



Research Article

SIRT2 Suppression Increases Responsiveness of Glioma Cells to Radiation Treatment via FoxO3a Inhibition

Zhihan Wang^{1#}, Jianyi Zhao^{2#}, Wenrui Zhang^{2#}, Keman Liao², Hao Xu¹, Zilong Wei¹, Li Ren^{1*}

¹Department of Neurosurgery, Shanghai Pudong Hospital, Fudan University, Shanghai, China

²Department of Neurosurgery, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

#Contributed equally to this work.

*Corresponding author: Li Ren, Department of Neurosurgery, Shanghai Pudong Hospital, Fudan University Pudong Medical Center, Shanghai, 201399, China

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Abstract

Due to the resistance to radiotherapy, the prognosis of patients with glioblastoma multiforme (GBM) is poor despite standard treatment. Forkhead Box O3 (FoxO3a) is reported to mediate multiple pathological processes involved in proliferation, apoptosis, and DNA repair processes, and associated with poor prognosis of GBM. Further work is required to explore the role of FoxO3a in GBM radioresistance. RT-qPCR indicated that FoxO3a was significantly up-regulated in GBM cells in response to radiation. Knockdown of FoxO3a enhances the responsiveness of GBM cells to radiotherapy. After radiation stimulation, the clonogenic capacity of the cells with FoxO3a knockdown (KD) was much weaker than that of the normal control group (NC), while the degree of DNA damage detected by the comet assay was significantly higher in the cells with FoxO3a knockdown. Moreover, combining recent research, we demonstrated that suppression of SIRT2 down-regulated FoxO3a with radiation therapy by Western Blot. DNA damage assay and comet assay indicated that the degree of DNA damage increased in the cells of SIRT2 knockdown compared to the normal control (NC). The study indicated that suppression of SIRT2 increased the sensitivity of GBM cells to radiotherapy via FoxO3a inhibition and provided a novel idea for overcoming radioresistance in GBM.

Keywords: DNA double-strand breaks; FoxO3a; Glioblastoma; radioresistance; SIRT2.

Introduction

Glioblastoma multiforme (GBM) is a common malignant intracranial tumor. Currently, core treatment for patients with GBM consists of maximal surgical removal with the fewest neurological complications, radiotherapy and chemotherapy (Chinot et al., 2014; Lozada-Delgado, Grafals-Ruiz, & Vivas-Mejia, 2017; Vaquero et al., 2006). However, the overall survival is still only lower than 15 months (Stupp et al., 2005). Radiotherapy is the main way of

postoperative adjuvant therapy for GBM patients (Ghia, 2018; Han, Xue, Zhou, & Zhang, 2017). But different GBM patients show heterogeneous radiosensitivity and would develop resistance towards radiotherapy gradually. Thus, it is urgent to clarify the underlying mechanisms of GBM progression and radioresistance.

FoxO3a, which belongs to the Forkhead box O (FoxO) transcriptional factor family, was first identified in human placental cosmid. As a central transcription factor, FoxO3a was expressed among a variety of tissues and mediates multiple pathological processes by transcription regulation of genes involved in proliferation, apoptosis, cell cycle and DNA repair processes

(Coomans de Brachene & Demoulin, 2016; Gomes, Zhao, & Lam, 2013; Li, Zhang, Chen, Fan, & Fang, 2012; Liu et al., 2018; Ni et al., 2014; Osuka et al., 2013; L. Zhang et al., 2017). These diverse functions of FoxO3a are highly associated with a series of malignancies (Calnan & Brunet, 2008; Daitoku, Sakamaki, & Fukamizu, 2011). Several recent reports have suggested that FoxO3a might be a biomarker for predicting response to radiotherapy in cervical carcinoma, breast cancer and esophageal cancer (M. F. Chen et al., 2008; Kim et al., 2017; Rehman et al., 2018). However, the role of FoxO3a in GBM radioresistance is unclear.

SIRT2 is a nicotinamide adenine dinucleotide-dependant (NAD) deacetylase (Imai, Armstrong, Kaeberlein, & Guarente, 2000; Landry et al., 2000; Smith et al., 2000). SIRT2 deacetylates many vital substrates, such as P53, NF- κ B and participates in aging and stress response (Langley et al., 2002; Luo et al., 2001; Yeung et al., 2004). Besides, many researches showed that SIRT2 plays an important role in multiple central nervous system diseases, such as Parkinson's disease (PD) and Huntington's disease (HD) (Chopra et al., 2012; Outeiro et al., 2007). Intriguingly, recent studies found that SIRT2 could bind to FoxO3a specifically and reduce its acetylation level (Wang, Nguyen, Qin, & Tong, 2007). Thus, we aimed to investigate whether SIRT2 mediates radiotherapy tolerance via FOXO3a up-regulated.

