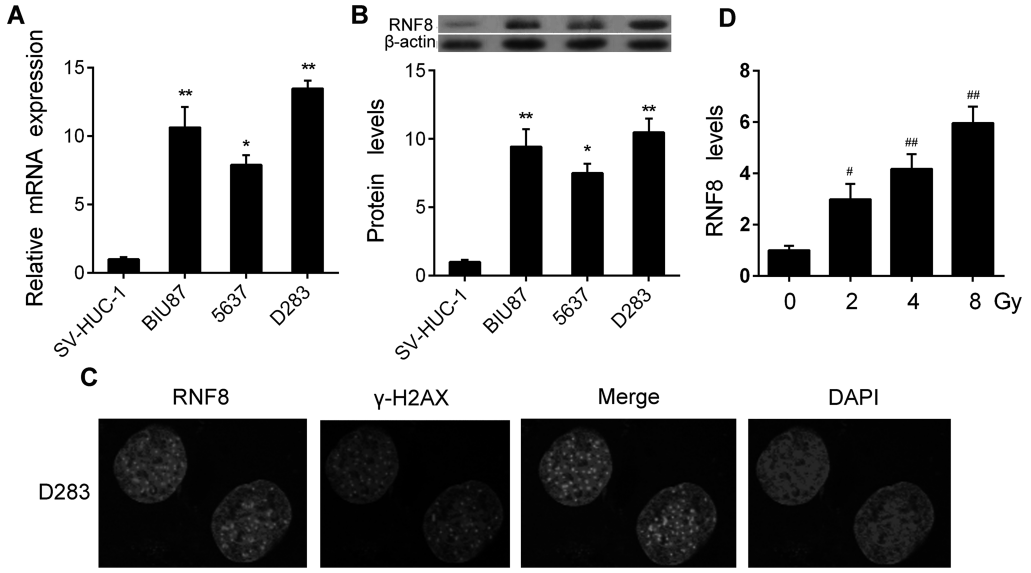

Figure 1. Ring finger protein 8 (RNF8) is upregulated in medulloblastoma cells. (A) RT-PCR and (B) Western blot were performed to detect the expression of RNF8 in cultured medulloblastoma BIU87, 5637, and D283 Med cells as well as in urothelial SV-HUC-1 cells. (C) D283 Med cells were treated with γ-ray irradiation (4 Gy) for 1 h and immunofluorescence stained with anti-RNF8 and anti-γ-H2AX antibodies. The nucleus was counterstained with DAPI. Scale bar: 5 μm. (D) The expression change in RNF8 was detected in cultured medulloblastoma D283 Med cells within the increase in γ-ray doses. *$p<0.05$, **$p<0.05$ versus SV-HUC-1; #$p<0.05$, ##$p<0.05$ versus 0 Gy.

