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Activation of Sonic Hedgehog Signaling Is Associated with Human Osteosarcoma Cells Radioresistance Characterized by Increased Proliferation, Migration, and Invasion

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Radioresistance restricts the application of radiotherapy in human osteosarcoma (OS). This study investigated the molecular mechanism of radioresistance in OS, which may provide clues to finding ideal targets for genetic therapy.

Materila/Methods: The human OS cell line MG63 was employed as parent cells. After repeat low-dose X-ray irradiation of MG63, the radioresistant OS cell line MG63R was produced. Colony formation assay was used to assess the radioresistance. Cell viability was evaluated by CCK-8 assay. Flow cytometry was used to detect cell apoptosis, and wound healing assay was used to evaluate invasive capacity. The nuclear translocation was evaluated by fluorescent immunohistochemistry. Protein expression levels were assessed by Western blotting. Specific siRNA against Shh was used to silence Shh.

Results: More survival colony formation, elevated cell viability, less cell apoptosis, and increased wound closure were found in MG63R than in MG63 cells exposed to irradiation. The nuclear translocation of Gli, expression levels of Shh, Smo, Ptch1, Bcl2, active MMP2, and active MMP9 were increased in MG63R cells compared with MG63 cells. Transfection of Shh-siRNA suppressed expression levels of Shh, Smo, Ptch1, Bcl2, active MMP2, and active MMP9, as well as the nuclear translocation of Gli in MG63R cells. The cell viability, survival colony formation, and wound closure were impaired, whereas cell apoptosis was increased, in siRNA-transfected MG63R cells than in control MG63R cells exposed to irradiation.

Conclusions: Activation of Shh signaling was involved in radioresistance of OS cells. Blocking this signaling can impair the radioresistance capacity of OS cells.

MeSH Keywords: Hedgehog Proteins • Osteosarcoma • Radiotherapy, Adjuvant

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Background

Osteosarcoma (OS) is the most frequent primary malignant tumor in bone system [1]. Currently, combining therapies of radical excision, adjuvant chemotherapy and multi- agent neoadjuvant chemotherapy are applying in OS patients [2]. The 5-year survival rate of patients receiving these therapies is 65% in localized disease [3]. However, the 5-year survival rate is as low as 25% in patients with axial/inoperable OS [4]. Radiotherapy has been utilized for malignant tumor for decades. Radiotherapy is applied to a small proportion of OS patients such as inoperable OS and painful bone metastasis [5]. Local control could be achieved by radiotherapy which is used as an adjuvant method in OS patients receiving intralesional resection [6]. Unfortunately, the utility of radiotherapy in OS is restricted because OS is considered relatively radioresistant [7]. Understanding the molecular mechanisms of radioresistance of OS would be helpful in developing radiosensitizing methods which could improve the efficacy of radiotherapy.

Ionizing radiation (IR) is the major method of irradiation used in radiotherapy which causing cell damage leading to cancer cell death and limitation of invasion. Several malignant cell types including OS are resistant to radiotherapy. DNA damage/repair, signaling pathway alterations, microenvironment change, cell apoptosis and cell cycle arrest were suggested [8]. However, the molecular mechanisms regulating the radioresistance in OS are still not clearly understood. Sonic hedgehog (Shh) was firstly identified in *Drosophila* as a protein conducting secretory signaling, which were associated with cell proliferation, apoptosis and migration [9]. Upon activation, Shh binds to patched (Ptch) at cell surface which further relives the inhibition of smoothened (Smo). Then Smo perform translocation and thereby activates downstream transcriptional factors of the glioma-associated oncogene homolog (Gli) family to which Shh belongs, including Gli1, Gli2, and Gli3 [10]. Gli1 is the most important member, which translocates to the nucleus after activation and further initiates transcription of target genes regulating cell cycle, apoptosis, migration, and signaling transductions [11].

The dysregulations of Shh signaling were found in several malignant human cancers such as liver cancer, breast cancer and gastric cancer [12,13]. The overexpression of Shh was reported to be correlated with the poor prognosis of malignant cancer [14]. It was also reported that up-regulation of Shh signaling protected human hepatocellular carcinoma against ionizing radiation [15]. In the present study, the radioresistant OS cell line MG63R was established by repeated irradiation by using MG63 as parental cells. The role of Shh signaling in the radioresistant capacity of MG63R as characterized by increased capacities of proliferation, migration, and invasion were investigated. Small interference RNA targeting Shh was used to treat MG63R. The effect of siRNA of impairing radioresistance of MG63R was also investigated. We believe that results from this study will not only be helpful in further understanding the mechanisms of radioresistance of OS, but also providing clues for therapeutic molecular targets for sensitizing radiotherapy of OS.