In the present study, Foxo3a-knockdown GBM cells and SIRT2-knockdown GBM cells were constructed using a lentivirus-mediated expression knockdown technique. Radiation treatment resulted in decreased cell clone formation, increased DNA damage, increased levels of apoptosis in FoxO3a-knockdown cells and reduced radiation tolerance in SIRT2-knockdown cells. The present study preliminarily suggested that inhibition of SIRT2 contributes to the sensitivity of GBM radiotherapy by reducing the level of FoxO3a.

Materials and Methods

Cell lines and maintenance

The two human GBM cell lines (U87-MG and T98G) were purchased from the Cell Bank of the Shanghai Branch of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan City, UT) supplemented with 10 % fetal bovine serum (FBS; Gibco, Carlsbad CA, USA), 100 units/ml penicillin and 100 μ g/ml streptomycin (Gibco, Carlsbad CA, USA) at 37 °C in a humid environment containing 5 % CO₂.

Radiation treatment

Cells in the culture medium were treated with an irradiator (GE 3000; GE Healthcare Life Sciences) using a ¹³⁷Cs source at an exact dose of 0 or 6.0 Gy. During irradiation, the culture was

stored in the cell culture incubator (5% CO₂ at 37°C). The cells were harvested or used in further experiments exactly at the end of the irradiation.

RNA extraction and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA extraction and further qRT-PCR were referred to the previous article (Milani et al., 2019). The TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA. The cDNA was synthesized using the oligo-dT primer and M-myeloblastosis virus reverse transcriptase XL (Promega, USA). Quantitative RT-PCR was performed with Thunderbird SYBR qPCR Mix (Toyobo, Japan) using LightCycler 480 II Fast PCR instrument (Roche Molecular Systems, Inc., Pleasanton, CA, USA). The primers for FoxO3a used in this study were: forward primer: 5'-TCTACGAGTGGATGGTGCCTTG-3'; reverse primer: 5'-CTCTTGCCAGTCCCTCATTCTG-3'. GAPDH was used as the internal parameter, forward primer, 5'-GAAGGACTCATGACCACAGTCCA-3', reverse primer, 5'-GCAGGGATGATGTTCTGGAGAG-3'.

Lentivirus, plasmid construction and transfection

FoxO3a-knockdown lentivirus was synthesized by Hanyin Biotech (Shanghai, China). Target sequences of FoxO3a short hairpin RNA (shRNA) were 5'-GCTCTGGTGGATCATCAA-3' (FoxO3a-KD). The FoxO3a-KD lentivirus and the negative control (NC) lentivirus (Hanyin Co. Shanghai, China) were prepared to the titer of 10⁹ TU/ml (transfection unit). After a 48-h incubation, the knockdown efficiency was confirmed via qRT-PCR. For stable construction, U87-MG and T98G cells were transfected with the lentivirus according to the manufacturer's instructions.

Western blot analysis

Isolated proteins (30 μ g) were electrophoresed on 10 % SDS-polyacrylamide gel (YEASEN Biotechnology Co., Ltd, Shanghai, China) and transferred onto PVDF membranes (Millipore, USA). The membranes were blocked with 5 % skim milk for 1 h at room temperature and incubated with the appropriate dilution of primary antibodies at 4°C overnight. Primary antibodies used in this study were listed as follows: anti-FoxO3a antibody (cat#12829, Cell Signaling Technology); anti-caspase-3 antibody (cat#9662, Cell Signaling Technology); anti-caspase-7 antibody (cat#9492, Cell Signaling Technology); anti-Bax antibody (cat#2772, Cell Signaling Technology); anti- β -actin antibody (cat#ab8227, Abcam). The membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse antibody (1:10,000 dilution; Sigma-Aldrich, USA) for 2h at RT after being washed with Tris-buffered saline-Tween (TBST) three times. Next, the protein bands were identified using the detection reagent Clarity Western ECL substrate (Bio-Rad Laboratories).

