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Original Article

IGF-1 partially counteracts X-ray-induced damage in brain cells proliferation in Tibet minipigs

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Abstract: The victims radiated by high-dose in a short time are often accompanied by symptoms of systemic reaction, such as systemic inflammation, nausea, slower growth and so on. X-ray exposure produce persistent difficulties in memory through an unknown mechanism. Insulin-like growth factor 1 (IGF-1), a protein encoded by this gene is similar to insulin in function and structure and is a member of a family of proteins involved in mediating growth and development. Here, in order to study the relationship between IGF-1 expression pattern and X-ray exposure in brain of Tibet Minipigs, not castrated male Tibetan minipig were chose as animal model, and exposed to the total body X-ray radiation at 0 and 10 Gy. The expression levels of IGF-1 were quantified in brain tissues of Tibetan minipig by qRT-PCR. Protein levels of IGF-1 and related signalling pathways were determined by western blot analysis. U251 cells were used for vitro experiments. Lentiviral vector had been successfully constructed for transfecting the cell line of IGF-1 over-expression and low-expression. The cell proliferation was evaluated by MTT assay, and the apoptosis was evaluated by flow-cytometry. We found that the proliferation of IGF-1 overexpression cell line was higher than the control group after X-ray irradiation ($P<0.05$). IGF-1, a molecule implicated in suppressing apoptosis, and partially counteracted the effects of high doses of X-ray exposure on brain cell proliferation.

Keywords: Radiation, Tibetan minipig, brain, proliferation, IGF-1

Introduction

Radiotherapy has been commonly used in treating cancer with the capacity to kill cancer cells through ionizing radiation [1-3]. However, the effectiveness of radiotherapy is usually limited by the potential injuries to normal tissue by ionizing radiation. To date, a lot of studies have been carried out to explore radiation-protective agents [4, 5]. Unfortunately, few agents have been approved by FDA for clinical practice [6]. It is therefore important to have a better understanding of which biological mechanisms play a pivotal role after the total body X-ray radiation in order to minimize normal tissue toxicity.

Radiation causes the loss of structure and function of organic macromolecular, including DNA and protein [7]. This loss of structure of the DNA molecule includes nucleotide changes or deletion, single-strand breaks or double-strand breaks [8]. During evolution, the cells have developed some mechanisms to repair the DNA damages and thus increase their own chance of survival. However, sometimes cells start the process of programmed death if their own DNA damages cannot be repaired. And during this process, radiation promotes a cellular state in which multiple DNA damages are made. Growth factors, hormones, neurotransmitters, and environmental factors influence the survival, proliferation, and differentiation of cells [9]. Insulin-like growth factor 1 (IGF-1), a protein encoded by this gene is similar to insulin in function and structure and is a member of a family of proteins involved in mediating growth and development. Although the association between IGF-1R expression and radiation res-

Table 1. Primer sequences of products expression

Gene name	Primer name	Primer sequence
IGF-1	Forward primer	5' ACAAGAACTACAGAATGTAGGAAGA 3'
	Reverse primer	5' AAGACAATGTTGGAATGTTTACT 3'
IGF-1R	Forward primer	5' ATGCTGTTTGAACTGATGCGCA 3'
	Reverse primer	5' CCGCTCGTTCTTGCGGCCCCCG 3'
GAPDH	Forward primer	5' CCTTCATTGACCTCAACTACAT 3'
	Reverse primer	5' CCAAAGTTGTCATGGATGACC 3'

ponse seems to exist [10-12], little is known about the association between IGF-1 expression and radiation response and the mechanisms behind this association, and the implication of IGF-1 in radiation response remains unclear. In earlier research, IGF-1 has been implicated in the radiation resistance of lung carcinoma in previous reports [13]. As a mitogen, IGF-1 can activate both signaling pathways through ligand binding to receptors (tyrosine kinase insulin-like growth factor receptor, IGF-1R). Once IGF-1 is bound to the IGF-1R, the progression of tumor is induced through activation of these pathways [14, 15].

Compared with small mammals such as mice, large mammal's models are superior in many aspects for the study of human diseases and pre-clinical therapies. The miniature pig is similar to human in anatomy, development, physiology, pathophysiology and disease occurrence, etc [16, 17], and thus had been chosen for this study. In this study, we aimed to investigate the potential molecular mechanism of the IGF-1 involve in X-ray radiation resistance in *vivo* and *vitro*, and to confirm whether IGF-1 can counteract X-ray-induced damage in brain cells proliferation in Tibet Minipigs.