Material and Methods

Cell culture and establishment of MG63R

The human osteosarcoma cell line MG63 was purchased from the China Center for Type Culture Collection (CCTCC). Cells were cultured in DMEM (Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco) and antibiotics mixture (Sigma-Aldrich, penicillin and streptomycin, Invitrogen). A cell incubator providing a humidified environment with 37°C and 5% CO_2/95% fresh air was used to incubate the cells.

MG63R was generated by repeat low-dose irradiation. MG63 cells in the period of logarithmic phase were irradiated with X-ray at 6 MV using a Varian Cx irradiator (Varian Medical System). The cells received a single X-ray irradiation at a dosage of 2 Gy for 1 min. The irradiated cells were irradiated again until reaching another logarithmic phase. This procedure was repeated 30 times and the total irradiation dosage was 60 Gy. The 10th–20th passages of the resulting cells were named MG63R cells, which were stable and radioresistant.

Colony formation assay

The radiosensitivity of MG63 and MG63R was assessed by colony formation assay. This assay was carried out by using OptiCell culture chambers (Thermo Scientific) according to previous descriptions [16]. Cell culture medium containing 400–600 cells at a volume of 10 ml was added to each chamber. Ws delivered 0, 2, 4, and 6 Gy irradiation to each chamber at a depth of 0 cm. The cells were cultured for 7 days until survival colonies were visualized. A colony consisting of over 50 cells was identified as a survival colony. Survival fractions were calculated.

Cell apoptosis evaluation

Cell apoptosis was evaluated by Annexin V-FITC (BD) and propidiumiodide (PI, BD) double staining with flow cytometry. Briefly, 5 µl Annexin V-FITC (BD) and 5 µl PI (BD) were used to incubate HAECs in binding buffer for 15 min in a dark chamber. Then, a flow cytometer (FACS Calibur, BD) was used to analyze the apoptosis and the apoptotic percentage was calculated.

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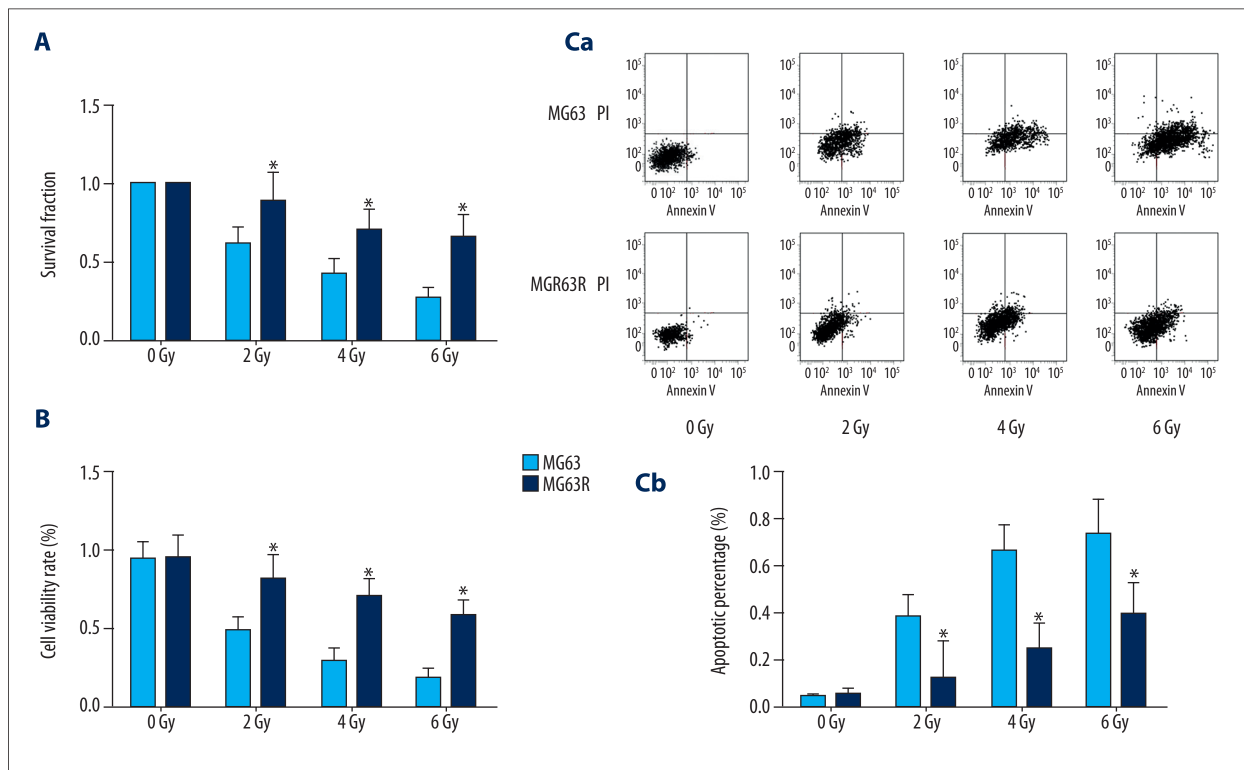