Plate clone formation assay

Fully suspended viable cells were seeded in a six-well tissue culture plate at the density of 300 cells per well and incubated at 37 °C for 24 h. The cells in the experimental group were treated with a radiation dose of 6 Gy and then grown until the visible size. The supernatant was discarded, then washed with PBS 3 times, and next dyed with 0.5 % crystal violet in 50 % methanol. Finally, wash it again with PBS and dry it, ≥ 50 cells were considered an effective clone.

Flow cytometry cell apoptosis analysis

Annexin V-FITC/PI Apoptosis Detection Kit (YEASEN, Shanghai, China) was utilized to detect apoptotic cells. Cells treated with 0 or 6 Gy radiation were harvested and washed three times with cold PBS. Then resuspended and incubated with Annexin V-FITC fluorescent labeled solution for 10 min. After centrifuging and washing with PBS, PI buffer was added and incubated for 20 min in the dark at 4 °C. The results were detected by Cell Quest software on a FACS Aria Flow Cytometer (BD Inc., USA). The detected fluorescence wavelength was 480 nm.

Comet assay

Comet assay, also named single-cell gel electrophoresis assay (SGEA) was carried out using Comet Assay Kit (Trevigen, USA) following the experiment instruction. 6 Gy radiation-treated GBM cells or untreated cells were first configured as a single-cell suspension and then mixed with low melting agarose in a ratio of 1 to 10 at 37°C. Next, pipetted the agarose/cell mixtures onto the top of the base layer which was prepared by pipetting comet agarose onto the comet slide in advance. After that, slides were treated with lysis buffer and alkaline solution and electrophoresed for 20 min at 4°C with the alkaline condition. Subsequently, the cells were visualized by staining with DNA dye (Goldview, SBS Genetech, Co., Ltd). A confocal laser microscope was applied to obtain the images. Tail DNA% = Tail DNA Intensity/Cell DNA Intensity x 100%.

Immunofluorescence staining

Cells were permeabilized with 0.3% Triton X-100-PBS for 15 minutes at 25°C after 0 or 6 Gy radiation with or without SIRT2 knockdown. Cells were then blocked with 3% BSA for 60 minutes and incubated in γ -H2AX antibody (CY6572, Abways, 1:100) overnight at 4°C with gentle shaking. After rinsing with PBS, γ -H2AX antibody was recognized with Alexa Fluor-label antibody. We counterstained nuclei of cells with DAPI in PBS, and the coverslips were placed on slides using Prolong Antifade Reagent (Molecular Probes). Images were then acquired using a Nikon Eclipse Ti fluorescent microscope (Nikon Instruments, NY) and processed by ImageJ (NIH) analysis software.

Statistical analysis

All data obtained in this study were analyzed by the SPSS for Windows v.17.0 (SPSS, Chicago, IL). All of the experiments were conducted in triplicate and described as the mean \pm standard error. The Tukey test was combined with One-way ANOVA for multiple comparisons. Results were considered significant at two-sided $P < 0.05$.

Results

FoxO3a is markedly upregulated in GBM cells in response to radiation

Functions of FoxO3a in GBM progression were explored in previous work (Qian et al., 2017). Analogously, Western Blot was performed to detect protein levels of FoxO3a in T98G and U87-MG cells treated with or without 6 Gy radiation. The results suggested that FoxO3a was significantly upregulated after 6 Gy radiation in both GBM cell lines compared with cells without radiation treatment (Fig 1).

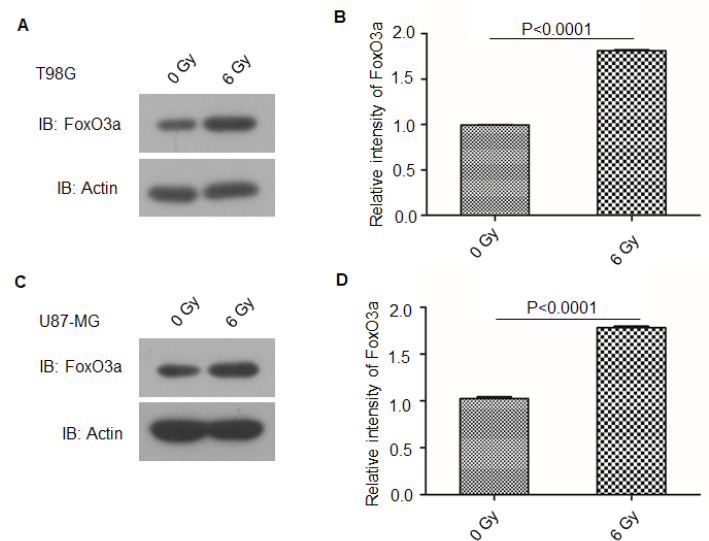


Figure 1. FoxO3a was significantly up-regulated in GBM cells after radiation. (A, B) Relative FoxO3a protein levels in T98G cells treated with 0 or 6 Gy radiation. (C, D) Relative FoxO3a protein levels in U87-MG cells treated with 0 or 6 Gy radiation. The values are expressed as the mean \pm SD from three independent experiments.

