Suppression of FoxO3a Increases Responsiveness of Glioma Cells to Radiation Treatment

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Abstract

Background Glioblastoma multiforme (GBM) is the most deadly and common cerebral malignant tumors in adults. Radiotherapy is one of the major treatments for GBM patients. Further work is urgently required to discover the mechanisms conferring GBM radioresistance.

Methods Reverse transcription-quantitative PCR was applied to detect the relative expression of FoxO3a in glioma cell lines after radiation. Lentivirus mediated FoxO3a knockdown cells were constructed and were confirmed by RT-qPCR and western blot. Plate clone formation assay and flow cytometry was used to determine the colony formation ability and cell apoptosis following radiotherapy, respectively. DNA damage and levels of apoptotic proteins were determined by comet assay and western blot.

Results FoxO3a was significantly up-regulated in GBM cells in response to radiation. Knockdown of FoxO3a enhances the responsiveness of glioma cells to radiotherapy, the cell clonogenic formation and DNA repair process with elevated the percentage of apoptotic cells. Moreover, suppression of FoxO3a increased pro-apoptotic proteins Caspase 3, Caspase 7 and Bax levels after radiotherapy.

Conclusions The study indicated that suppression of FoxO3a increased radiosensitivity in glioma cells, which might be a potential diagnostic and therapeutic target. Inhibition of FoxO3a expression enhances the responsiveness of glioma cells to radiotherapy, and its proliferation ability is weaker than that of normal Foxo3 cells.

Introduction

Glioblastoma multiforme (GBM) is a common malignant intracranial tumor. Currently, core treatment for patients with GBM consists of maximal surgical removal with fewest neurological complications, radiotherapy and chemotherapy (1, 2). However, the overall survival is still only 14.6 months (3). Radiotherapy is the main way of postoperative adjuvant therapy for GBM patients (4, 5). The heterogeneous of GBM patients’ different radiosensitivity might related to molecular biology of tumor cells. Thus, it is important and urgent to clarify underlying mechanism 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 mediates multiple pathological processes by inducing transcription of genes involved in proliferation, apoptosis, cell cycle and DNA repair processes (6-12). These diverse functions of FoxO3a is highly associated with a series of malignancies and mainly regulate aberrant post-translational modifications in various types of cancer (13, 14). Several recent reports have suggested that FoxO3a might be a biomarker for predicting response to the radiotherapy in cervical carcinoma, breast cancer and esophageal cancer (15-17). However, the role of FoxO3a in GBM radiosensitivity remains unclear.
Therefore, in this study we examined whether FoxO3a play a role in the radiosensitivity of GBM. Additionally, we studied several apoptotic proteins which might be regulated by FoxO3a after radiotherapy. This study illustrates the role of FoxO3a in the radiosensitivity of GBM and explores the mechanisms preliminarily.

**Materials And Methods**

**Cell lines and maintenance**

The two GBM cell lines U87-MG and T98G were purchased from the Cell Bank of the Shanghai Branch of the Chinese Academy of Sciences. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in a humid environment containing 5% CO₂.

**RNA extraction and qRT-PCR**

RNA extraction and further quantitative reverse transcriptase polymerase chain reaction (real-time quantitative PCR detection were referred to the previous article (18). The TRizol reagent (Invitrogen) was used to extract total RNA. And the cDNA was synthesized using the oligo-dT primer and M-myeloblastosis virus reverse transcriptase XL (Promega). Quantitative RT-PCR was performed with Thunderbird SYBR qPCR Mix (Toyobo) using LightCycler 2.0 (Roche Molecular Systems, Inc., Pleasanton, CA, USA). The primers for FoxO3a used in this study were: F: 5’-TCTACGAGTGGATGGTGCGTTG-3’; R: 5’-CTCTTGCCAGTTCCCTCATTCTG-3’. GAPDH was used as the internal parameter, forward primer, 5’-GAAGGACTCATGACCACAGTCCA-3’, reverse primer, 5’-GCAGGGATGATGTTCTGGAGAG-3’.

**Western blot analysis**

Isolated proteins (30 μg) were electrophoresed on 8-10 % SDS-polyacrylamide gel and transferred onto Hybond ECL membranes (Amersham). The membranes were blocked with 5 % skim milk for 1 h at room temperature and then incubated with the appropriate dilution of primary antibodies at 4°C overnight. Primary antibodies used in this study were listed as below: 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) for 2 h at RT after washed with Tris-buffered saline-Tween (TBST) for three times. Next, the protein bands were identified using the detection reagent Clarity Western ECL substrate (Bio-Rad Laboratories).

