ORIGINAL ARTICLE

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Synergistic effect of TRAIL and irradiation in elimination of glioblastoma stem-like cells

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Abstract

Glioblastoma multiforme (GBM) is the most common malignancy in central nervous system. A small subpopulation of GBM cells known as GBM stem-like cells (GSLCs) were supposed to be the most malignant cells among GBM cells as they are resistant to multiple therapies including radiotherapy. In this study, we set up two GSLCs cell lines from the two parental U87 and U251 glioma cell lines, and studied the expression of apoptosis-related genes alteration in GSLCs before and after irradiation. We found that one of the receptors of TNF-related apoptosis-inducing ligand (TRAIL), DR5, was dramatically up-regulated in GSLCs after irradiation (IR). Although GSLCs are resistant to both TRAIL and radiation treatment alone, the combined treatment with TRAIL and irradiation achieved maximum killing effect of GSLCs due to inducing the expression of DR5 and inhibiting the expression of cFLIP. Therefore, TRAIL and IR combined treatment would be a simple but practical therapeutic strategy for clinical application.

Keywords Glioblastoma · Stem-like cells · Radioresistance · TRAIL · Irradiation · DR5

Introduction

Glioblastomas (GBMs) are the most malignant type of gliomas, accounting for 60–70% of them [1]. In the last decades, increasing evidence has shown that a small subset of glioblastoma cells known as glioblastoma stem-like cells (GSLCs) [2, 3] possess capacity of self-renewal, multi-differentiation and oncogenicity [4, 5]. GSLCs are resistant to multiple therapies including radiotherapy and chemotherapy. Furthermore, this type of cell is the source of recurrence of malignant gliomas. Therefore, eradication of GSLCs is an imperative problem to be solved in the treatment of GBMs [6].

Radiotherapy is one of the most commonly used anti-cancer therapy methods. Since GSLCs are demonstrated to be resistant to irradiation (IR), the killing effects of radiotherapy are limited on these cells [2, 7]. Abundance of studies have clarified the underlying mechanisms involved in the radioresistance of GSLCs including preferential activation of DNA damage checkpoint [2], stem cell specific pathway like Notch [7], Wnt [8] and Hedgehog [9], pro-survival pathway [10], and so on. However, apoptosis-related signaling pathway has not been comprehensively studied in the research on the radioresistance of GSLCs, although cell apoptosis is the primary manner of radiation-induced cell death [11–13]. For the purpose of exploring the mechanisms of radioresistance of GSLCs through apoptosis pathway, we used human apoptosis PCR array to detect the expression of apoptosis-related genes in GSLCs after irradiation, and found that the mRNA level of TNFRSF10B (DR5) was the most impressively increased gene after irradiation in GSLCs.

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DR5 is one of the receptors of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). TRAIL has been demonstrated to have killing effects on malignant tumors [14–19]. TRAIL receptors include death receptor 4, 5 (DR4 and DR5) and decoy receptor 1, 2 (DcR1 and DcR2). DR4 and DR5 preferentially distribute in tumor cells, whereas DcR1 and DcR2 commonly express in normal cells [20]. When TRAIL binds with DR4 or DR5, it could induce cell apoptosis in tumor cells through activation of caspase-8 and caspase-3 [21], but it would be sequestered by DcR1 or DcR2 in normal cells [20]. Therefore, TRAIL has the merits that it could kill tumor cells without affecting normal cells [22]. Because of such merits, TRAIL has long been studied as a potential anti-cancer drug. However, many tumor cells including GSLCs are resistant to TRAIL [14, 16–18, 23]. Consequently, the researchers should focus on the mechanisms of TRAIL resistance and pursue a strategy to increase the sensitivity of GSLCs toward TRAIL.

Considering the DR5 mRNA was significantly up-regulated after irradiation in GSLCs, and it was the receptor of TRAIL, in this study, we investigated whether TRAIL in combination with irradiation could effectively eliminate GSLCs. Our data demonstrated that TRAIL in combination with irradiation not only reversed the resistant characteristics of GSLCs to irradiation and TRAIL, but also achieved synergistic sensitization to irradiation-induced apoptosis.

Materials and methods

Cell lines and cell culture

The glioblastoma cell lines U87 and U251 were originally obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) containing 10% (*v/v*) fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) at 37 °C in a humidified incubator with 5% CO_2 as previously described [24].

The primary glioblastoma stem-like cell lines, NCH-421k and NCH-644, were obtained from the Department of Neurosurgery at Heidelberg University. They were cultured in serum-free DMEM/F12 medium (Hyclone, Logan, UT, USA) comprising 20 ng/ml epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ, USA), 20 ng/ml basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, NJ, USA) and 20% BIT serum-free supplement (STEMCELL Technologies Inc., Vancouver, BC, Canada).

Enrichment, culture and identification of glioma stem-like cells

U87 and U251 (parental cell lines) were cultured in serum-free DMEM/F12 medium (Hyclone, Logan, UT, USA)

supplemented with bFGF (20 ng/ml) (Peprotech, Rocky Hill, NJ, USA), EGF (20 ng/ml) (Peprotech, Rocky Hill, NJ, USA), recombinant human leukemia inhibitor factor (rhLIF, 10 ng/ml) (Millipore, Billerica, MA, USA) and 1×B27 supplement (Gibco, Grand Island, NY, USA). They were cultured in an incubator with 5% CO_2 at 37 °C and passaged every 4–5 days. After 6 weeks, stem cells markers were analyzed by flow cytometry (FCM) and RT-PCR.

Reagents and antibodies

Recombinant human TRAIL/Apo2 Ligand was purchased from Peprotech (Rocky Hill, NJ, USA). PE-conjugated human CD133/1 antibody was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). PE-conjugated human DR5 and DR4 antibodies were purchased from BioLegend (San Diego, CA, USA). The following antibodies were used in western blot: anti-NF-κB/p65 1:1000 (Cell Signaling Technology (CST), Danvers, MA, USA), anti-p-NF-κB/p65 1:1000 (CST), anti-IκBα 1:1000 (CST), anti-p-IκBα 1:1000 (CST), anti-cFLIP (cellular FLICE-like inhibitory protein) 1:1000 (Abcam ab167409, Cambridge, MA, USA), anti-caspase-8 1:1000 (CST), anti-caspase-3 1:1000 (CST), anti-DR5 1:1000 (CST), anti-TNFRSF10A (DR4) 1:500 (ABGENT AP13702b, San Diego, CA, USA), anti-GAPDH 1:500 (Boster, Wuhan, China) and anti-β-actin 1:500 (Boster, Wuhan, China).