Suppression of FoxO3a confers sensitivity of GBM cells to radiation treatment

Since FoxO3a shows responsive upregulation after radiation treatment, we propose that FoxO3a may be involved with the radiosensitivity of GBM cells. We infected two GBM cell lines

T98G and U87-MG with FoxO3a shRNAs or control shRNA, FoxO3a mRNA and protein expression levels were efficiently inhibited (**Fig 2A and B**). The results of the plate clone formation assay suggested that the colony number of cells with FoxO3a knockdown was strikingly reduced compared to the cells of negative control (**Fig 2C and D**). Next, we evaluated the single and double-strand DNA breaks of cells with or without FoxO3a knockdown upon 6.0 Gy-radiation by single-cell electrophoresis assay. The results showed that the tail DNA (%) increased in FoxO3a-KD cells, especially after 6.0-Gy radiation (**Fig 2E and F**). The apoptotic rate of tumor cells was an important measure of the effectiveness of radiation therapy. Therefore, we examined the capacity of FoxO3a knockdown to induce GBM cell apoptosis in response to radiation. The apoptotic rates of T98G and U87-MG cells with or without FoxO3a knockdown following radiation were detected by flow cytometry. The results showed that cells with FoxO3a knockdown presented a higher apoptosis rate (**Fig 3A and B**). Further detection of the apoptosis-related proteins indicated that cells with FoxO3a knockdown expressed higher Caspase 3, Caspase 7 and Bax compared with cells with FoxO3a normal (**Fig 3C and D**).