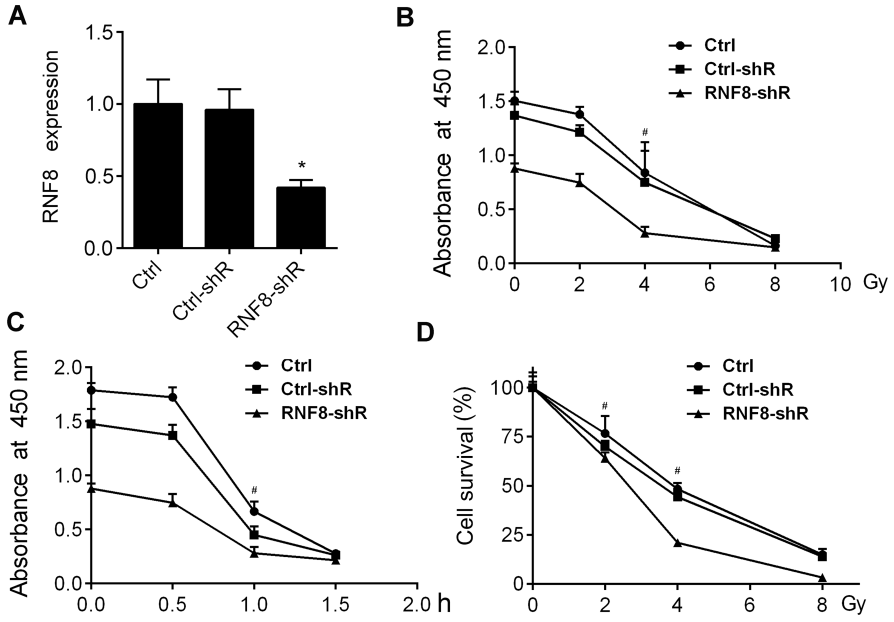

Figure 2. RNF8 silencing enhances sensitivity of ionizing radiation (IR) in cells. (A) D283 Med cells were cultured and treated with RNF8 short hairpin RNA (shRNA), negative shRNA, or not (Ctrl) for 48 h, and the expression of RNF8 was measured by RT-PCR. MTT assay was performed to detect the proliferation of D283 Med cells treated with (B) (2, 4, and 8 Gy) γ-ray irradiation for 1 h or (C) (4 Gy) γ-ray irradiation for 0.5, 1, and 1.5 h. (D) Colony formation assay was performed in cells with increasing γ-ray doses. *$p<0.05$ versus Ctrl; #$p<0.05$ versus 0 Gy or 0 h.

no obvious alteration between untreated and negative shRNA-treated cells after exposure to several doses of IR or at different times (Fig. 2B and C). A similar change was observed in the survival of medulloblastoma cells as determined by the colony formation assay (Fig. 2D). Taken together, these results suggest that RNF8 knockdown enhances the sensitivity of medulloblastoma cells to ionizing irradiation.

RNF8 Silencing Enhances Apoptosis and Cell Cycle Arrest

We further investigated the effect of RNF8 on apoptosis and cell cycle arrest in medulloblastoma cells after exposure to IR. Compared to the control, expression of RNF8 was significantly suppressed by shRNA in D283 Med cells, as shown in Figure 2A. There was a low level of apoptosis in cultured D283 Med cells, and the apoptosis capacity of medulloblastoma cells was dramatically increased by RNF8 suppression after IR treatment (Fig. 3A). The data from caspase 3, a key apoptosis factor, were similar to those for cell apoptosis. Inhibition of endogenous RNF8 dramatically upregulated the levels of caspase 3 in D283 Med cells (Fig. 3B). Additionally, the enhanced cell cycle arrest was observed in D283 Med cells treated by RNF8 shRNA after exposure to IR compared to the control cells (Fig. 3C). These data determine that the elevated RNF8 levels may have a negative effect on the sensitivity of IR in medulloblastoma cells by regulating cell cycle progression and inhibiting the apoptosis.

RNF8 Is Associated With DNA Repair Response

To determine the role of RNF8 in the DNA repair response after exposure to IR in medulloblastoma cells, we further investigated the effect of RNF8 on the DDR as well as DNA repair. BRCA1 is a crucial protein involved in the IR-induced DNA damage repair being recruited at the site of DNA damage and facilitating the transduction of the DDR[5]. γ-H2AX activity has been reported as a sensitive marker of DSBs induced by γ-ray radiation[12]. We found that the protein levels of BRCA1 were not affected by RNF8 inhibition in medulloblastoma cells after exposure to IR (Fig. 4A). On the contrary, RNF8 inhibition significantly decreased the recruitment of BRCA1 and increased the levels of γ-H2AX at DNA damage sites compared to the control, as shown in Figure 4B. Furthermore, we performed neutral comet assay to detect the change in DNA damage repair in medulloblastoma cells. As seen in Figure 4C, a significant increase in OTM, a DNA damage parameter, was observed in medulloblastoma cells treated by RNF8 shRNA after exposure to IR, indicating