**FoxO3a knockdown by lentiviral vector**

Target sequences of FoxO3a short hairpin RNA (shRNA) were 5’-GCTCTTGGTGGATCATCAA-3’ (FoxO3a-KD). The recombinant lentivirus and the negative control (NC) lentivirus (Hanyin Co. Shanghai, China)
were prepared to the titer of $10^9$ TU/ml (transfection unit). After a 48-h incubation, the knockdown efficiency was confirmed via qRT-PCR. For stable construction, GBM cells were seeded in six-well dishes at a density of $2 \times 10^5$ cells per well. The cells were then infected with the same titre virus with 8 μg/ml Polybrene on the following day. 72 h after viral infection, the culture medium was replaced and infected cells were selected with 4 μg/ml of puromycin.

**Plate clone formation assay**

Fully suspended viable cells were seeded in a six-well tissue culture plate at the density of 300 cells/well and incubated at 37 °C for 24 h. The cells in the experimental group were treated with a radiation dose of 6Gy and then grown until the visible size, the supernatant was discarded, then washed with PBS for 3 times, 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 washed 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 480nm.

**Comet assay**

Comet assay also named as single-cell gel electrophoresis assay (SGEA) was carried out using Trevigen Comet Assay Kit following the experiment instruction. 6Gy 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 treated with lysis buffer and alkaline solution and electrophoresed for 20 min at 4°C with 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%.

**Statistical analysis**

All data obtained in this study were analyzed by the SPSS for Windows v.17.0 (SPSS, Chicago, IL), and described as mean ± 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 highly expressed in GBM cells in response to radiation.
Functions of FoxO3a in GBM progression were explored in previous work (19). Analogously, western blot was performed to detect protein levels of FoxO3a in T98G and U87-MG cells treated with or without 6Gy radiation. FoxO3a was upregulated after 6Gy radiation in both cells compared with cells without radiation treatment (Figure 1).

**Suppression of FoxO3a alerts GBM cells response to radiation therapy**

To investigate the function of FoxO3a in GBM radiotherapy, two GBM cell lines T98g and U87-MG with FoxO3a stably knockdown were constructed. FoxO3a mRNA and protein expression level were efficiently inhibited in FoxO3a-KD cells compared to the negative control (NC) (Figure 2). The results of plate clone formation assay suggested that colony number and size of FoxO3a-KD cells was strikingly reduced compared to the NC cells after 6.0Gy radiation treatment (Figure 3). Single cell electrophoresis assay could be utilized to evaluated single and double-strand DNA breaks in vitro. The “comet tail” became longer as X-ray irradiation performed (Fig. 4A and 4C), indicating that the DNA strand breakage were induced by X-ray irradiation. The tail DNA (%) increased in FoxO3a-KD cells, especially after 6-Gy radiation, as shown in Fig. 4B and 4D. Significant differences were indicated (p <0.01). To further investigate whether FoxO3a impacted radiosensitivity through regulation of apoptosis, we examined apoptosis following radiation. The results displayed that there was statistically significant increased in apoptotic rate in FoxO3a-KD cells (Figure 5).

**FoxO3a regulates factors associated with apoptosis.**

Since FoxO3a could regulated apoptosis-related protein in other diseases(20-25), Western blot analysis was performed to measure protein levels of caspase-3, -7 and Bax in both FoxO3a-KD and NC cells. As shown in Figure 6, caspase-3, -7 and Bax increased remarkably in the FoxO3a-KD group after radiation (P<0.001). These findings indicated that suppression of FoxO3a results in an increase in protein levels of GBM cells after 6-Gy radiation.

**Discussion**

Many researches have indicated that FoxO3a exerted pivot biological functions in respect to the resistance against radiotherapy of cancer (8, 26, 27). For instance, Chen et al found that nuclear accumulation of FoxO3a in tumor cells was linked with the increased radiosensitivity of esophageal carcinoma (15). Meanwhile FoxO3a, which could be regulated by miRNAs, will notably induce radiation resistance in several cancer (28-32). Based on the early findings, we postulated that functional inhibition of FoxO3a expression may attenuate the radioresistance of malignant glioma.