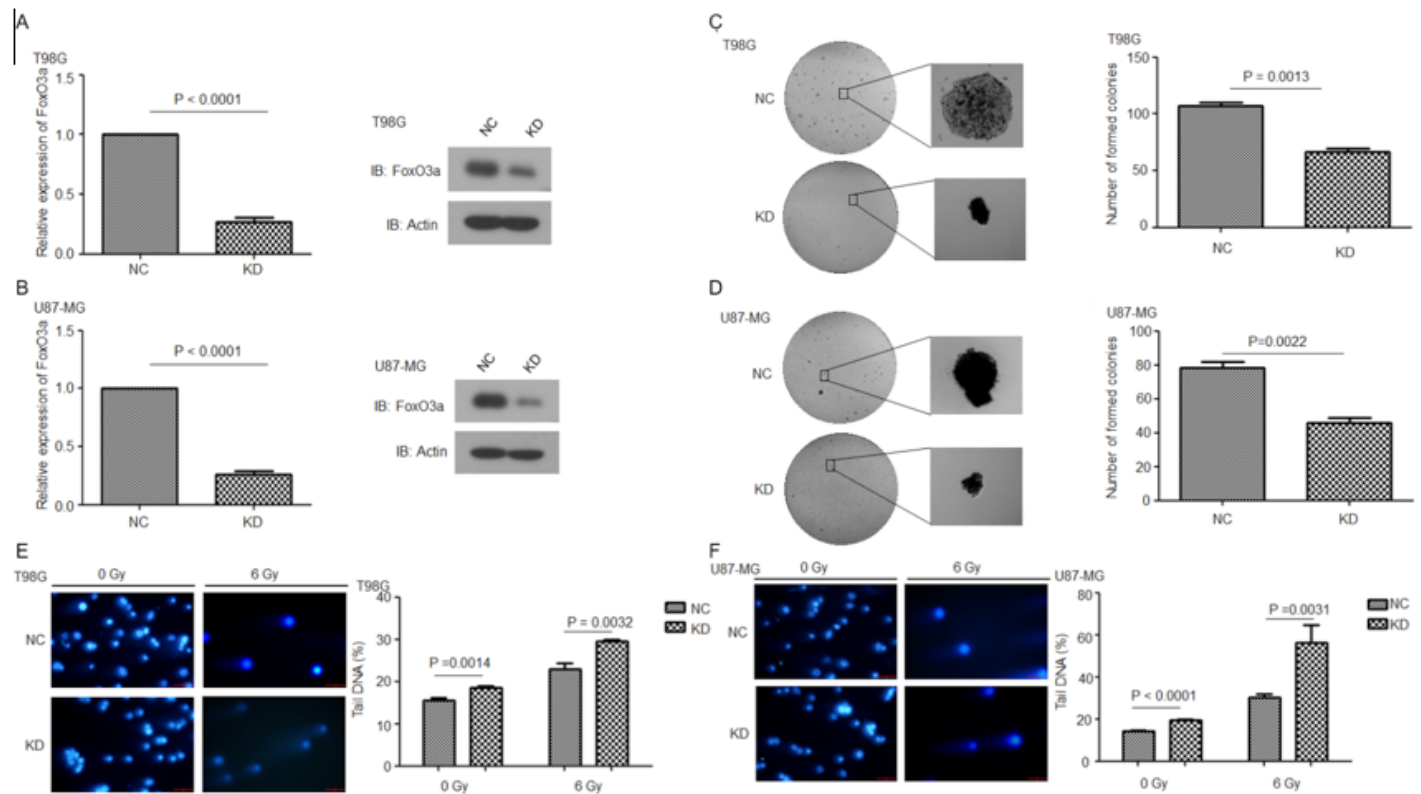


Figure 2. Suppression of FoxO3a confers sensitivity of GBM cells to radiation treatment. (A, B) Relative FoxO3a mRNA levels and protein levels in T98G cells and U87-MG cells with or without FoxO3a KD. (C, D) The number of formed colonies of T98G cells and U87-MG cells with or without FoxO3a KD. (E, F) T98G and U87-MG cells treated with 6-Gy radiation present longer comet tail DNA in cells with FoxO3a KD and Tail DNA (%) were measured in T98G and U87-MG cells with or without FoxO3a KD treated with 0 or 6 Gy radiation. Error bars, 20 μ m. The values are expressed as the mean \pm SD from three independent experiments. NC, negative control; KD, knockdown.