Quantitative RT-PCR

Total RNA from cultured cells was isolated with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols and quantified spectrophotometrically. cDNAs were synthesized from 1 μg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Quantitative real-time PCR analysis was performed with SYBR Green Real-Time PCR Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. DNA primer sequences were designed as follows: for human CD133, sense 5′-GGACCC ATTGGCATTCTC-3′ and antisense 5′-CAGGACACAGCA TAGAATAATC-3′; for human nestin, sense 5′-CTGCTA CCCTTGAGACACCTG-3′ and antisense 5′-GGGCTCTGA TCTCTGCATCTAC-3′; for human Sox-2, sense 5′-CCA ACTTTCCATTTTGTTCAGATAA-3′ and antisense 5′-CAG AGCCGAATCTTTTAAAATACAA-3′; for human GFAP (Glial fibrillary acidic protein), sense 5′-GCACGCAGTATG AGGCAATG-3′ and antisense 5′-TAGTCGTTGGCTTCG TGCTT-3′; for human GAPDH, sense 5′-CACCAGGGC TGCTTTTAACTCTGGTA-3′ and antisense 5′-CCTTGA CGGTGCCATGGAATTTGC-3′; for human DR5, sense 5′-CAACGCTTCCAACAATGA-3′ and antisense 5′-ACG TGCCTTCTTTACACTGA-3′; and for human DR4, sense

5′-TGTACGCCCTGGAGTGACAT-3′ and antisense 5′-CAC CAACAGCAACGGAACAA-3′. Each sample was run in triplicate. The real-time PCR reactions were performed as follows: one cycle of 95 °C for 60 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s. A data analysis of the quantitative real-time PCR was performed using CFX Connect Real-Time System (Bio-Rad Laboratories, Berkeley, CA, USA). Mean cycle threshold (Ct) values for the target genes were normalized to the endogenous control GAPDH. The relative levels of mRNA expression were quantified using $2^{-\Delta\Delta Ct}$ method.

Flow cytometry analysis

Flow cytometry was conducted on U87 and U87-sph cells to analyze the expression of CD133, DR5 and DR4. Cells were harvested and washed with PBS and resuspended with 100 µl PBS containing 0.5% bovine serum albumin (BSA). Then, the PE-conjugated antibodies were added to the cell suspension and incubated at 4 °C for 10 min. After twice washing with 0.5% BSA/PBS, the mean fluorescence intensity of cells was analyzed using FACS Calibur from BD Biosciences.

X-ray treatment

The cells were irradiated using RS2000 Biological X-ray Irradiator (Rad Source Technologies, Boca Raton, FL, USA) at a dose rate of 100 cGy/min. The medium was routinely changed after irradiation.

Apoptosis analysis by annexin V-FITC/PI staining

The cells including those suspended in the medium were harvested 24 or 48 h after treatment with TRAIL or irradiation. Then, the harvested cells were stained with an Annexin V-FITC/Propidium Iodide Kit (KeyGEN Biotech, Nanjing, China) and prepared for flow cytometry analysis according to the manufacturer's instructions. The cells treated with dose escalation of TRAIL were harvested for staining 48 h after treatment. The cells treated with X-ray with different doses were harvested 24 and 48 h after irradiation.

Cell counting kit-8 assay

The proliferation rates of the cells treated with irradiation were measured by cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). Cells were seeded at a density of 5×10^3/wells into poly-L-lysine (PLL)-coated 96-well plate. At different time points (0.25, 0.5, 1, 2, 3, 4, 5, and 6 day) after treatment with X-ray with different doses (2, 4, 6, and 8 Gy), CCK-8 was added to the medium of each well to incubate for 3 h at 37 °C. Then, the absorbance was measured by microplate reader at a wavelength of 450 nm with subtraction of baseline reading. Each time point was repeated five times. Cell viability was calculated by the following formula: cell viability (%) = A irradiated well/A unirradiated control well × 100%, where A is absorbance.

Real-time PCR apoptosis array

U87 and U87-sph were irradiated with a dose of 8 Gy. Twenty-four hours after irradiation, irradiated U87, irradiated U87-sph, untreated U87 and untreated U87-sph were harvested and total RNA was extracted and purified. Then, the total RNA was reverse-transcribed into complementary DNA (cDNA) using RT2 First Strand Kit (SABiosciences/ QIAGEN, Fredrick, MD, USA). Subsequently, the expressions of 84 apoptosis-related genes were analyzed using Human Apoptosis RT^2 Profiler™ PCR Array (SABiosciences/QIAGEN, Fredrick, MD, USA) following the manufacturer's instructions.

Western blot analysis

After treatment, the cells were lysed in pre-cold RIPA buffer (Beyotime, Shanghai, China) with protease inhibitor PMSF (1:100) and phosphatase inhibitor cocktail (1:100) at 0 °C for 30 min. The protein concentration of each sample was measured using BCA protein assay kit (Beyotime). The protein samples were added into 5 × loading buffer and subjected to heat denaturation at 100 °C for 5 min. Then, the protein (40 µg) was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Following blocking with TBST (Tris-buffered saline with 0.1% Tween-20) containing 5% nonfat milk at room temperature for 1 h to block nonspecific binding, the membranes were incubated with primary antibodies at 4 °C overnight. Then, the membranes were washed with TBST three times and incubated with corresponding second antibodies (horseradish peroxidase-conjugated goat anti-rabbit/mouse antibodies, Proteintech, Chicago, IL, USA) at a dilution of 1:2000 at room temperature for 1 h. After being washed with TBST for three times, the protein–antibody complexes were visualized using an enhanced chemiluminescent (ECL) detection kit (Menlo Park, CA, USA). The images were quantified by Quantity One software (Bio-Rad).

Caspase activity assay

Caspase-3 and caspase-8 activities were measured by Caspase 3 or Caspase 8 Activity Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocols. Briefly, cells were lysed in lysis buffer and the cell lysates were incubated with caspase-3 specific substrates (Ac-DEVD-pNA) or caspase-8 (Ac-IETD-pNA) specific substrates at 37 °C

for 60 min. The absorbance was measured by microplate reader at a wavelength of 405 nm with subtraction of baseline reading. Caspase activity was calculated according to the standard curves and protein concentration.

Clonogenic assay

The cells were seeded at a specific density according to the dose of X-rays received onto six-well plates coated with laminin and incubated 12 h prior to irradiation. Twenty-four hours after irradiation with 0, 2, 4, 6, 8 and 10 Gy, cells were treated with or without 50 ng/ml TRAIL. Then, the cells were cultured at 37 °C for 14 days, fixed with methanol and stained with 0.5% (w/v) crystal violet subsequently. Colonies consisting of 50 cells or more were counted to calculate plating efficiency (PE) and survival fraction (SF). SF was defined as follows: SF = (mean colonies number)/(initial number of plating cells × plating efficiency). Cell survival curves were determined with a multi-target single-hit model using GraphPad Prism software (San Diego, CA, USA).

TRAIL and irradiation combined treatment

The cells were seeded into six-well plates and separated into four groups: non-treated group (control), TRAIL (50 ng/ml) alone group, irradiation (IR) (8 Gy) alone group and TRAIL (50 ng/ml) combined IR (8 Gy) group. For TRAIL alone group and IR alone group, cells were harvested 48 h after treatment for protein extraction or apoptosis measurement. For TRAIL combined IR group, cells were treated with TRAIL 24 h after irradiation and harvested for experiments 48 h after irradiation [25].