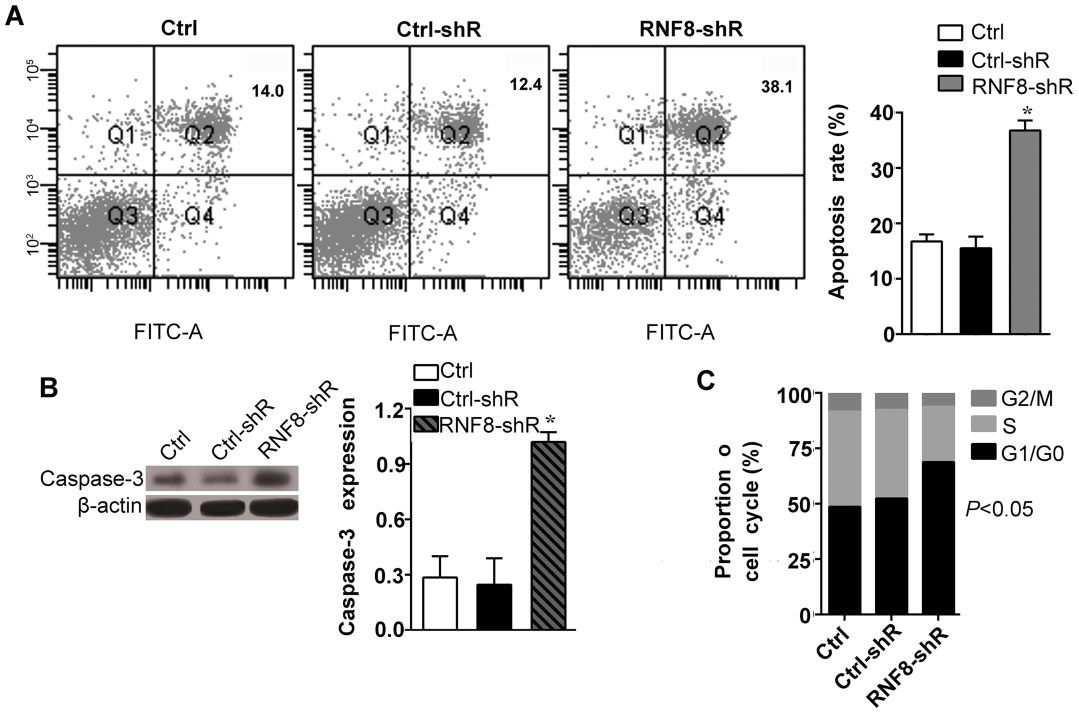

Figure 3. RNF8 silencing enhances apoptosis and cell cycle arrest. D283 Med cells were cultured and treated with RNF8 shRNA, negative shRNA, or not (Ctrl) for 48 h, and were exposed to 4 Gy γ-ray irradiation for 1 h. (A) The apoptosis of D283 Med cells was measured and quantified. (B) Western blots were performed to detect the expression of caspase 3 in D283 Med cells. (C) The cell cycle of the D283 Med cells was detected by flow cytometric analysis. *$p<0.05$ versus Ctrl.

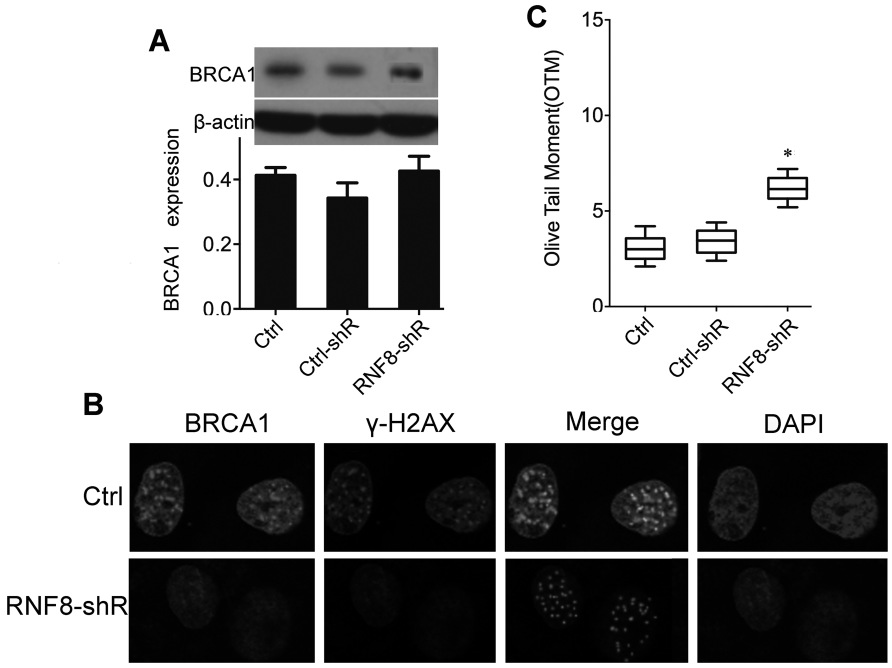

Figure 4. RNF8 is associated with the DNA repair response. D283 Med cells were cultured and treated with RNF8 shRNA, negative shRNA, or not (Ctrl) for 48 h and were exposed to 4 Gy γ-ray irradiation for 1 h. (A) Western blots were performed to detect the expression of BRCA1 in D283 Med cells. (B) Cells were immunofluorescently stained with anti-BRCA1 and anti-γ-H2AX antibodies. The nucleus was counterstained with DAPI. Scale bar: 5 μm. (C) Neutral comet assay analysis was performed to detect the change of DNA damage repair in medulloblastoma cells. $*p < 0.05$ versus Ctrl.

the enhanced levels of DNA damage and generation of DSBs. These results imply that RNF8 has a complicated regulatory role in the DNA repair response by inhibiting the DNA damage and promoting the DNA repair.