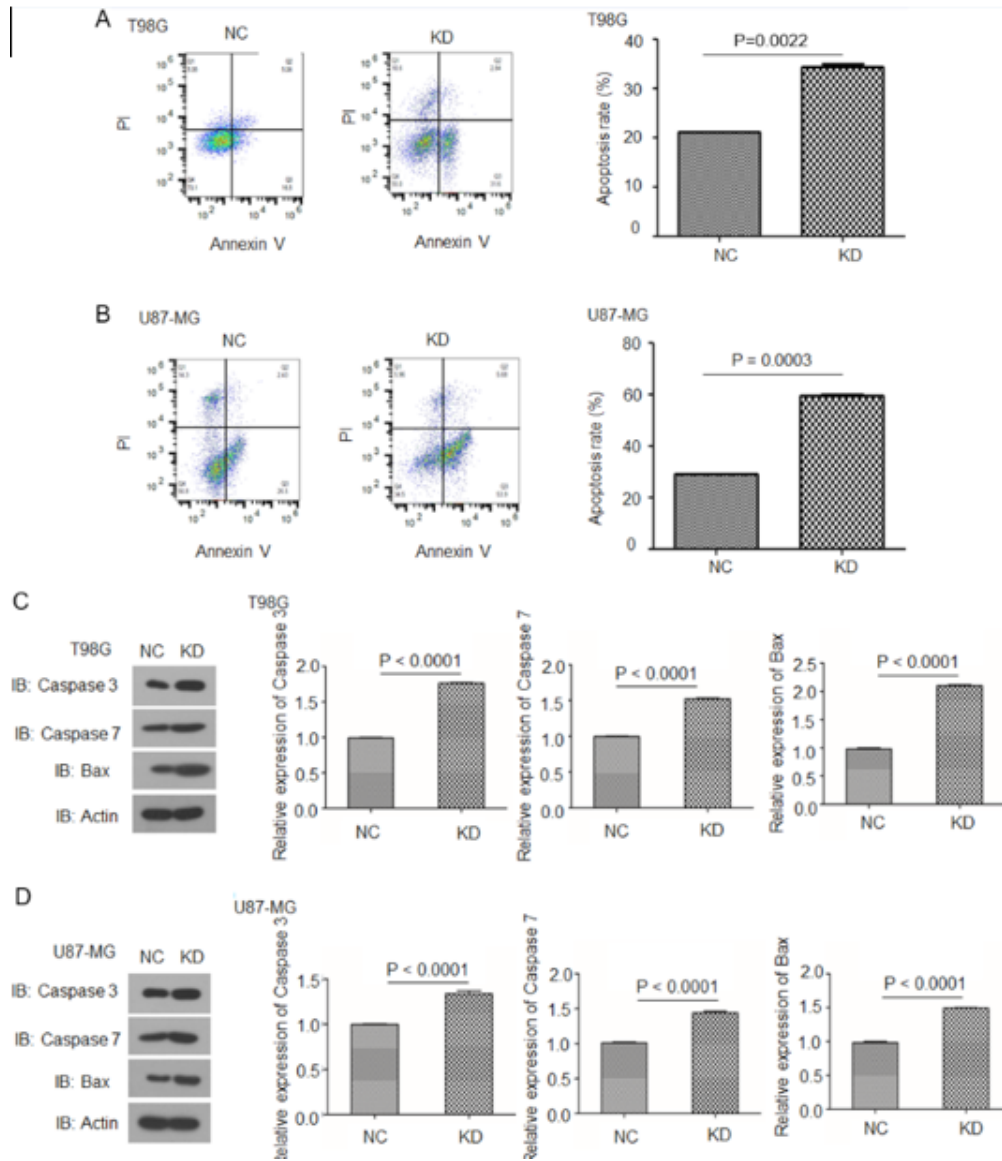


Figure 3. Suppression of FoxO3a increased apoptosis rate and expression levels of apoptosis protein in GBM. (A, B) Flow cytometry analysis and apoptosis rate of T98G and U87-MG cells with or without FoxO3a KD treated with 0 or 6 Gy radiation. The upper right and lower right corners represent late apoptotic, early apoptotic, respectively. (C, D) The amount and relative expression levels of Caspase 3, Caspase 7, Bax in T98G and U87-MG cells with or without FoxO3a KD treated with 0 or 6 Gy radiation. The values are expressed as the mean \pm SD from three independent experiments. NC, negative control; KD, knockdown.

Suppression of SIRT2 downregulates FoxO3a

Next, we investigated the relationship between SIRT2 and FoxO3a to explore the upstream mechanisms of FoxO3a to mediate radioresistance. Similarly, we infected T98G and U87-MG cells with SIRT2 shRNAs respectively and one of the most effective shRNAs was chosen for further experiments (Fig 4A and B). Western blot was performed to detect protein levels of Sirt2 and FoxO3a in T98G and U87-MG cells with or without SIRT2 knockdown after 0 or 6 Gy radiation treatment. FK866 is an acknowledged inhibitor of NMPRTase and is supposed to inhibit the activity of SIRT2. The result showed that the expression of SIRT2 was upregulated in cells with 6 Gy compared to cells without radiation and with SIRT2 knock-down, the expression of Foxo3a decreased (Fig 4C).

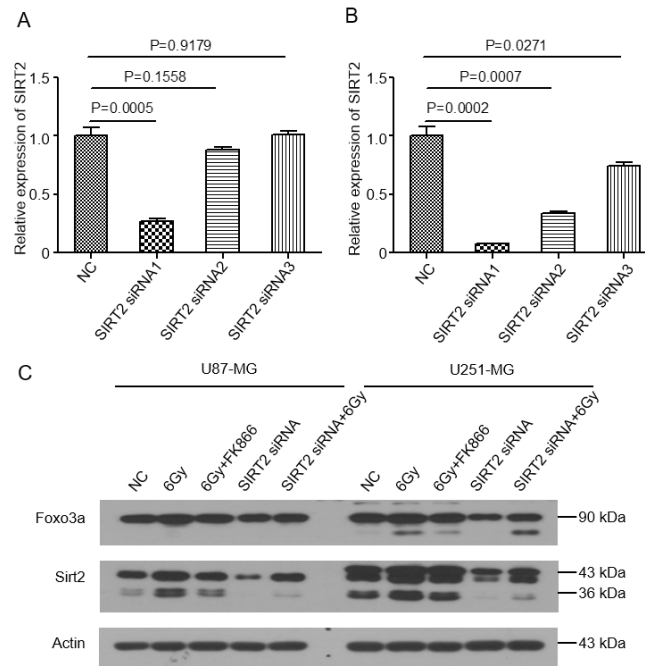


Figure 4. Suppression of SIRT2 downregulates FoxO3a. (A, B) Relative expression of SIRT2 in U87 cells and U251 cells with SIRT2 knockdown by various primers was measured by qRT-PCR. (C, D) Relative protein expression levels of Sirt2 in U87-MG and U251-MG cells were measured by Western Blot. NC, negative control.