Statistical analysis

The results are presented as mean ± standard error. Differences between groups were evaluated for significance by two-tailed Student's t test or one-way analysis of variance (ANOVA) test using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Probability (P) values < 0.05 were considered statistically significant.

Results

Enrichment of glioma stem-like cells from glioblastoma cell lines

Both U87 and U251 cells were turned into sphere formation 24 h after they cultured in serum-free medium supplemented with EGF, bFGF, B27 and LIF (GSLC medium) (Fig. 1a). These spheres could stably proliferate in a time-dependent manner with a growing number and volume. In contrast, the parental U87 and U251 cells under the traditional serum-grown culture condition do not form spheres, indicating that the spheres but not serum-grown cultures retain the self-renewal capacity of cancer stem cells. Six weeks after GBM cell lines cultured in GSLC medium, quantitative real-time RT-PCR showed that the expression of stemness-related genes including CD133, Nestin and Sox-2 was significantly higher in U87-sph and U251-sph than in their parental GBM cell lines; however, GFAP, a differentiated marker of stem cell, was significantly lower in U251-sph than in their parental GBM cell (P < 0.05) (Fig. 1b). To further examine the stemness of spheres, we analyzed the expression of the stem cell surface markers CD133. Flow cytometry analysis showed that the percentages of CD133 positive cells in U87-sph and U251-sph were (9.42 ± 0.25) % and (10.38 ± 0.38) % (Fig. 1c), which showed significantly higher than their parental glioma cells (P < 0.01) (Fig. 1d).

### Glioma stem-like cells are resistant to irradiation-induced apoptosis

As the property of resistance to irradiation therapy is one of the major clinical criteria to characterize "tumor stem-like cells," we next determined the effect of irradiation on glioma stem-like cells. Because the baseline apoptotic rates are significantly different between GSLCs and their parental cells, we set the ratio of post-radiation apoptotic rates/baseline apoptotic rates as the indicator of radiation effect. As data shown in Fig. 2a, b, the irradiation effect was significantly lower in U87-sph and U251-sph compared with their parental cells (P < 0.05), no matter the different time (24 or 48 h) and the different doses (4 or 8 Gy), which further confirmed that GSLCs are resistant to irradiation-induced apoptosis.

We also detected the cell viabilities after irradiation in U87-sph, U251-sph and their parental cells. The cell viabilities of both U87 cells and U251 cells were predominantly decreased when irradiated with 8 Gy X-ray on Day 1, and were sharply decreased at all doses on Day 2 or later (P < 0.05). However, the decreased cell viabilities of both U87-sph and U251-sph were taken place much later than their parental cells, indicating that both U87-sph and U251-sph are more resistant to irradiation than their parental cells (Fig. 2c, d).

DR5 was down-regulated in glioma stem-like cells

To explore the mechanisms of the radioresistance of GSLCs, we used real-time PCR apoptosis array to compare the difference of apoptosis-related genes expression between GSLCs and their parental cells after irradiation. Based on the Ct values of the 84 apoptosis-related genes, we found that the expression of apoptosis-related genes in U87-sph was different from that in U87 cells after

Fig. 1 Enrichment and identification of glioblastoma stem-like cells (GSLCs). **a** Representative images of U87, U87-sph, U251 and U251-sph. (scale bar, 50 μm). **b** The expression of CD133, nestin, Sox-2 and GFAP in GSLCs was measured in triplicate using real-time PCR. U87 and U251 served as a control, respectively. Expression levels are presented as the fold changes of the corresponding control values (**$P < 0.01$, *$P < 0.05$, vs. control). **c** Representative flow cytometry histograms showing expression of CD133 in U87, U87-sph, U251 and U251-sph. **d** Bar charts represent mean rates of CD133 positive cells ± SEM from three independent experiments (**$P < 0.01$, vs. U87 or U251)
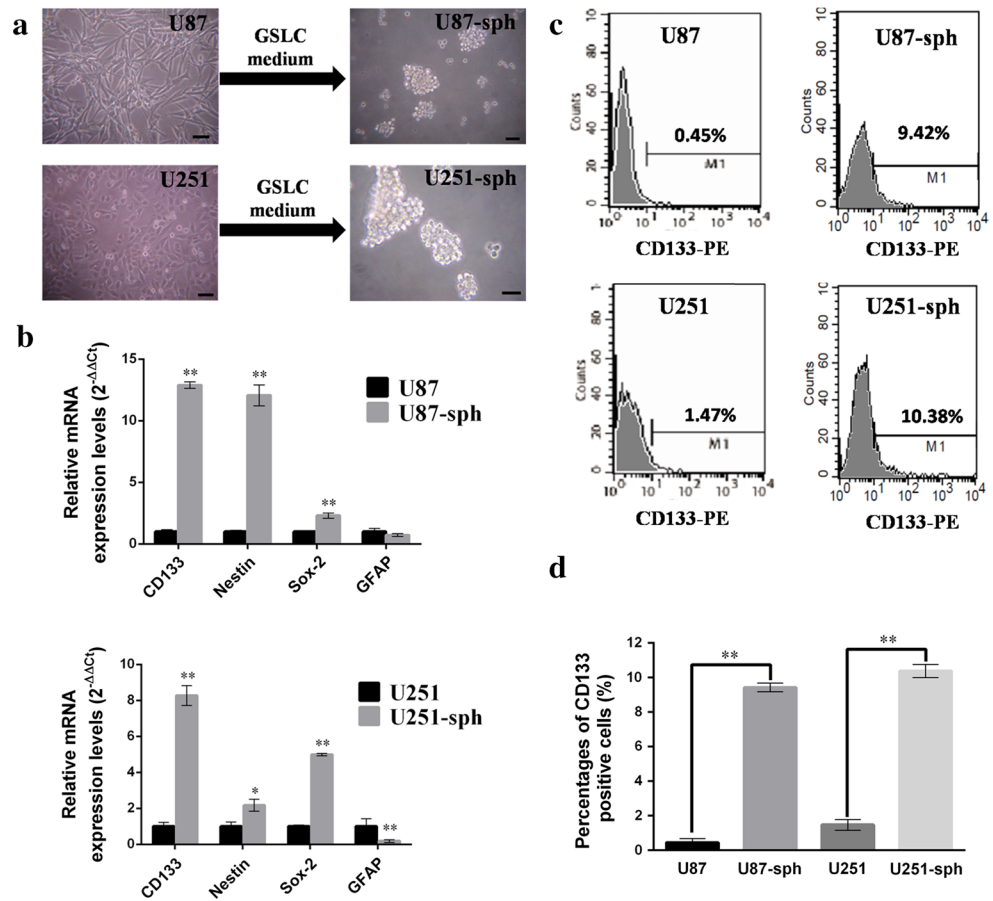
radiation (Online Resource 1) (Fig. 3a). Of note, the most dramatic alteration was the expression of TNFRSF10B (DR5) gene, which was increased ($6.335 ± 0.333$)-folds in irradiated U87-sph and ($1.210 ± 0.210$)-folds in irradiated U87 cells, compared with the untreated control group (Fig. 3b). This discrepant increase in DR5 between U87-sph and its parental cells after irradiation suggested that DR5 may involve in the radioresistance of GSLCs.