Figure 1. (**A**) Columns indicate the survival fraction of MG63 and MG63R cells receiving irradiation at dosages of 0, 2, 4, and 6 Gy, respectively. (**B**) Columns indicate the cell viability rate of MG63 and MG63R cells receiving irradiation at dosages of 0, 2, 4, and 6 Gy, respectively. (**C**) **a**) Charts of flow cytometry demonstrate the apoptosis of MG63 and MG63R cells receiving irradiation at dosages of 0, 2, 4, and 6 Gy, respectively. **b**) Columns indicate the calculated apoptotic rate of MG63 and MG63R cells receiving irradiations at dosages of 2, 4, and 6 Gy, respectively. [* differences were significant when compared with MG63 ($p<0.05$)].

Cell viability assay

Cell viability was determined by a use of a Cell Counting kit-8 (CCK-8) assay kit (Beyotime) following the manufacturer's instructions. Cultured cells were seeded into a 96-well plate and were incubated with CCK-8 reagent at 25°C for 2 h. A plate reader was used to determine the absorbance at 450 nm (A450). Viability was determined relative to the control group measurement.

Cell migration and invasion assessment

The migration ability of OS cells was evaluated by wound healing assay. Briefly, the OS cells were cultured on a culturing dish (Corning) with 60-mm diameter to reach 90% confluence. A 2-mm razor blade was used to make the wound and the injury line was marked. After removing the peeled-off cells, the remaining cells were continually cultured for 24 h to migrate to heal the wound. Cells were then fixed by acetone and then subjected to DAPI fluorescence staining with a DAPI labeling kit (Beyotime) following the manufacturer's instructions. The cells were observed with an inverted fluorescence microscope and the images were captured. The invasion ability was assessed by Transwell assay by using Matrigel-coated Transwell chambers (BD). The Transwell assay was carried out according to the manufacturer's instructions. The invasion ratio was calculated by the formula: invasion ratio=(invasive cell count/total cell count)×100%.

Small interference RNA transfection

Shh in MG63 and MG63R was silenced by small interfering RNA (siRNA) transfection. Shh-siRNA (h) (Cat. sc-29477, Santa Cruz Biotechnology) was used to knock down Shh. SignalSilence Control siRNA (Cat.6568S, Cell Signaling Technology) was used as control. MG63 and MG63R cells at confluence above 70% were transfected with siRNAs by using Mirus TransIT-TKO reagent (Mirus) for 48 h per the manufacturer's instructions.

Gli1 nuclear translocation assessment

Fluorescent immunohistochemistry was used to assess Gli1 nuclear translocation. Cultured MG63 and MG63R cells were fixed and then incubated with primary antibody against Gli1 (Cell

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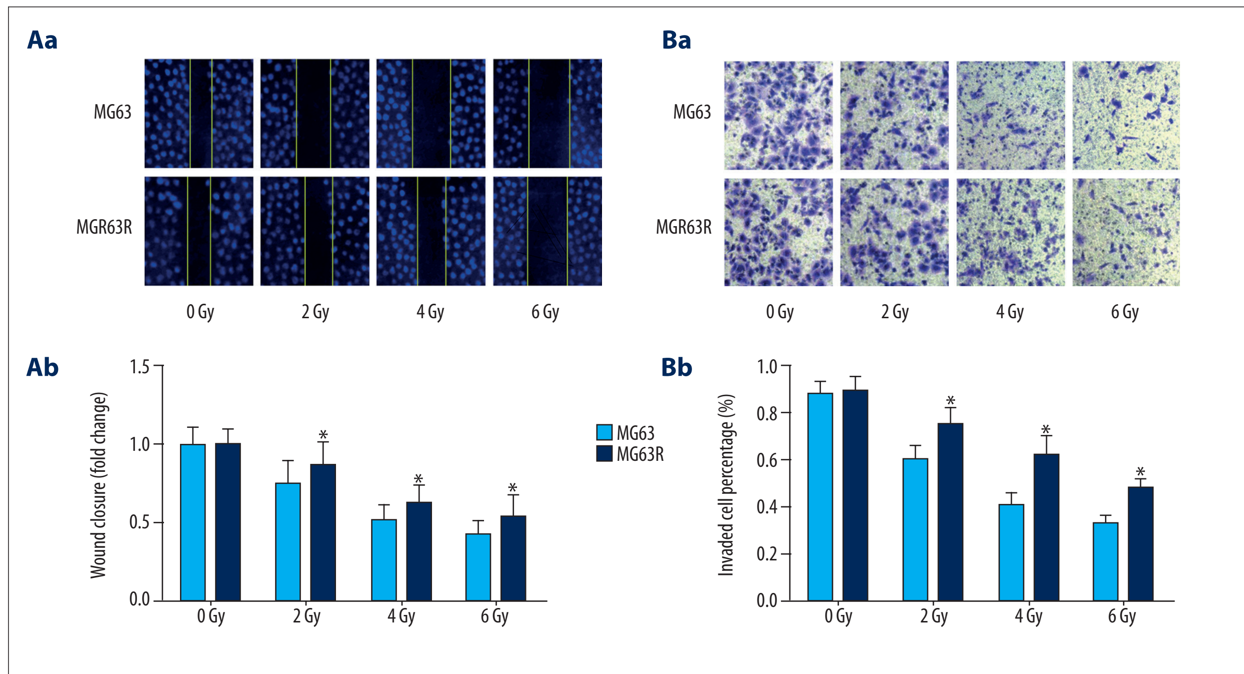