PCNA Is Monoubiquitinated by E3 Ligase RNF8 in IR-Induced Medulloblastoma Cells

PCNA plays a critical role in DNA synthesis and DSB repair at the replication fork. It has been shown that PCNA is a major target for Ub modification during DDR signaling pathways[13,14]. We then intended to evaluate whether PCNA ubiquitination was involved in RNF8-mediated DDR. After irradiation, the level of monoubiquitinated PCNA (Lys164) was upregulated following incremental time of post-IR exposure, especially at 6 h post-IR exposure, but PCNA protein expression had no substantial change compared with the control (Fig. 5A and B). Next, we transfected pcDNA3.1/USP7, an inhibitor of PCNA ubiquitination (Lys164), into medulloblastoma cells to suppress PCNA ubiquitination (Lys164). USP7 overexpression is considered as an indirect and exogenous PCNA-Ub inhibitor[15]. As seen in Figure 5C, OTM was dramatically elevated in those cells in which PCNA-Ub was inhibited, indicating that PCNA ubiquitination may be involved in blocking the DDR.

We further examined whether the E3 ligase RNF8 was responsible for the Ub modification of PCNA. We found that the expression of RNF8 was elevated following γ-ray irradiation in a dose-dependent manner in medulloblastoma cells (Fig. 1D). Knockdown of RNF8 significantly repressed the level of PCNA ubiquitination with no statistical influence on the expression of PCNA protein (Fig. 5D and E). Based on these results, we concluded that PCNA is monoubiquitinated by E3 ligase RNF8 after DNA damage induced by IR exposure, which might contribute to the low radiosensitivity of medulloblastoma cells.

DISCUSSION

In this study, we identified E3 ligase RNF8 as a crucial participator and regulator of the DDR during IR exposure in human medulloblastoma cells. Our findings suggested that RNF8 facilitates the recruitment of DNA damage repair-associated proteins at the site of DNA damage by regulating the ubiquitination of PCNA, and RNF8 silencing could enhance the IR sensitivity of medulloblastoma cells.

Radiotherapy is a common strategy for medulloblastoma treatment, but the therapeutic effect is limited. IR-induced DNA damage can be repaired by cells via the homologous recombination (HR) and nonhomologous end-joining (NHEJ) pathways, resulting in the reduction of cell damage and IR sensitivity[15]. DNA damage can cause base mutation or mismatch, intrastrand