Materials and methods

Tibetan minipig

Tibetan minipigs were purchased from the Laboratory Animal Center of Southern Medical University of China at 8-15 months of age. The average weight and height of the pigs were 22.36±7.74 kg and 82.88±9.13 cm, respectively. They were anesthetized with ketamine (0.05 ml/kg i.v.) before radiation exposure. The control group (n=5) was not exposed to radiation without any further processing. The radiation treatment groups (n=5) were irradiated with single doses of 10 Gy TBI using an 8-MV X-ray linear accelerator (Elekta Synergy Platform, ELEKTA Ltd, Sweden) at a dose rate of 255 cGy/min. The IGF-1 treatment groups (n=5) were injected through a needle placed into a vein with IGF-1 (25 μg/kg, Millipore, USA) before radiation. The Tibetan minipigs were killed by cardiac puncture after data collection and the brain tissue were dissected for the following experiment. The experimental protocol was approved by Institutional Animal Care and Use Committee of the Southern Medical University of China.

Weight and physical appearance

We measured the Tibetan minipig weights in all animals receiving X-ray radiation. Tibetan minipig was weighed prior to their X-ray radiation. And we also measured the Tibetan minipig for changes in activity and hair loss but these were not quantified.

Quantitative real-time PCR

The total RNA was isolated from Tibetan minipig brain tissue under X-ray radiation using Trizol reagent (Takara, Japan) according to the instructions. Quantitative real-time PCR (qRT-PCR) was carried out using SYBR PremixExTaq™ (Takara, Japan). SYBR Green qRT-PCR was performed and monitored with a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The primer sequences are shown in **Table 1**. The qRT-PCR results were analyzed and the relative CT (threshold cycle) values were converted into expression fold alterations.

Immunohistochemistry

To evaluate the expression of immune cell antigens using immunohistochemistry, 3 μm brain tissue sections of paraffin-embedded, formalin-fixed tissues were deparaffinized in xylene and rehydrated in a series of graded ethanol. Endogenous peroxidase activity was blocked by immersing sections in 3% H_2O_2 for 5 minutes. The sections were blocked in 10% fetal bovine serum for 10 minutes at room temperature and then incubated with primary antibodies to IGF-1 and IGF-1R (Cell Signaling) for 60 minutes of staining. Immunostaining was carried out using the IHC Kit (Beyotime, China) for 15 minutes

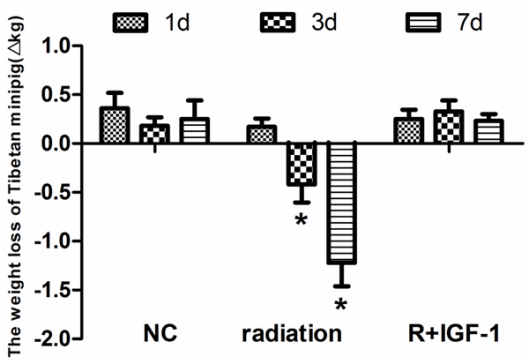

Figure 1. The effect of X-ray radiation to Tibetan minipig weight loss. *indicate $P<0.05$.

according to the manufacturer's protocol and finally visualized with diaminobenzidine. In addition, sections were then counterstained with hematoxylin. The normal tissue served as the positive control.

Cell culture and transfection

The human malignant glioma cell lines U251 were purchased from CICLR (Beijing, China). Cells were cultured with DMEM (Hyclone, USA) and supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma, USA), at 37°C, in 5% CO_2 incubator (Thermo, USA). Cells were seeded in a 100 mm^2 plate at 5×10^6 cells per plate 24 hours before transfection.

Lentiviral plasmid construction and cell infection

The human IGF-1 (Gen-Bank Gene ID: 3479) specific small interfering RNA (siRNA) sequence, which was designed with online software from Invitrogen, and the sequence of shRNA-IGF-1 sense: CCGGTGTTCAGGAAACAAGAACTACTCGAGTAGTTCTTGTTTCCTGAACTTTTTTG; and shRNA-IGF-1 antisense: AATTCAAAAAAGTTCAGGAAACAAGAACTACTCGAGTAGTTCTGTTTCCTGAACA. The shRNA-IGF-1 fragment was cloned into the pGLV3-GFP vector. The fragment of IGF-1 CDS was cloned into the Pglv5-GFP vector for overexpression. Cells were transfected using Lipofectamine 2000 (Invitrogen) and lentivirus vector. Cells were cultured in transfection media for 6 hours then in McCoy's 5A media with 10% FBS. 24 hours after the completion of transfection, cells were harvested for radiation treatment. The cells were irradiated with single doses of 5 Gy TBI using an 8-MV X-ray linear accelerator (Elekta Synergy Platform, ELEKTA Ltd, Sweden) at a dose rate of 255 cGy/min after lentivirus vector infection.