Figure 2. (**A**) **a**) Captured fluorescent images of wound healing assay. The nuclei of MG63 and MG63R cells were stained by DAPI. Yellow lines indicate the edges of wounds. **b**) Columns indicate the wound closure of MG63 and MG63R cells exposed to irradiation at dosages of 0, 2, 4, and 6 Gy, respectively. (**B**) **a**) Captured images of Transwell assay. **b**) Columns indicate the invaded cell percentage of MG63 and MG63R cells exposed to irradiation at dosages of 0, 2, 4, and 6 Gy, respectively. [* differences were significant when compared with MG63 ($p<0.05$)].

Signaling Technology, 1: 500) at 4°C for 8 h. Then, the secondary antibody conjugated with Alexa 488 (Invitrogen) and DAPI (Invitrogen) were used to incubate the cells. Quenching of fluorescence was alleviated using the SlowFade Light Antifade kit (Molecular Probes). Samples were excited at 519 nm and observed at 442 nm and 49 5nm with an inverse fluorescence microscope (Axio Imager 2, Zeiss). The fluorescent intensity was determined by using Zeiss Physiology software (Ver. 3.2, Zeiss).

Western blotting

Cultured cells were lysed by use of the RIPA lysis buffering system (Santa Cruz Biotechnology) supplemented with 1% PMSF. Total protein was extracted by Protein Extraction Reagents (Pierce) according to the manufacturer's instructions. The protein concentration was detected by BCA method with a BCA protein assay kit (Beyotime). Protein samples were separated by SDS-PAGE. Then, the separated proteins were transferred to PVDF membranes electrically. Specific antibodies against Shh (Cell Signaling Tech, 1: 500), Ptch1 (Abcam, 1: 500), Smo (Abcam, 1: 500), active MMP2 (Abcam, 1: 1000), active MMP9 (Abcam, 1: 500), caspase3 (Abcam, 1: 500), cleaved caspase3 (Abcam, 1: 250), Bcl2 (Sigma-Aldrich, 1: 500), and GAPDH (Sigma-Aldrich, 1: 500) were used to incubate the membranes at 4°C for 12 h. Corresponding second antibodies conjugated to HRP (Cell Signaling Technology) were used to incubate the membranes at room temperature for 2 h. The immunoblots were visualized on X-ray films with Super Signal West Pico chemiluminescence reagent (Pierce).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical significance was determined by use of the independent-samples *t* test and one-way analysis of variance (ANOVA). Tukey's post hoc tests were then performed. The analysis was carried out by using SPSS (ver.16.0, SPSS). $P<0.05$ was considered to indicate a statistically significant difference.

Results

MG63R cells showed stronger proliferative capacity than MG63 cells

The MG63 and MG63R cells received irradiation of 0, 2, 4, and 6 Gy. As demonstrated in Figure 1, the survival fractions were significantly higher in MG63R cells than MG63 cells at irradiations at dosages of 2, 4, and 6 Gy. Results of CCK-8 assay indicated that the cell viabilities of MG63R cells were significantly higher than in MG63 cells receiving irradiations at dosages of 2, 4, and 6 Gy. Moreover, the irradiation-induced cell apoptosis was