To clarify this, we examined the expression of DR5 in the U87-sph and U251-sph compared with their parental cell lines in both mRNA and protein levels. Real-time PCR showed that the mRNA level of DR5 in U87-sph and U251-sph was both significantly decreased compared to their parental glioma cells (Fig. 3c). This result was further confirmed by flow cytometry analysis, as the data shown that sixfolds decrease in U87-sph and 2.4-folds decrease in U251-sph compared with their parental cells (Fig. 3d, e). Furthermore, the sharply down-regulated DR5 expression in U87-sph and U251-sph was clearly seen by western blot assay (Fig. 3f).

Glioma stem-like cells are resistant to TRAIL-induced apoptosis

Since it was shown the expression of DR5 was lower in GSLCs, we further studied the effects of TRAIL, the ligand of DR5, exerted on GSLCs. When treated with TRAIL at concentrations of 25, 50, 100 and 500 ng/ml, the apoptosis rate was shown in a dose-dependent increased manner in the parental GBM cells, but not in the two GSLCs. There was a significant difference between the GSLCs and their parental cell lines when treated with TRAIL at a concentration more than 50 ng/ml (Fig. 4a). These results indicated that GSLCs are resistant to TRAIL-induced apoptosis.

Then, we performed western blot to further study the underlying mechanisms. As the literature showed that NF-κB pathway, cFLIP and caspase proteins involved in the TRAIL-induced apoptosis [26, 27], we then detected the expression of these proteins by western blot. The data showed that after treatment with dose escalation of TRAIL, the expression of p-NF-κB/p65 and p-IκB was
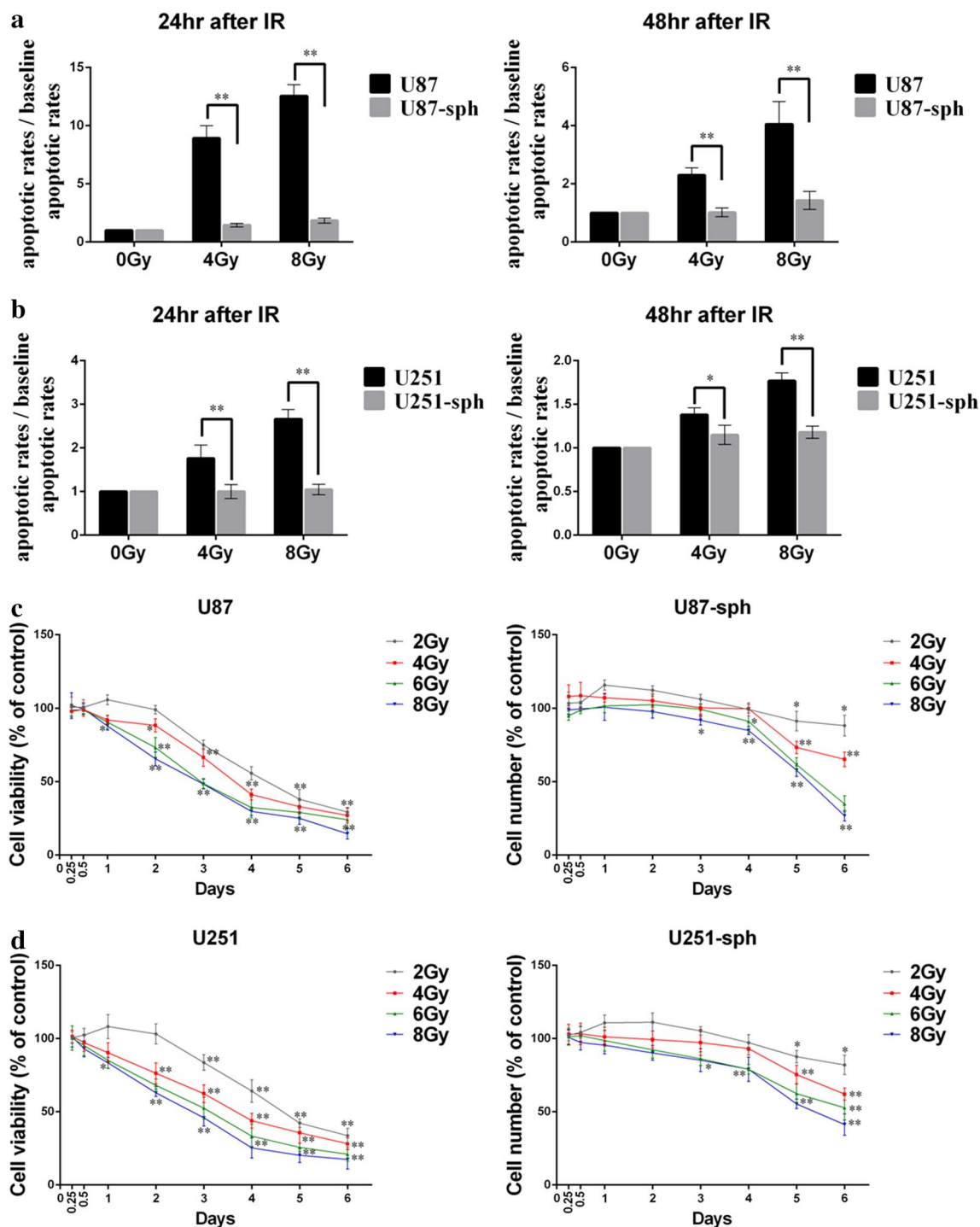
Fig. 2 Glioblastoma stem-like cells are resistant to irradiation-induced apoptosis. **a**, **b** The cells were irradiated with X-ray with a dose of 4 or 8 Gy and harvested 24 or 48 h after treatment. Then, the cell apoptosis was assessed by Annexin V-FITC/PI assay. The bar charts show the mean cell apoptosis of three independent experiments. The vertical axis represents apoptotic rates/baseline apoptotic rates. Unirradiated cells served as a control (**$P < 0.01$, *$P < 0.05$, vs. U87 or U251). **c**, **d** The cell viability was measured by CCK-8 assay at different time points after treatment with X-ray with different doses (2 Gy, 4 Gy, 6 Gy, 8 Gy). The relative cell viability (%) is represented as a percentage in comparison with the control group (unirradiated group) (**$P < 0.01$, *$P < 0.05$, vs. control, $n = 3$)