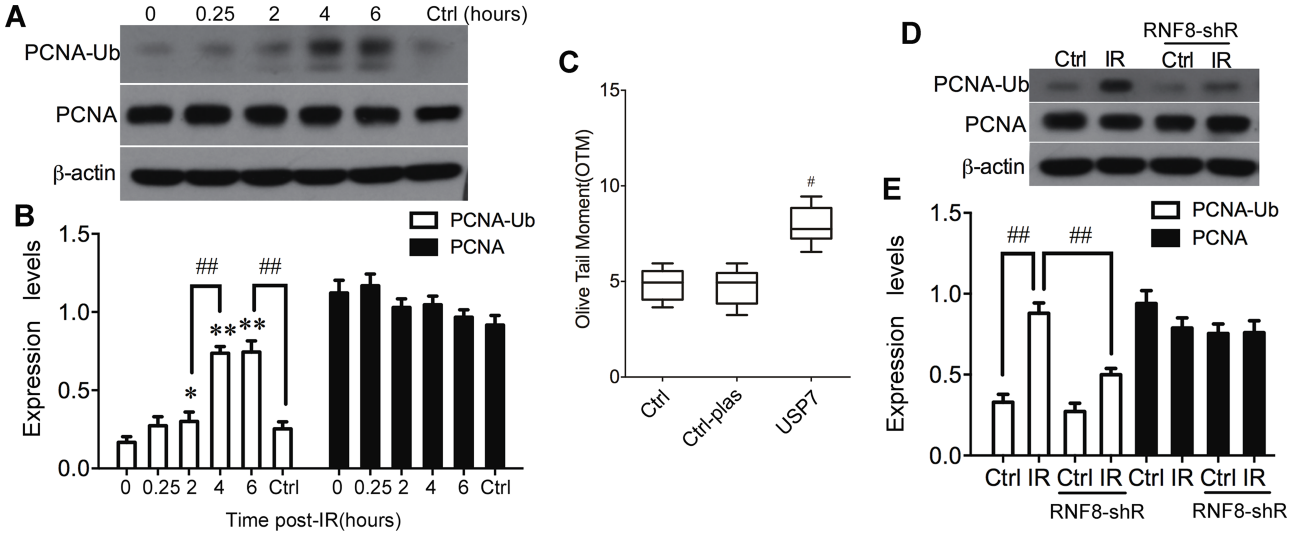

Figure 5. Proliferating cell nuclear antigen (PCNA) is monoubiquitinated by E3 ligase RNF8 in IR-treated medulloblastoma cells. (A) Expression and (B) quantification of PCNA and PCNA-ubiquitin (Ub) were evaluated in D283 Med cells post-IR exposure. (C) The pcDNA3.1/USP7 as well as a control plasmid (Ctrl-plas) were transfected into D283 Med cells for 48 h or not (Ctrl) and were exposed to 4 Gy γ-ray irradiation for 1 h. Then neutral comet assay analysis was performed. D283 Med cells were cultured and treated with RNF8 shRNA, negative shRNA, or not (Ctrl) for 48 h and were exposed to 4 Gy γ-ray irradiation for 1 h. Then the (D) expression and (E) quantification of PCNA and PCNA-Ub were detected in cells. $*p<0.05$, $**p<0.01$ versus 0 h; $\#p<0.05$, $\#\#p<0.01$ versus Ctrl.

or interstand cross-linking, DNA single-strand break or DSB, and multiple other forms of DNA damage[16]. DNA damage is sensed by the DDR, which can transmit signaling to a series of highly ordered responses to protect cells from injuries[17]. The γ-ray radiation induces mainly DSBs in genomic DNA. It has been reported that DSBs arise through the direct action of IR, and γ-ray radiation can induce DNA damage, chromosomal aberrations, and mutations in human blood cells, the pale grass blue butterfly, and fish larvae[18–20]. The DDR induced by IR is regulated by various key factors. RNF8 is an E3 Ub ligase and plays a role in the process of DNA damage repair by promoting the recruitment of regulatory proteins at the sites of DNA damage. The aberrant expression of RNF8 is found in a variety of diseases, including cancers[6,21]. Consistently, our data showed that RNF8 is upregulated in medulloblastoma cells and can be significantly increased by γ-ray irradiation in a dose-dependent manner.

As the pivotal function of RNF8 in DDR, we hypothesized that the high level of RNF8 may be associated with the limited sensitivity of medulloblastomas to radiotherapy[22]. By specific shRNA technique, we found that RNF8 silencing actually could inhibit the survival and affect the apoptosis and cell cycle arrest of medulloblastoma cells after IR exposure, contributing to the enhancement of sensitivity to IR in medulloblastoma cells. The role of RNF8 might be correlated with the process of DNA damage repair. Once DSBs occur, sensor proteins such as γ-H2AX immediately recognize DSB formation and move close to the DSB site (H2AX-containing nucleosomes)[23]. Moreover, phosphorylated H2AX recruits mediator of DNA damage checkpoint 1 (MDC1) and initiates a series of biological responses to modify the chromatin flanking[23]. We found that RNF8 inhibition significantly increased the recruitment of γ-H2AX at DNA damage sites in medulloblastoma cells after exposure to IR. The recruitment of BRCA1, another protein that is involved in IR-induced DNA repair[24], was observed to be decreased by RNF8 silencing.