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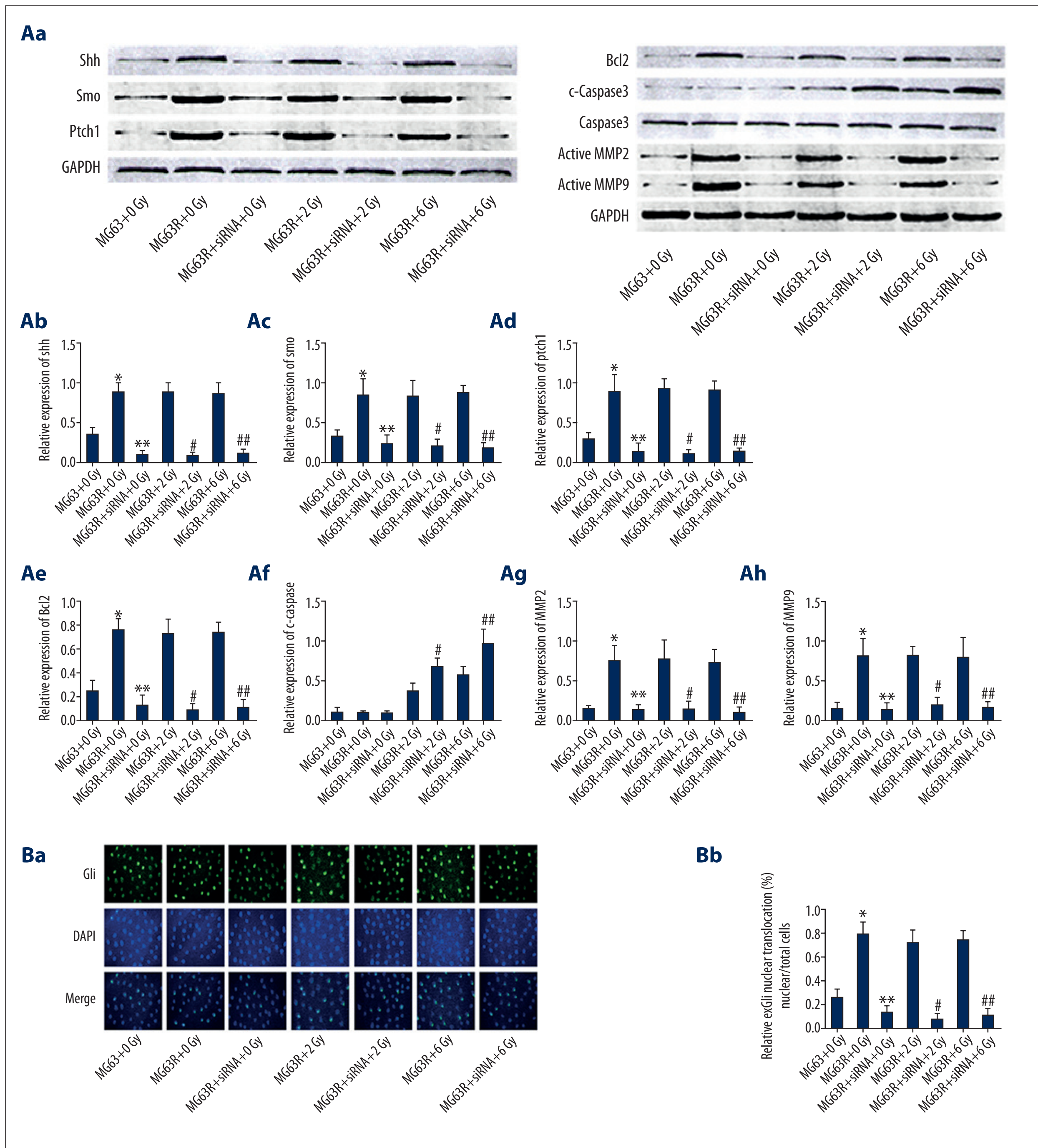

Figure 3. (**A**) **a**) Immunoblots of Shh, Smo, Ptch1, Bcl2, c-caspase3, caspase3, active MMP2, active MMP9, and GAPDH in MG63 and MG63R cells. Columns in **b**), **c**), **d**), **e**), **f**), **g**), and **h**) indicate the relative expression levels of Shh, smo, ptch1, bcl2, cleaved caspase3, MMP2, and MMP9 in MG63, MG63R, and MG63R cells transfected with siRNA against Shh and/or MG63R cells receiving irradiations. (**B**) **a**) Captured images of fluorescence staining of Gli, DAPI, and their merged images of MG63 and MG63R cells. **b**) Columns indicate Gli nuclear translocation rate in MG63, MG63R, and MG63R cells transfected with siRNA against Shh and/or MG63R cells receiving irradiations. [* differences were significant when compared with MG63+0Gy ($p<0.05$); ** differences were significant when compared with MG63R+0Gy ($p<0.05$); # differences were significant when compared with MG63R+2Gy ($p<0.05$); ## differences were significant when compared with MG63R+6Gy ($p<0.05$)].