Suppression of SIRT2 enhances the effect of radiotherapy

At last, we evaluated the effect of radiotherapy with or without SIRT2 knockdown upon 0 or 6.0 Gy-radiation by immunofluorescence staining. The results showed that the γ -H2AX foci formation was elevated in both GBM cells with SIRT2 knockdown after radiation therapy (Fig 5). A comet array was also performed and the DNA in the tail (%) increased in SIRT2-KD cells after 6.0-Gy radiation (Fig 6).

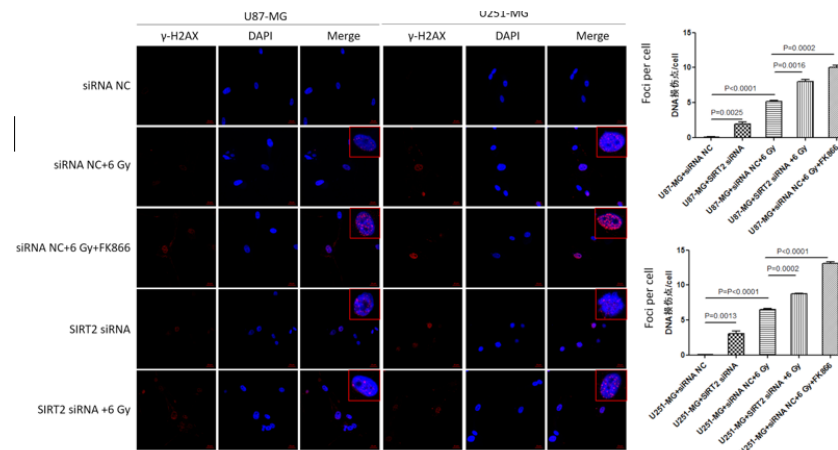


Figure 5. Suppression of STRT2 confers sensitivity of GBM cells to radiation treatment. Cells treated with 6-Gy radiation present more foci of the formation of γ -H2AX (red) and DAPI (blue) in T98G and U87-MG cells with or without SIRT2 KD treated with 0 or 6 Gy radiation. Quantitative analysis of foci per cell in U87-MG and U251-MG was measured in U87-MG and U251-MG. The values are expressed as the mean \pm SD from three independent experiments. NC, negative control.

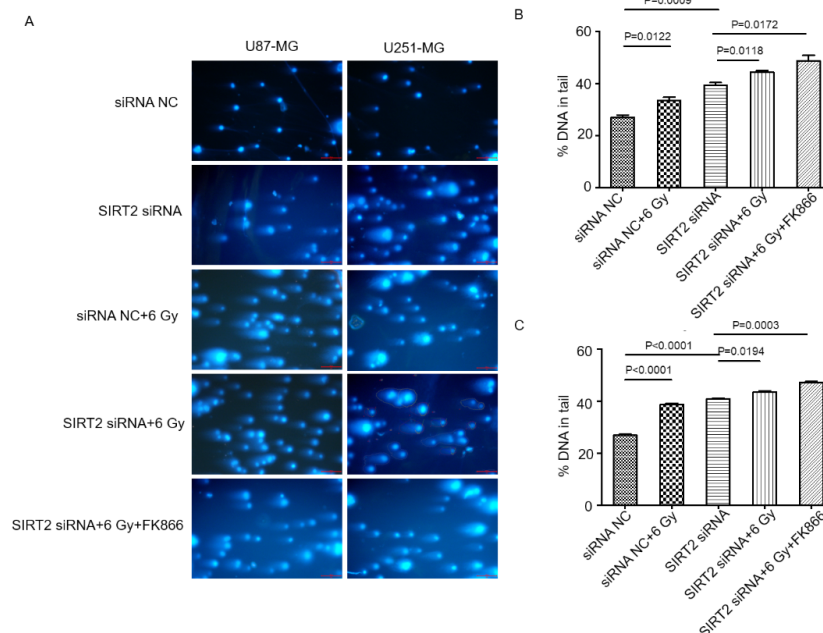


Figure 6. Suppression of SIRT2 decreased DNA repair ability of GBM cells after radiation (A) Cells treated with 6-Gy radiation present longer comet tails DNA damage in U87-MG and U251-MG cells with or without SIRT2 KD treated with 0 or 6 Gy radiation. Error bars, 20 μ m. (B, C) Tail DNA (%) was measured in U87-MG and U251-MG cells with or without SIRT2 KD treated with 0 or 6 Gy radiation. The values are expressed as the mean \pm SD from three independent experiments. NC, negative control.