Regarding differences in the DNA repair pathway, regulation of PCNA ubiquitination also plays a crucial role in the DDR induced by IR. PCNA, an indispensable component of the eukaryotic DNA replication machinery, controls several metabolic pathways, including DNA repair, DNA synthesis, chromatin remodeling, apoptosis, and cell cycle regulation through its interaction with various partners[25]. Posttranslational monoubiquitination of PCNA is an important event in the regulation of DNA damage[26]. The previous study reported that RNF8 depletion suppressed both UV- and MNNG-stimulated monoubiquitination of PCNA, indicating that the RNF8-dependent pathway for PCNA ubiquitination may play a role in DDR[27]. Similarly, we found that the expression of RNF8 was upregulated after IR exposure, and RNF8 knockdown inhibited IR-stimulated PCNA ubiquitination. Once ubiquitinated, PCNA is recognized by RNF168 that initiates Ku80 ubiquitination. The Ku70/Ku80 heterodimer is the central component of the NHEJ

pathway of DSB repair[28]. Ku forms a ring through the DSB threads, which are then topologically attached to DNA during repair[29,30]. Additionally, several studies have reported that PCNA modulates the cell cycle and DNA replication in response to DNA damage[31]. In the present study, we found that PCNA is monoubiquitinated by E3 ligase RNF8 in IR-induced medulloblastoma cells, which might contribute to the low radiosensitivity in medulloblastoma cells.

In summary, we demonstrated that E3 ligase RNF8 is upregulated in medulloblastoma cells and can be significantly increased by γ-ray irradiation in a dose-dependent manner. RNF8 silencing actually can inhibit the survival and affect the apoptosis as well as cell cycle arrest of medulloblastoma cells after IR exposure, contributing to the enhancement of the sensitivity of medulloblastoma cells to IR. Furthermore, we found that PCNA ubiquitinated by E3 ligase RNF8 is involved in the RNF8-mediated sensitivity of medulloblastoma cells to IR. These findings may provide a novel target for the improvement of radiotherapy in medulloblastoma.

ACKNOWLEDGMENT: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The authors declare no conflicts of interest.