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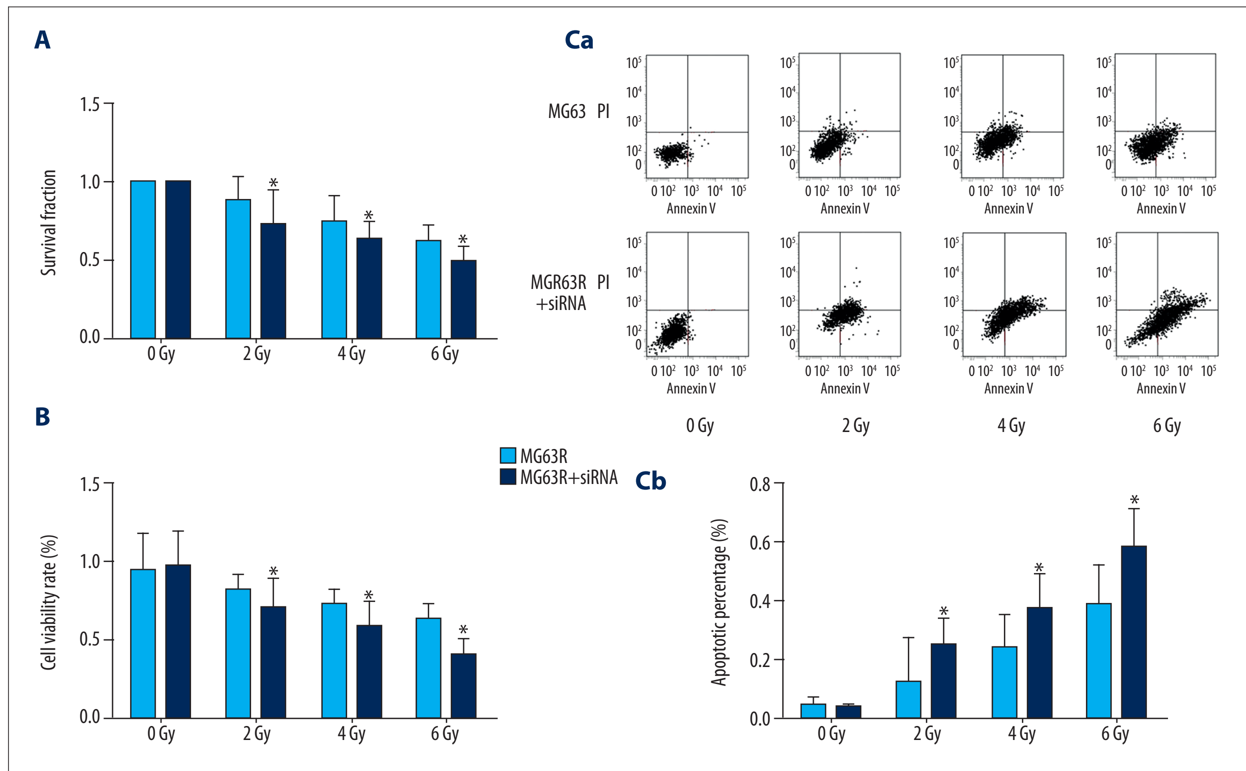

Figure 4. (**A**) Columns indicate the survival fraction of MG63R and Shh-silenced MG63R cells receiving irradiation at dosages of 0, 2, 4, and 6 Gy, respectively. (**B**) Columns indicate the cell viability rate of MG63R and Shh-silenced MG63R cells receiving irradiation at dosages of 0, 2, 4, and 6 Gy, respectively. (**C**) **a**) Charts of flow cytometry detection of apoptosis of MG63R and Shh-silenced MG63R cells receiving irradiation at dosages of 0, 2, 4, and 6 Gy, respectively. **b**) Columns indicate the calculated apoptotic rate of MG63R and Shh-silenced MG63R cells receiving irradiations at dosages of 2, 4, and 6 Gy, respectively. [* differences were significant when compared with MG63R ($p<0.05$)].

dramatically lower in MG63R cells compared with MG63 cells receiving irradiations at dosages of 2, 4, and 6 Gy, respectively.

MG63R cells showed more potent migration and invasion ability than MG63 cells

As demonstrated in Figure 2, the migration and invasion ability of OS cells were evaluated by wound healing assay and Transwell assay, respectively. Compared with MG63 cells, the wound healing percentage and cell of number that invaded through the membrane were up-regulated MG63R cells receiving irradiations at dosages of 2, 4, and 6 Gy, respectively, indicating the migration and invasion ability of MG63R cells was stronger than in MG63 cells undergoing irradiations.