Fig. 3 Expression of DR5 in glioblastoma stem-like cells (GSLCs) and their parental glioblastoma (GBM) cell lines. **a** U87 and U87-sph were irradiated with 8 Gy. Twenty-four hours after irradiation, irradiated U87-sph (U87-sph IR), irradiated U87 (U87 IR), untreated U87-sph and untreated U87 were all subjected to real-time PCR apoptosis array. The expressions of 84 apoptosis-related genes were analyzed and a heatmap was generated to display the differentially expressed genes. **b** The expression of DR5 mRNA in the cells in (**a**) was detected in triplicate using real-time PCR. Untreated cells served as a control. Expression levels are presented as the fold changes of the corresponding control values (**$P < 0.01$, vs. control). **c** The expression of DR5 was detected in triplicate using real-time PCR. U87 or U251 served as a control. Expression levels are presented as the fold changes of the corresponding control values (**$P < 0.01$, vs. control). **d** Representative flow cytometry histograms showing expression of DR5 in U87, U87-sph, U251 and U251-sph. **e** Bar charts represent mean rates of DR5 positive cells ± SEM from three independent experiments (**$P < 0.01$, vs. U87 or U251). **f** The total cell lysates of U87, U87-sph, U251 and U251-sph were subjected to western blot using DR5 antibody. GAPDH served as an internal loading control
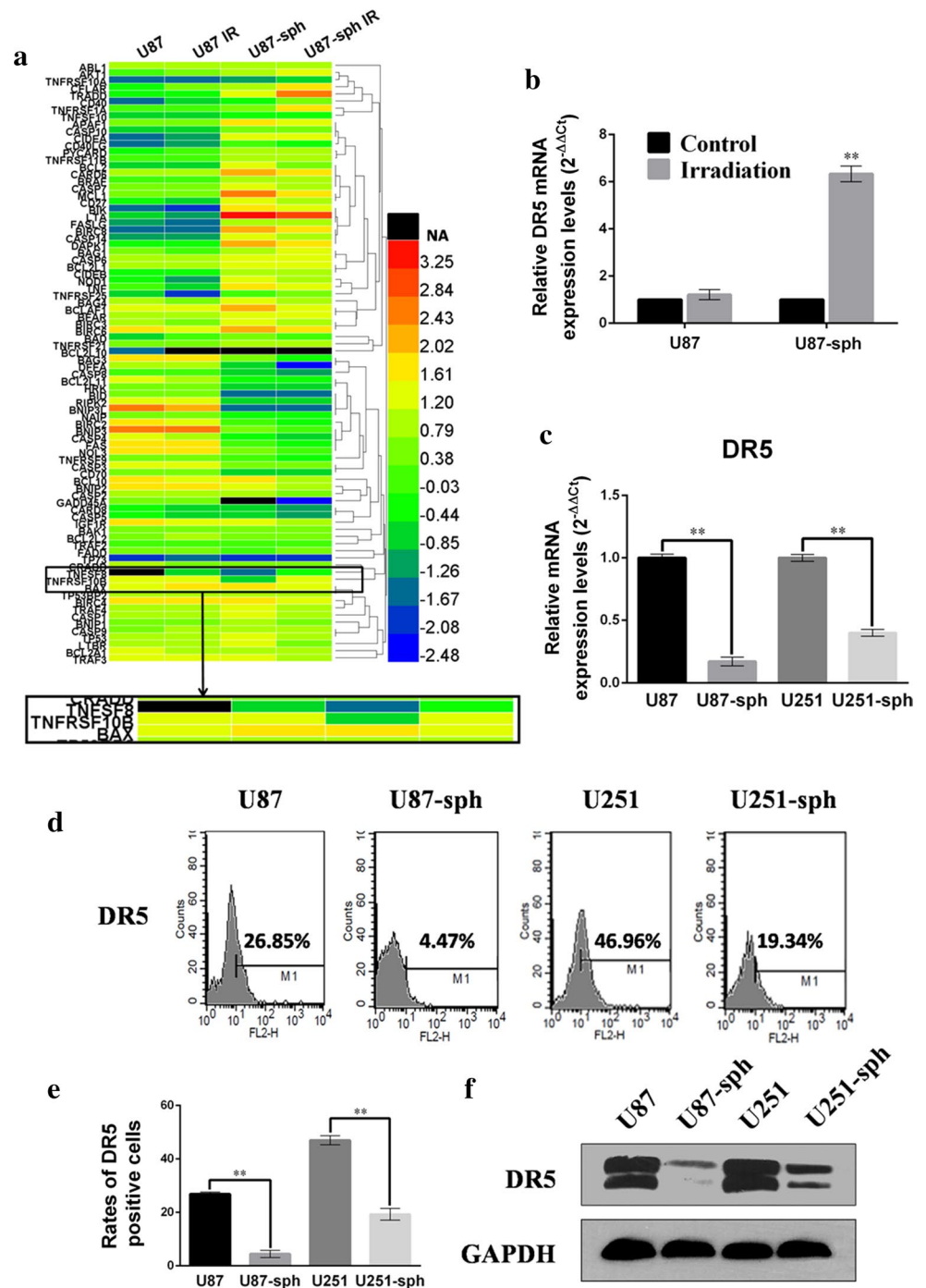
clearly up-regulated in U87-sph, suggesting that NF-κB pathway was activated in U87-sph after treatment with TRAIL. Compared with U87-sph, the activation of NF-κB pathway was not obviously in U87 cells after TRAIL treatment. On the contrary, cFLIP was decreased dose dependently in U87 cells compared with unchanged in U87-sph cells followed by TRAIL treatment, which was the same trend as the expression of caspase-3 and caspase-8 in