Cell proliferation assays

The Cell Proliferation Reagent Kit I (Roche, USA) was used to evaluate the cell proliferation after radiation treatment. cells were harvested 72 h after transfection and radiation treatment, and grown in the 48-well plates (5000 cells/well). Cell proliferation was recorded every 24 h according the manufacturer's protocol.

Flow-cytometric analysis of apoptosis

The cells were harvested 2 d after transfection by trypsinization and radiation treatment, then washed twice with washing buffer , and resuspended in binding buffer at 1×10^5 cells/ml. Cells double stained by FITC and PI underwent flow cytometry for apoptosis detection. All cells were divided into living cells, necrosis cells and apoptotic cells.

Western blotting

Different treated cells were cultured in 6-well plates at a density of 1×10^6 cells, cells were collected in lysis buffer, and the total protein was extracted. Approximately 50 μg of protein were electrophoresed by 12% SDS-PAGE and transferred to nitrocellulose membranes and incubated with rabbit anti-human monoclonal antibody against IGF-1, P53, Caspase3 and GAPDH (1:5000 dilution, Abcam, USA). HRP-labeled secondary antibodies (1:5000 dilution) were added for two hours at room temperature, followed by ECL. Quantity One v4.4.0 software (Bio-Rad, USA) was used to assay optical density of the IGF-1, P53 and Caspase3 bands and results were normalized to the expression of GAPDH.

Statistical analyses

Data in the tables and figures are expressed as means and their standard errors. All statistical analyses were performed using SPSS version 19.0 (SPSS Inc. Chicago, IL) and GraphPad Prism version 5.0 (GraphPad, San Diego, Calif) softwares. In all cases, *P* value less than 0.05 was considered statistically significant.