This present study assessed the expression pattern and function of FoxO3a in GBM radiosensitivity. First, in comparison to radiation-untreated GBM cell lines, there was a significant overexpression of FoxO3a in cell treated with radiation, which suggested that the overexpression of FoxO3a might be an important biomarker for predicting response to radiotherapy in GBM.
Next several in vitro experiments were conducted in GBM cells infected with lentivirus-mediated FoxO3a-siRNA. To evaluate the function of FoxO3a in GBM radiosensitivity. A clonogenic assay was conducted and revealed that knockdown of FoxO3a had strikingly decreased both the numbers and size of colonies after radiation. The results of SGEA confirmed that DNA damage of GBM cells were aggravated in group of FoxO3a-KD compared with control, especially after radiation, which was consistent with our scenario. Normally, DNA damage will induce or enhance cell apoptosis, and could also lead to cell cycle arrest. We validated these changes by several apoptosis analysis that GBM cells in the FoxO3a-KD groups with radiation treatment had elevated apoptotic rate compared with the FoxO3a-NC groups. While with respect to the expressions of caspase-3, -7 and Bax, which were linked to the apoptosis pathway, they were significant upregulated in the FoxO3a-KD groups. This is in line with apoptosis assay.

Although, radiotherapy resistance of GBM is actuated by a complicated signaling network with functional redundancy. This study addressed whether FoxO3a participating in GBM radioresistance and the results established that suppression of FoxO3a increased radiosensitivity in GBM cells for the first time. Thus, it is conceivable that FoxO3a might be a potential diagnostic and therapeutic target for GBM radioresistance, which enriched the molecular mechanism of GBM radiotherapy resistance.

**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 author request.

**Competing interests**

There is no conflict of interest.

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**Authors' contributions**
LR and YL conceived and designed the research. ZHW and BC conducted most of the experiments. KL, HX, JX and ZW contributed new reagents or analytical tools and analyzed data and helped with some of the experiments. BC 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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Not applicable

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**Figures**

**Figure 1**

FoxO3a was significantly up-regulated in glioma cells after radiation. (A,B) Relative FoxO3a protein levels in T98G cells treated with 0 or 6Gy radiation. (C,D) Relative FoxO3a protein levels in U87-MG cells treated with 0 or 6Gy radiation. Error bars represent the mean±S.D. values.
Knockdown of FoxO3a in glioma cells. (A) Relative FoxO3a mRNA levels in T98G cells with or without FoxO3a KD. (B) Relative FoxO3a protein levels in T98G cells with or without FoxO3a KD. (C) Relative FoxO3a mRNA levels in U87-MG cells with or without FoxO3a KD. (D) Relative FoxO3a protein levels in U87-MG cells with or without FoxO3a KD. Error bars represent the mean±SD. values.
Figure 3

FoxO3a depletion reduced the clone formation ability of GBM cells after radiotherapy. (A-D) Clonogenic assays of T98G and U87-MG cells with or without FoxO3a knockdown were performed. The number of colonies (B, D) were determined. A multiple comparison test adjusted P-value of <0.05 was considered statistically significant. Error bars represent the mean±SD values.
Figure 4

FoxO3a depletion decreased DNA repair ability of GBM cells after radiation. (A) Cells treated with 6 Gy radiation present longer comet tails DNA damage in the T98G-NC/T98G-KD cells treated with 0 or 6 Gy radiation. (B) DNA damage in the U87-MG-NC/U87-MG-KD cells treated with 0 or 6 Gy radiation. More cells had comet tails in the KD group than in the NC groups. Error bars represent the mean±S.D. values.
Figure 5

FoxO3a depletion elevated the percentage of apoptotic cells after radiotherapy. (A,C) Upper right and low right corners represent late apoptotic, early apoptotic, respectively. (B,D) Apoptosis rate of FoxO3a-NC or KD T98G and U87-MG cells after radiation.
Figure 6

FoxO3a depletion increased apoptotic proteins after radiotherapy. (A, C) The amount of Caspase 3, Caspase 7, Bax and internal reference protein β-actin extracted from T98G or U87-MG cells with or without FoxO3a depletion treated with radiation calculated by gray scanning. (B, D) Relative expression of Caspase 3 in T98G or U87-MG cells with or without FoxO3a depletion treated with radiation.