these two paired cell lines. However, the basal level of caspase-8 was significantly lower in U87-sph than in its parental glioma cells. Cleaved (activated) caspases were significantly increased in U87 cells after TRAIL treatment, but not detectable in U87-sph (Fig. 4b). Caspase activity assay also showed that when treated with dose escalation of TRAIL, caspase-3 and caspase-8 activities in U87 cells were much higher than those in U87-sph cells (Fig. 4c).
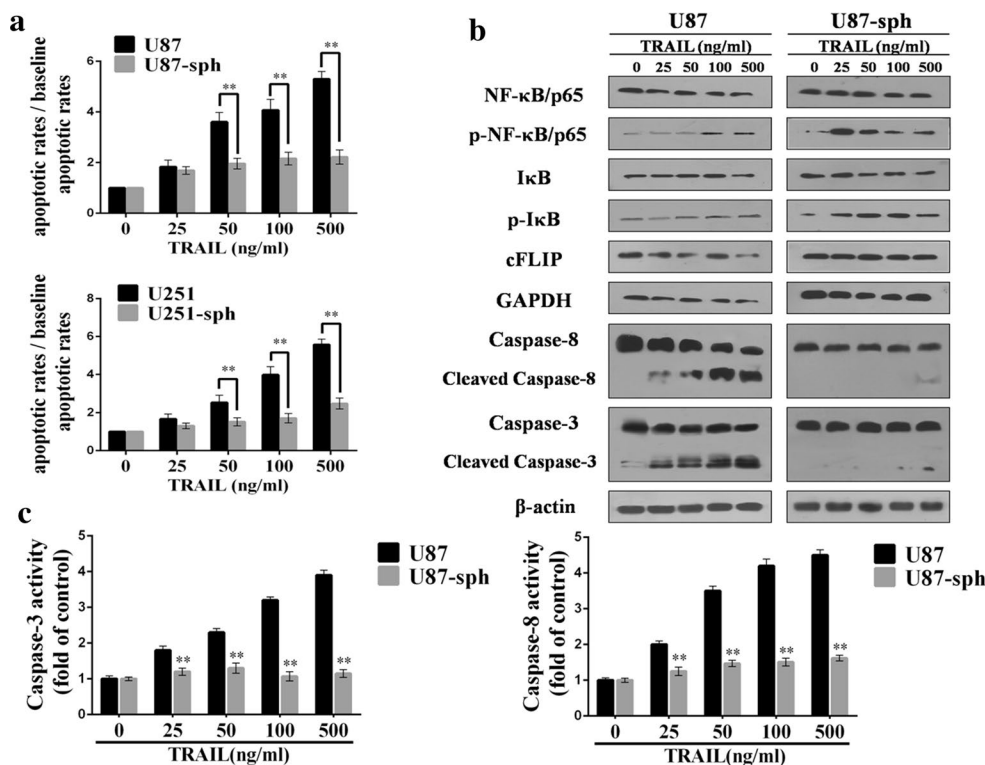
Fig. 4 Glioblastoma stem-like cells are resistant to TRAIL-induced apoptosis. **a** The cells were treated with dose escalation of TRAIL and harvested 48 h after treatment. Then, the cell apoptosis was assessed by Annexin V-FITC/PI assay. The bar charts show the mean cell apoptosis of three independent experiments. The vertical axis represents apoptotic rates/baseline apoptotic rates. Untreated cells served as a control (**$P < 0.01$, vs. U87 or U251). **b** The cells were treated with dose escalation of TRAIL. After cells were cultured for

48 h, the cell lysates were extracted and detected by western blot for the expression of NF-κB/p65, p-NF-κB/p65, IκB, p-IκB, cFLIP, caspase-8, cleaved caspase-8, caspase-3 and cleaved caspase-3. GAPDH and β-actin served as an internal loading control. **c** U87 and U87-sph cells were treated with dose escalation of TRAIL. After cells were cultured for 48 h, caspase-3 and caspase-8 activities were measured. Untreated cells served as a control. Data from three independent experiments are presented as the mean ± SEM (**$P < 0.01$, vs. U87)

DR5 was up-regulated in glioma stem-like cells after irradiation

Although the baseline of DR5 is very low in GSLCs compared with their parental cells, it was increased much higher in GSLCs than in the parental cells after irradiation using PCR apoptosis array analysis and real-time PCR. The expression of DR5 was further confirmed by western blot after 8 Gy irradiation 12–120 h (Fig. 5a). As shown in Fig. 5b, d, e, expression of DR5 was gradually increased in the parental cells; moreover, it was sharply increased in GSLCs both by western blot and FACS analyses. However, another receptor of TRAIL, DR4 expression, was very low in both GSLCs and non-GSLCs, especially lower expression was seen in the cell surface and was decreased after irradiation in U87-sph cells (Online Resource 2). In order to further determine the expression of DR5 in GSLCs after irradiation, we studied the expression of DR5 after irradiation by western blot in three other GSLCs including two primary GSLCs. The results also showed that DR5 was up-regulated after irradiation in all of the three GSLCs (Fig. 5c).

Following by determined the signaling-related protein, we found that the expression of p-NF-κB/p65 and p-IκB was both up-regulated with time after irradiation in U87-sph and the parental cells, although they were very low in U87 cells. And the expression of cFLIP was down-regulated with time-dependent manner in U87, while up-regulated in U87-sph cells after irradiation. The expression of cleaved caspase-8 was apparently up-regulated in U87, but was barely detectable in U87-sph after irradiation (Fig. 5a).

TRAIL synergistically sensitized irradiation-induced apoptosis in GSLCs by increasing DR5 expression and decreasing cFLIP expression

Considering that DR5 was down-regulated in GSLCs which was one possible reason of the resistance to TRAIL, and it could be induced dramatically after irradiation, we hypothesized that the combined treatment with irradiation and TRAIL could possibly sensitize GSLCs to apoptosis. Indeed, significant cytotoxicity was observed when combined with irradiation and TRAIL treatment in GSLCs (Fig. 6a), and
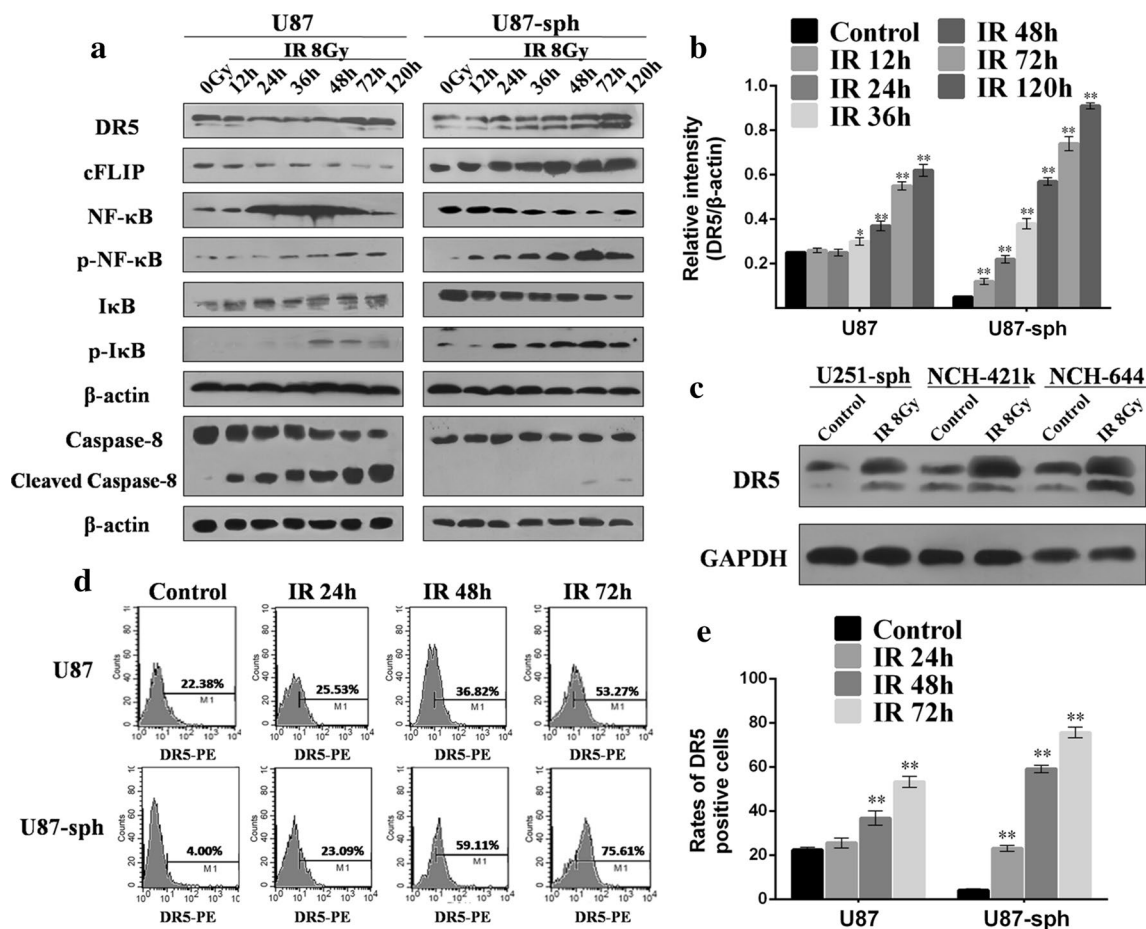
Fig. 5 Expressions of DR5 and signaling-related proteins in glioblastoma stem-like cells after irradiation. **a** Cell lysates were extracted at different time points after treatment with 8 Gy X-ray and were analyzed by western blot for the expression of DR5, cFLIP, NF-κB, p-NF-κB, IκB, p-IκB, caspase-8 and cleaved caspase-8. β-actin served as an internal loading control. The experiments were performed three times. **b** The bar chart shows quantification of the bands intensity from figure a. Data from three independent experiments are presented as the mean ± SEM (**$P < 0.01$, *$P < 0.05$, vs. control, $n = 3$). **c** Cell lysates of U251-sph, NCH-421k and NCH-644 were extracted 48 h after treatment with 8 Gy X-ray and were analyzed by western blot for the expression of DR5. GAPDH served as an internal loading control. The experiments were performed three times. **d** The cells (U87, U87-sph) were subjected to flow cytometry analysis at different time points after irradiated with 8 Gy X-ray. Representative histograms show expression of DR5 in irradiated cells and control cells (unirradiated cells). **e** Bar chart represents mean rates of DR5 positive cells ± SEM from three independent experiments (**$P < 0.01$, vs. control, $n = 3$)