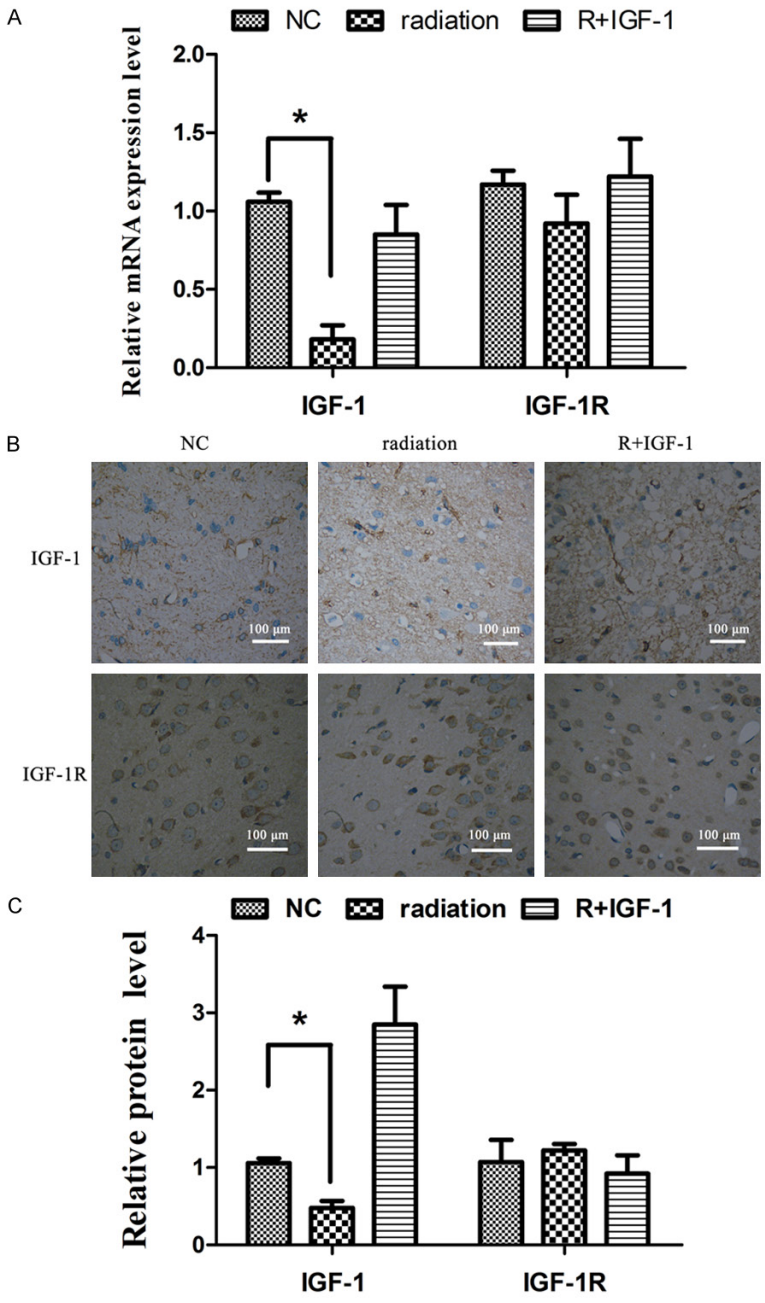

Figure 2. Effect of X-ray irradiation administration on IGF-1 expression. A. The relative mRNA expression level of IGF-1 and IGF-1R after X-ray irradiation administration on Tibetan minipig brain tissue. B and C. The relative protein expression of IGF-1 and IGF-1R were detected on Tibetan minipig brain tissue after X-ray irradiation administration. *indicate *P*<0.05. *Scale bars*, 100 μm.

Results

X-ray radiation causes weight loss

We compared weights of Tibetan minipigs receiving X-ray radiation over days 1, 3, and 7 in control-treated animals (**Figure 1**). Tibetan minipigs receiving the X-ray radiation experienced significant weight loss from day 1 to day 7 as revealed by treatment effect ($P<0.05$).

For the more, we observed Tibetan minipigs twice daily to determine other physical characteristics of Tibetan minipigs receiving X-ray radiation and control treatments in all experiments. None experienced hair loss or had apparent decrease in overall activity regardless of treatment group. None died following radiation.

IGF-1 is downregulated in Tibetan minipigs brain tissue after radiation treatment

The expression levels of IGF-1 in Tibetan minipigs brain tissue was assayed with qRT-PCR and immunohistochemistry, IGF-1 expression in brain tissue after radiation treatment at 10 Gy was significantly decreased (**Figure 2A-C**) in comparison to control. But there were no obvious differences about the expression of IGF-1R after radiation treatment. These findings support the hypothesis that downregulated IGF-1 may play a key role in X-ray irradiation.

IGF-1 suppress cell apoptosis in vitro

The flow cytometer was used to evaluate the apoptosis rate of U251 cells transfected by IGF-1 overexpression and low expression lentivirus vector after radiation treatment. The results shown in **Figure 3A**, **3B** demonstrated a significantly higher percentage of apoptotic cells for IGF-1 shRNA-treated cells compared to NC ($P<0.05$), and a significantly lower percentage of apoptotic cells for IGF-1 overexpression-treated cells compared to NC ($P<0.05$).