Taken together, the present study explored the roles of FoxO3a and SIRT2 in GBM radiation tolerance. Suppression of FoxO3a increased the responsiveness of GBM cells to radiation therapy, decreased clonogenic capacity, increased DNA damage and promoted cell apoptosis. SIRT2 inhibition down-regulated expression of FoxO3a and thus increased DNA damage of GBM cells. This study uncovers the underlying mechanism between SIRT2 and FoxO3a in GBM radioresistance, and provides a novel idea for overcoming GBM radiotherapy resistance.

Discussion

Many researchers have indicated that FoxO3a exerted pivot biological functions concerning the resistance against radiotherapy of cancer (G. Chen, Yu, Dong, Liu, & Sun, 2018; Matt & Hofmann, 2016; Osuka et al., 2013). Being regulated by miRNAs, FoxO3a notably induce radiation resistance in several cancer (Babar et al., 2011; Feng, He, & Wang, 2018; Khoshinani et al., 2017; Yan et al., 2017; H. Zhang, Zhang, Wang, Zhang, & Bi, 2018). Functionally, it may be related to the aggregation of FoxO3a in the nucleus of tumor cells (M. F. Chen et al., 2008). SIRT2 is a NAD-dependent tubulin deacetylase and distributes mainly in the cytoplasm (North, Marshall, Borra, Denu, & Verdin, 2003). Recent research showed that SIRT2 can migrate to the nucleus and play a vital role during mitosis to deacetylate α -tubulin at the lysine-40 (Vaquero et al.,

2006). Based on the early findings, the present study assessed the function of FoxO3a and the relationship between FoxO3a and SIRT2 in GBM radioresistance. First, in comparison to radiation-untreated GBM cell lines, there was significant overexpression of FoxO3a in cells treated with radiation, which suggested that the overexpression of FoxO3a might be an important biomarker for predicting response to radiotherapy in GBM. Next, FoxO3aKD was conducted in GBM cells infected with lentivirus. The functional assay revealed that the knockdown of FoxO3a strikingly decreased the number of colonies and promoted DNA damage after radiation. Flow cytometry and pro-apoptotic proteins detection revealed that FoxO3a-KD causes elevated levels of apoptosis in GBM cells after radiotherapy. Then, recent researches showed that SIRT2 could bind to Foxo3a and we demonstrated that SIRT2 up-regulated after radiotherapy and suppression of SIRT2 down-regulated the expression level of FoxO3a. At last, immunofluorescence staining and comet assay revealed that suppression of SIRT2 promoted DNA damage in GBM cells after radiation.

In fact, the molecular mechanisms of GBM radioresistance are extremely complex. All these previously reported mechanisms have identified several key factors, including tumor microenvironment, tumor heterogeneity, glioma stem cells, DNA damage and repair, hypoxia, and metabolic alteration (Ali

et al., 2020). Specifically, the following molecules, hypoxia-inducible factors (HIFs) (Li et al., 2009), reactive oxygen species (ROS) (Gao et al., 2008), reductant NADPH (Wahl et al., 2017), Tumor protein P53 (Peña-Rico et al., 2011), epidermal growth factor receptor variant III (EGFRvIII) (Mukherjee et al., 2009), Checkpoint Kinase 1/2 (CHK1/2) (Patties et al., 2019), Histone deacetylase (HDAC)-4 and -6 (Marampon et al., 2017), together form a complex regulatory network involved in GBM resistance to radiotherapy. This study addressed FoxO3a participating in GBM radioresistance for the first time and dug out its upstream mechanism. The results established the interaction of FoxO3a and SIRT2 in GBM radioresistance and enriched the molecular mechanisms of GBM radioresistance which may provide a novel idea to overcome radiotherapy resistance in GBM.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: All authors agreed on the manuscript.

Availability of data and material: The original data of this study are available from the corresponding authors upon reasonable request.

Competing interests: There is no conflict of interest.

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Authors' contributions

L.R., Z.H.W., B.H.C conceived and designed the research. Z.H.W and B.H.C conducted most of the experiments. K.L., H.X., J.X. and Z.W contributed new reagents or analytical tools and analyzed data and helped with some of the experiments. B.H.C wrote the manuscript. All authors read the manuscript, agree with the manuscript, and are willing to take responsibility for the research contents.

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