it was further confirmed by flow cytometry and clonogenic assay. The ratio of apoptotic rates/baseline apoptotic rates in TRAIL combined IR treatment group was predominantly higher than that in TRAIL treatment alone group and IR treatment alone group in U87-sph cells ($P < 0.01$), and the same result was seen in U251-sph (Fig. 6c). Furthermore, TRAIL treatment reduced the cell survival fraction after irradiation at all doses in both GSLCs and their parental cells ($P < 0.01$). Similar results were obtained in two primary GSLCs (Fig. 6b).

To explore the molecular mechanisms of TRAIL in increasing the sensitivity of GSLCs to irradiation, cells were separated into four groups as mentioned above. In both U87-sph and U251-sph cells, although 50 ng/ml TRAIL did not significantly

increase the expression of DR5, a significant increase in DR5 was found after combined TRAIL and irradiation treatments. Furthermore, expression of signaling-associated proteins such as cFLIP, NF-κB, and caspase was determined by western blot in these cells (Fig. 6d, e). Expression of the anti-apoptotic protein cFLIP was significantly lower in the combined treatment group than the group only treated with TRAIL or irradiation in U87-sph and U251-sph cell lines ($P < 0.01$). Expressions of caspase-8 and caspase-3, other important members known to the apoptosis pathway, were also significantly decreased in the combined treatment groups, and the expressions of activated (cleaved) caspases were significantly increased in the combined treatment group. However, expressions of p-NF-κB/p65 and p-IκB did not change among different treatment groups
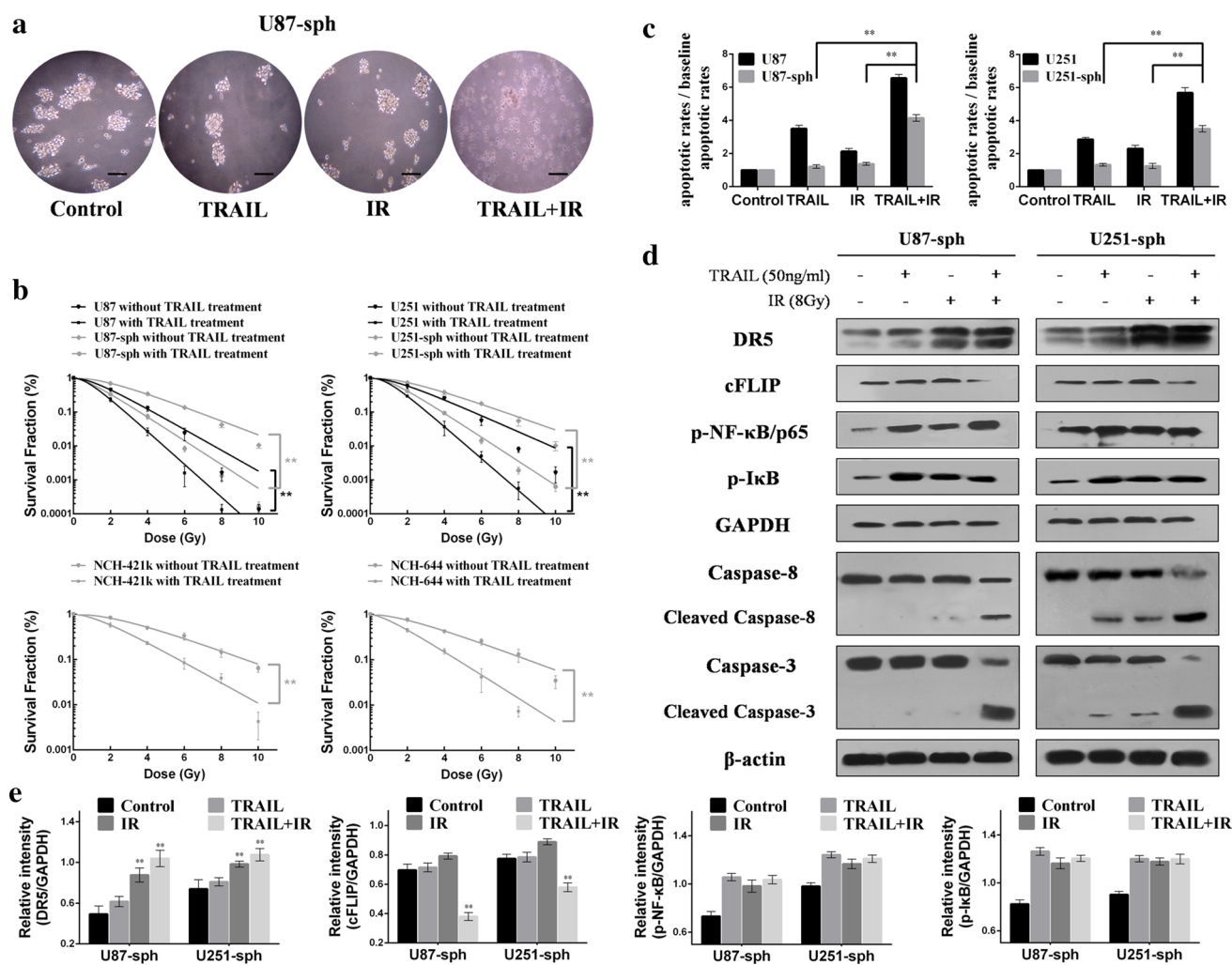
Fig. 6 Synergistic effects of TRAIL and irradiation (IR) in inducing apoptosis of glioblastoma stem-like cells. **a** The cells were divided into four groups: control group (without treatment), TRAIL alone group (50 ng/ml), IR alone group (8 Gy) and TRAIL+IR group. Forty-eight hours after treatment, representative images of the four cell groups were displayed. (scale bar, 50 μm). **b** Survival curves derived from clonogenic assay of the cells (U87, U87-sph, U251, U251-sph, NCH-421k and NCH-644) irradiated with different doses of X-ray (0–10 Gy) and treated with or without TRAIL (50 ng/ml). Error bars represent mean±SEM from three independent experiments (**$P < 0.01$, vs. cells without TRAIL treatment, $n = 3$). **c** The cell apoptosis of the four cell groups was assessed by Annexin