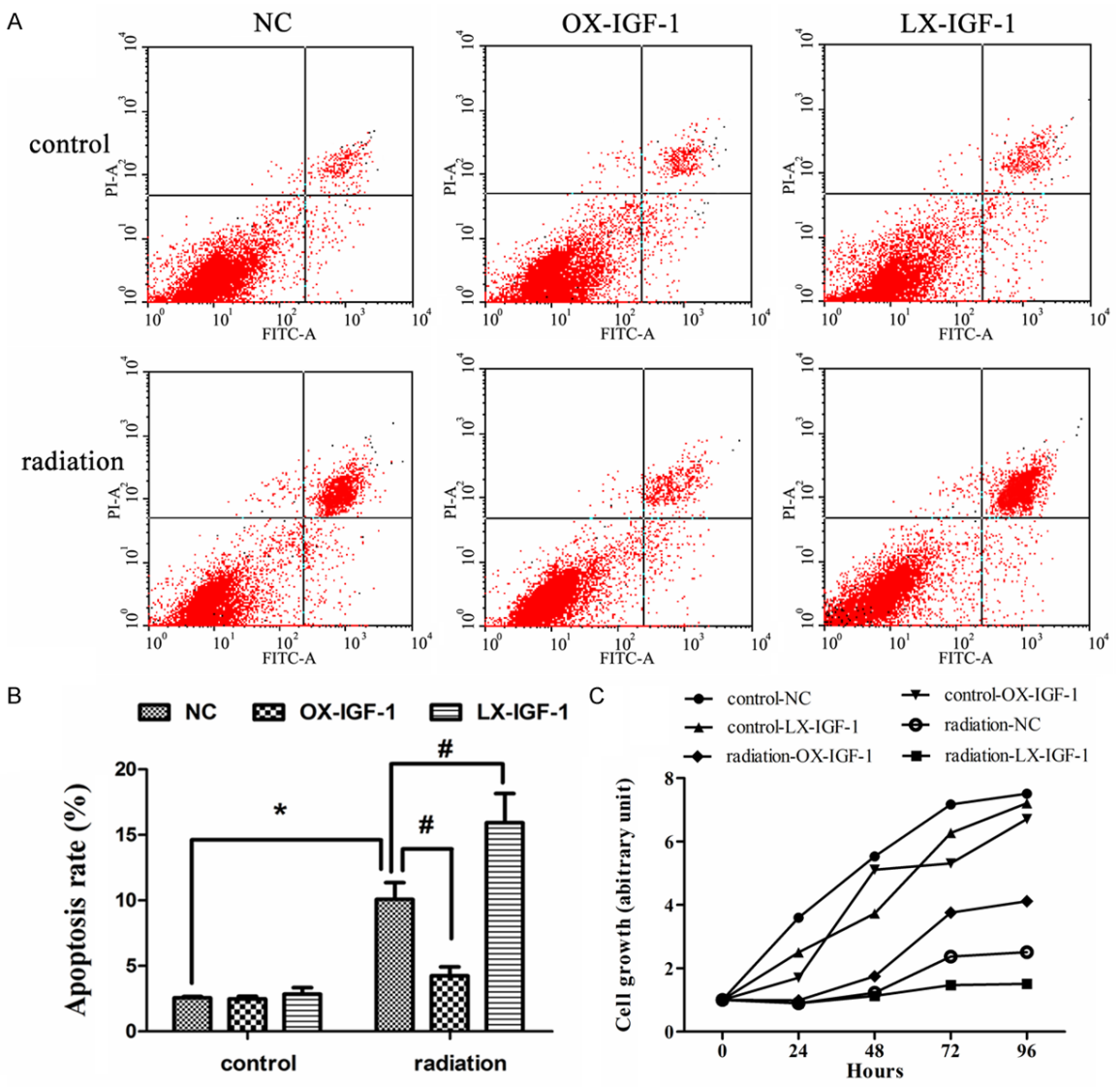

Figure 3. Effect of IGF-1 on cell apoptosis and proliferation after X-ray irradiation administration. A, B. Apoptotic rates were detected by flow cytometry in U251 cells. NC, normal control; OX-IGF-1, U251 cells with IGF-1 over-expression; LX-IGF-1, U251 cells with IGF-1 low-expression. Compared with control, $^{*}P<0.05$; compared with NC, $^{\#}P<0.05$. C. U251 cells was transfected with IGF-1 over-expression and IGF-1 low-expression vector, and MTT assays were performed to determine the proliferation in U251 cells. Experiments were performed in triplicate.

IGF-1 induce cell proliferation in vitro

For the more, we examined effect of IGF-1 overexpression and low expression on cell proliferation after radiation treatment. MTT assay showed that growth of U251 cells was affected by the transfection of IGF-1 overexpression and low expression lentivirus vector (**Figure 3C**). These results indicate that radiation treatment can suppress the growth and induce apoptosis in U251 cell, but IGF-1 can partially counteract X-ray-induced damage in U251 cell.

IGF-1 restores expression of P53 and Caspase3 after radiation treatment in vivo

The expression of P53 and Caspase3 was assayed by western blot and immunohistochemistry. The results suggested that the overexpression of IGF-1 can significantly inhibit protein expression of P53 and Caspase3. On the contrary, low expression of IGF-1 could significantly promote protein expression of P53 and Caspase3 (**Figure 4**). The findings further support the hypothesis that IGF-1 may affect the