REFERENCES

1. Dennis M, Spiegler BJ, Hetherington CR, Greenberg ML. Neuropsychological sequelae of the treatment of children with medulloblastoma. J Neurooncol. 1996;29(1):91–101.
2. Chetty C, Dontula R, Gujrati M, Dinh DH, Lakka SS. Blockade of SOX4 mediated DNA repair by SPARC enhances radioresponse in medulloblastoma. Cancer Lett. 2012;323(2):188–98.
3. MacDonald TJ, Rood BR, Santi MR, Vezina G, Bingaman K, Cogen PH, Packer RJ. Advances in the diagnosis, molecular genetics, and treatment of pediatric embryonal CNS tumors. Oncologist 2003;8(2):174–86.
4. Duan J, Yu Y, Li Y, Yu Y, Li Y, Zhou X, Huang P, Sun Z. Toxic effect of silica nanoparticles on endothelial cells through DNA damage response via Chk1-dependent G2/M checkpoint. PLoS One 2013;8(4):e62087.
5. Tan X, Peng J, Fu Y, An S, Rezaei K, Tabbara S, Teal CB, Man YG, Brem RF, Fu SW. miR-638 mediated regulation of BRCA1 affects DNA repair and sensitivity to UV and cisplatin in triple-negative breast cancer. Breast Cancer Res. 2014;16(5):435.
6. Zhao MJ, Song YF, Niu HT, Tian YX, Yang XG, Xie K, Jing YH, Wang DG. Adenovirus-mediated downregulation of the ubiquitin ligase RNF8 sensitizes bladder cancer to radiotherapy. Oncotarget 2016;7(8):8956–67.
7. Witko-Sarsat V, Ohayon D. Proliferating cell nuclear antigen in neutrophil fate. Immunol Rev. 2016;273(1): 344–56.
8. Zhao H, Ho PC, Lo YH, Espejo A, Bedford MT, Hung MC, Wang SC. Interaction of proliferation cell nuclear antigen (PCNA) with c-Abl in cell proliferation and response to DNA damages in breast cancer. PLoS One 2012;7(1): e29416.
9. Zhu Q, Chang Y, Yang J, Wei Q. Post-translational modifications of proliferating cell nuclear antigen: A key signal integrator for DNA damage response. Oncol Lett. 2014;7(5):1363–69.
10. Olive PL. DNA damage and repair in individual cells: Applications of the comet assay in radiobiology. Int J Radiat Biol. 1999;75(4):395–405.
11. Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nat Protoc. 2006;1(5):2315–19.
12. Ivashkevich A, Redon CE, Nakamura AJ, Martin RF, Martin OA. Use of the gamma-H2AX assay to monitor DNA damage and repair in translational cancer research. Cancer Lett. 2012;327(1–2):123–33.
13. Gazy I, Kupiec M. The importance of being modified: PCNA modification and DNA damage response. Cell Cycle 2012;11(14):2620–23.
14. Tsutakawa SE, Yan C, Xu X, Weinacht CP, Freudenthal BD, Yang K, Zhuang Z, Washington MT, Tainer JA, Ivanov I. Structurally distinct ubiquitin- and sumo-modified PCNA: Implications for their distinct roles in the DNA damage response. Structure 2015;23(4):724–33.
15. Kashiwaba S, Kanao R, Masuda Y, Kusumoto-Matsuo R, Hanaoka F, Masutani C. USP7 is a suppressor of PCNA ubiquitination and oxidative-stress-induced mutagenesis in human cells. Cell Rep. 2015;13(10):2072–80.
16. Ciccia A, Elledge SJ. The DNA damage response: Making it safe to play with knives. Mol Cell 2010;40(2):179–204.
17. Harper JW, Elledge SJ. The DNA damage response: Ten years after. Mol Cell 2007;28(5):739–45.
18. Karran P. DNA double strand break repair in mammalian cells. Curr Opin Genet Dev. 2000;10(2):144–50.
19. Sudprasert W, Navasumrit P, Ruchirawat M. Effects of low-dose gamma radiation on DNA damage, chromosomal aberration and expression of repair genes in human blood cells. Int J Hyg Environ Health. 2006;209(6):503–11.
20. Hiyama A, Nohara C, Taira W, Kinjo S, Iwata M, Otaki JM. The Fukushima nuclear accident and the pale grass blue butterfly: Evaluating biological effects of long-term low-dose exposures. BMC Evol Biol. 2013;13:168.
21. Rhee JS, Kim BM, Kim RO, Seo JS, Kim IC, Lee YM, Lee JS. Co-expression of antioxidant enzymes with expression of p53, DNA repair, and heat shock protein genes in the gamma ray-irradiated hermaphroditic fish Kryptolebias marmoratus larvae. Aquat Toxicol. 2013;140–141: 58–67.
22. Shibata A, Moiani D, Arvai AS, Perry J, Harding SM, Genois M-M, Maity R, van Rossum-Fikkert S, Kertokalio A, Romoli F, Ismail A, Ismalaj E, Petricci E, Neale MJ, Bristow RG, Masson J-Y, Wyman C, Jeggo PA, Tainer JA. DNA double-strand break repair pathway choice is directed by distinct MRE11 nuclease activities. Mol Cell 2014;53(1):7–18.
23. Wang Z, Yin H, Zhang Y, Feng Y, Yan Z, Jiang X, Bukhari I, Iqbal F, Cooke HJ, Shi Q. miR-214-mediated downregulation of RNF8 induces chromosomal instability in ovarian cancer cells. Cell Cycle 2011;13(22):3519–28.
24. Vignard J, Mirey G, Salles B. Ionizing-radiation induced DNA double-strand breaks: A direct and indirect lighting up. Radiother Oncol. 2013;108(3):362–9.
25. Chapman JR, Taylor MR, Boulton SJ. Playing the end game: DNA double-strand break repair pathway choice. Mol Cell 2012;47(4):497–510.

26. Maga G, Hubscher U. Proliferating cell nuclear antigen (PCNA): A dancer with many partners. J Cell Sci. 2003; 116(Pt 15):3051–60.
27. Hoege C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature 2002; 419(6903):135–41.
28. Zhang S, Chea J, Meng X, Zhou Y, Lee EY, Lee MY. PCNA is ubiquitinated by RNF8. Cell Cycle 2008;7(21):3399–404.
29. Postow L, Ghenoiu C, Woo EM, Krutchinsky AN, Chait BT, Funabiki H. Ku80 removal from DNA through double strand break-induced ubiquitylation. J Cell Biol. 2008; 182(3):467–79.
30. Yu SL, Kang MS, Kim HY, Gorospe CM, Kim TS, Lee SK. The PCNA binding domain of Rad2p plays a role in mutagenesis by modulating the cell cycle in response to DNA damage. DNA Repair 2014;16:1–10.
31. Szuts D, Christov C, Kitching L, Krude T. Distinct populations of human PCNA are required for initiation of chromosomal DNA replication and concurrent DNA repair. Exp Cell Res. 2005;311(2):240–50.