Signaling activation was identified in MG63R and was impaired by siRNA against Shh

The results were shown in Figure 3. Compared with MG63, expression levels of Shh, Smo, and Ptch1, as well as the nuclear translocation of Gli1, increased significantly in MG63R cells. As a result, expression levels of Bcl2, MMP2, and MMP9 were also dramatically up-regulated in MG63R cells. The specific siRNA was also transfected into MG63R cells to silence Shh expression. The results show that Shh silencing reduced expression levels of Shh, Smo, Ptch1, Bcl2, MMP2, and MMP9, as well as the nuclear translocation of Gli1, in MG63R cells receiving irradiation at dosages of 0, 2, and 6 Gy. In addition, the Shh silencing increased the cleavage of caspase3 in MG63R cells receiving irradiations at dosages of 2 and 6 Gy.

Shh silencing impaired proliferative capacity of MG63R

As shown in Figure 4, the survival fractions and cell viability were significantly decreased in MG63R cells treated with siRNA against Shh compared to control MG63R cells receiving irradiation at 2, 4, and 6 Gy. The irradiation-induced apoptosis was also significantly increased in MG63R cells transfected with Shh-siRNA when receiving irradiation at dosages of 2, 4, and 6 Gy.

Shh silencing impaired invasive ability of MG63R

The results of wound healing assay and Transwell assay indicating the migration and invasion abilities are demonstrated

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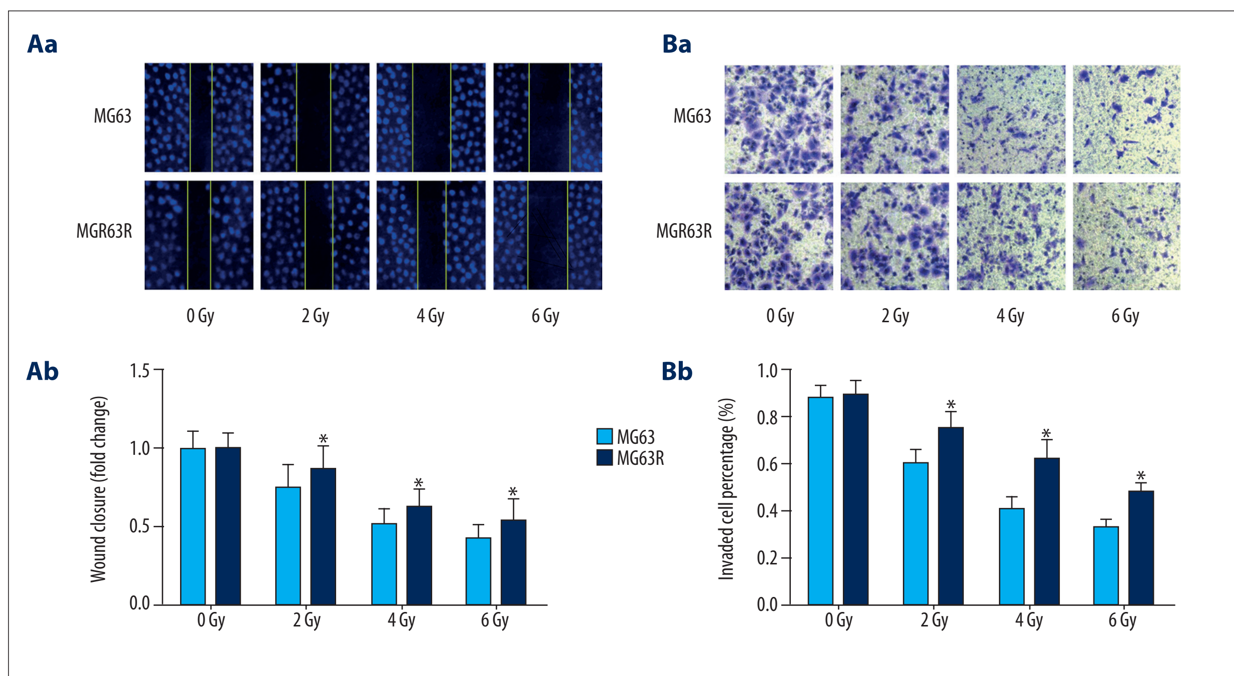

Figure 5. (**A**) **a**) the captured fluorescent images of wound healing assay. The nuclei of MG63R and Shh-silenced MG63R were stained by DAPI. Yellow lines indicated the edges of the wounds. **b**) Columns indicated the wound closure of MG63R and Shh-silenced MG63R exposed to irradiation at dosages of 0, 2, 4 and 6 Gy, respectively. (**B**) **a**) Captured images of Transwell assay of MG63R and Shh-silenced MG63R. **b**) Columns indicated the invaded cell percentage of MG63 and MG63R cells exposed to irradiation at dosages of 0, 2, 4, and 6 Gy, respectively. [* differences were significant when compared with MG63R ($p<0.05$)].

in Figure 5. The wound healing percentage and cell number invaded through membrane were significantly down-regulated in MG63R cells transfected with Shh-siRNA compared with MG63R control cells receiving irradiation at dosages of 2, 4, and 6 Gy.