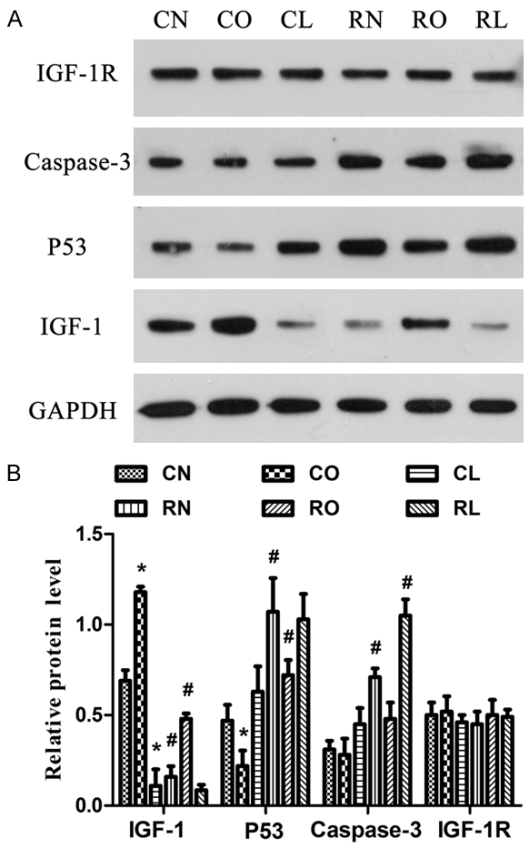

Figure 4. Effect of IGF-1 on protein expression after X-ray irradiation administration. CN, normal control; CO, U251 cells with IGF-1 over-expression; CL, U251 cells with IGF-1 low-expression; RN, U251 cells after X-ray irradiation administration; RO, U251 cells with IGF-1 over-expression after X-ray irradiation administration; RL, U251 cells with IGF-1 low-expression after X-ray irradiation administration. Compared with CN, $^{*}P<0.05$; compared with control, $^{\#}P<0.05$.

growth of U251 cells in vivo and restore X-ray-induced damage.

Discussion

Cell growth, cell cycle, apoptosis rate and DNA damage repair of mammals may be affected in the case of radiation exposure [18-20]. In recent years, Although more and more evidences have confirmed that IGF-1 may render cell stress responses [21-23], the mechanism inducing and stimulating apoptosis is very complex and still unknown. So, the specific expression of the IGF-1 may involve in response to radiation exposure.

The insulin-like growth factor 1 receptor (IGF-1R) is a cell membrane receptor widely distributed in human tissues. The structure and functions of IGF-1R have been deeply studied for the past 20 years [24]. IGF-1 binds with high affinity to IGF-1R [25]. Irradiation induced cell death is involving in gene activation, protein modification, ion concentration changes and many other factors. It is known that the radiation-induces cell death due to cellular DNA damage, which activates p53 gene expression and promotes apoptosis in thymocytes [26]. Further investigation demonstrated that the expression of P53 and Caspase3 can been suppressed after IGF-1 overexpression, and IGF-1 might responsible for the inhibition of U251 cells apoptosis. Our results suggest that IGF-1 affect the outcome of X-ray irradiation treatment. Due to their ease of culture, abundant availability, and low immunity, the regenerative capability of U251 cell have been applied extensively in medical fields. In this study, we demonstrated that IGF-1 could promote the proliferation and radioresistance of U251 cells in a manner which might be associated with the lower apoptosis.

Recent studies have demonstrated that IGF-1 could promote radioresistance of brain tumors [27]. However, the mechanisms underlying the relationship between IGF-1 and the radioresistance of cell remains unclear. IGF-1R is a transmembrane receptor tyrosine kinase involved in the development and progression of cancer whose activation strongly promotes cell growth and survival [28]. Studies indicated that IGF-1R has been associated with cell adhesion, cell motility, and tumor metastasis [29]. Additionally, IGF-1R activation has also been associated with enhanced radioresistance both in vitro and in vivo, which might be due to its overexpression post-irradiation [30]. In this study, we found that the expression of IGF-1 and IGF-1R can been suppressed after X-ray irradiation treatment. We also found that IGF-1 overexpression promoted proliferation and radioresistance in U251 cells, and induced the expression of IGF-1R. we revealed that there might be a potential molecular mechanism of the crosstalk between IGF-1 and the radioresistance. Our data showed that the IGF-1 expression was significantly downregulated after radiation both in Tibetan minipig brain tissue and U251 cells.

In summary, our results highlight the role of IGF-1 in the radioresistance of brain cells in *vitro* and *vivo*, and IGF-1 can induce U251 cell

proliferation and suppress U251 cell apoptosis by regulating the expression level of P53 and Caspase3 partially. However, further work is required to investigate the complex mechanism of IGF-1 and the radioresistance.

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Disclosure of conflict of interest

None.

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