V-FITC/PI assay. The bar charts show the mean cell apoptosis of three independent experiments. The vertical axis represents apoptotic rates/baseline apoptotic rates (**$P < 0.01$, vs. TRAIL alone group or IR alone group, $n = 3$). **d** The cell lysates (U87-sph and U251-sph) of the four cell groups were detected by western blot for the expression of DR5, cFLIP, p-NF-κB/p65, p-IκB, caspase-8, cleaved caspase-8, caspase-3 and cleaved caspase-3. GAPDH and β-actin served as an internal loading control. The experiments were performed three times. **e** The bar chart shows quantification of the bands intensity from figure **d**. Data from three independent experiments are presented as the mean±SEM (**$P < 0.01$, vs. TRAIL alone group or IR alone group, $n = 3$)

(Fig. 6d, e). Together, these findings indicate that TRAIL-sensitized irradiation-induced apoptosis might depend mainly on

inducing the expression of DR5 and inhibiting the expression of cFLIP in GSLCs.

Discussion

In this study, we tried to explore a new practical clinical application to eradicate glioblastoma stem-like cells. To the best of our knowledge, this is the first comprehensive report on the study of the synergistic effects of TRAIL and X-ray on elimination of GSLCs [25, 28]. GSLC was the most vicious cell type in GBM cells, involving in treatment resistance and recurrence of GBMs [2, 5]. In order to study the properties of GSLCs, we firstly enriched them from two GBM cell lines using serum-free medium supplemented with growth factors as the literature reported [29–31]. CD133 is still the most commonly used cell markers of glioma stem cells [6, 32] despite some disputes [33]. In our study, we also used nestin and sox-2, another two commonly used stem cell markers [34], to identify whether the enriched cells possessed properties of glioma stem cells. Considering that both RT-PCR and FCM results showed that the stem cell markers mentioned above were up-regulated, the enriched cells could be mainly considered as glioma stem-like cells.

Radiotherapy is one of the most clinical commonly used anti-cancer therapy methods. TRAIL has entered into phase II clinical trial in non-small cell lung cancer (NCT00508625) because it has the merits of inducing apoptosis in varieties of malignant tumors sparing normal tissues [14–18, 22]. However, these two commonly used treatments do not work for GSLCs. In this study, we broke through the restriction of traditional research on GSLCs and analyzed the discrepant alteration of apoptosis-related genes between GSLCs and its parental cells before and after irradiation. Of note, DR5 was the most predominantly up-regulated gene with time-dependent manner in GSLCs, but was only slightly up-regulated in non-GSLCs after irradiation. The modulation mechanisms of DR5 expression are still obscure at present. Previous reports have suggested that activation of NF-κB pathway could up-regulate DR5 expression [35]; therefore, irradiation possibly increases the expression of DR5 through activation of NF-κB pathway. This is possibly one mechanism of up-regulation of DR5 after irradiation in GSLCs. However, further more studies should be done. Moreover, it is very interesting that the expression of DR5 was much lower in GSLCs compared to non-GSLCs as previously reported [23]. Since TRAIL-induced apoptosis is mainly through TRAIL/DR5 pathway, low expression of DR5 could directly lead to TRAIL insensitivity [25], and this is probably the primary reason for TRAIL resistance in GSLCs. Considering that DR5 was expressed fairly low in GSLCs and could sharply increase after irradiation, the combined TRAIL with irradiation achieved the maximum killing effect on GSLCs. This simple but practical treatment not only reversed the resistant characteristics of GSLCs to irradiation and TRAIL, but also achieved synergistic sensitization to irradiation-induced apoptosis. Therefore, combined treatment with TRAIL and irradiation could be considered as a potential component of a comprehensive strategy for clinical application of tumor therapy.

Data show that DR5 expression was increased after irradiation in GSLCs, and it was increased even higher after combined treatment. Thus, DR5 up-regulation might be a mechanism for eradication of GSLCs through TRAIL and IR combined therapy. Although cFLIP expression was still high after treated with TRAIL or irradiation in GSLCs, in combined treatment with TRAIL and irradiation, its expression was apparently decreased in the two GSLCs. Similar results showed that the expressions of caspase-8 and caspase-3 were also decreased after combined treatment in GSLCs, which could be explained by the fact that cFLIP could inhibit activation of caspase-8 and the degradation of cFLIP could accelerate the cleavage of caspase-8 thus inducing cell apoptosis [36, 37]. Additionally, NF-κB pathway featured protein p-NF-κB and p-IκB was activated after either TRAIL or irradiation treatment. Activation of NF-κB pathway could inhibit apoptosis via expression of anti-apoptotic protein [38, 39], which could explain NF-κB pathway involved in the TRAIL resistance and irradiation resistance in GSLCs. However, when combined treatment with TRAIL and IR, the expression of p-NF-κB and p-IκB was neither decreased nor increased. Therefore, activation of NF-κB pathway was not involved in the mechanisms of combined therapy on eliminating GSLCs.

Taken together, GSLCs are resistant to both TRAIL and radiation treatment. TRAIL resistance in GSLCs probably results from low expression of DR5, activation of NF-κB and high expression of cFLIP. The dramatically up-regulated DR5 expression after irradiation could sensitize TRAIL treatment; thus, the combined treatment with irradiation and TRAIL significantly increased apoptotic rates and decreased survival fraction of GSLCs because of the enhanced DR5 expression and the down-regulated cFLIP expression. Therefore, in light of the effective elimination of GSLCs, TRAIL and IR combination treatment would be a simple but practical therapeutic strategy targeting GBMs. However, further studies should be done to explore the underlying mechanisms and an appropriate drug delivery approach.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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