Discussion

Radiotherapy is an important part of the combinatory therapies of OS [17]. It is believed that the incorporation of radiotherapy could improve the efficacy of chemotherapy with several drugs such as cisplatin and ifosfamide in OS [18]. With high-intensity radiotherapy, in some OS cases unresectable or partially resected lesions could achieve local complete cure. In the present study, the human OS cell line MG63 was used as parent cells to produce radioresistant OS cells, which was termed as MG63R. After repeat low-dose X-ray irradiation of MG63 cells, the resulting MG63R cells showed radioresistance characterized by more potent proliferation, migration, and invasion. Clinically, the radioresistance makes some inoperable OS patients lose their chances for further treatment. Understanding of the mechanism of OS radioresistance would be helpful in improving anti-cancer treatment efficacy by increasing the radiosensitivity of OS.

Shh signaling is composed of Shh ligand, transmembrane receptor complex (Ptch and Smo), and the downstream nuclear transcription factor Gli. The Shh signaling pathway plays crucial roles in regulating cell differentiation, adhesion, migration, transformation, and apoptosis [19]. It was found that Shh signaling was activated in many human cancers such as gastric cancer, liver cancer, breast cancer, and ovarian cancer [11,20]. Moreover, activation of Shh signaling was found to be highly associated with increased capacities of chemoresistance and radioresistance in human malignant tumors [21,22]. In the present study, compared with MG63 cells, in the radioresistant MG63R cells the expression levels of Shh, Smo, and Ptch1 were significantly up-regulated and Gli1 nuclear translocation was also increased, indicating Shh signaling was obviously activated. Specific siRNA against Shh was used to silence Shh signaling in MG63R. The results show that Shh silencing impaired the proliferative, migration, and invasive abilities of MG63R cells undergoing irradiations. These results indicate that the Shh signaling pathway activation is associated with the increased radioresistance of MG63R cells.

After binding with Shh, the inhibitory effect of Ptch on Smo is removed. Smo is released into cytoplasm to activate Gli nuclear translocation, which further initiates target gene transcription [23]. The transcription of anti-apoptotic factor Bcl2 was shown to be Gli-dependent [24]. Bcl2 stabilizes the membrane potential of mitochondria to reduce the release of cytochrome C [25]. As a result, the cleavage of caspase3 is inhibited,

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which would not initiate the activation of the caspase cascade. MMPs, including MMP2 and MMP9, were identified as targets of Gli [11]. Increased MMP2 and MMP9 expression and activities were highly correlated with migration and invasion abilities of cancer due to their ability to degrade extracellular matrix [26]. In the present study, we found that compared with its parent MG63 cells, expression levels of Shh, Smo, Ptch1, Bcl2, MMP2, and MMP9, as well as nuclear translocation of Gli, were significantly up-regulated in the radioresistant MG63R cells. As a result, the cleavage of caspase3 was inhibited. In addition, we used small RNA interfering technique to further confirm the involvement of Shh signaling. The Shh-siRNA significantly inhibited Shh signaling activation, which was evidenced by decreased expression levels of Shh, Smo, Ptch1, Bcl2, MMP2 and MMP9, as well as the nuclear translocation of Gli in MG63R cells. The Shh-siRNA transfection impaired the proliferation, migration, and invasion of MG63R cells exposed to irradiation.

Conclusions

In summary, compared with MG63 cells, MG63R cells showed stronger radioresistance characterized by more potent proliferation, migration, and invasion. Moreover, Shh-siRNA interference effectively impaired the radioresistant capacity of MG63R, indicating that Shh signaling activation was associated with the radioresistance of MG63R cells. These results not only provide evidence of the involvement of the Shh signaling pathway in radioresistance of OS, but also suggests that Shh signaling inhibition is a novel gene therapeutic target in genetic treatment of OS. However, there were also limitations to this study. For instance, only 1 OS cell line was studied. Investigation of more OS cell lines would further verify the conclusion that Shh signaling is the potential target for attenuating the radioresistance of human OS. Moreover, the application of RNAi technique inhibited the activation of Shh signaling by silencing the Shh expression. Application of Shh-specific inhibitors such as 22-NHC and Jervine would be also necessary because they inhibited the activation of Shh functionally.

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