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Emodin Impairs Radioresistance of Human Osteosarcoma Cells by Suppressing Sonic Hedgehog Signaling

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: This study aimed to investigate the possible involvement of sonic hedgehog (Shh) signaling in the radioresistance of human osteosarcoma cells and the inhibitory effects of emodin on radioresistance.

Material/Methods: Human osteosarcoma (OS) cell line MG63 was employed as parent cells. After 30-repeat low-dose (2 Gy) x-ray irradiation on MG63 cells, radioresistant OS cell MG63R cells were produced. Colony formation assay was used to assess the radioresistance. Cell viability was evaluated by CCK-8 assay. TUNEL assay was used to detect cell apoptosis. Protein expression levels and nuclear translocation were evaluated by western blotting.

Results: More survival colony formation, elevated cell viability, and less cell apoptosis were found in MG63R cells compared to MG63 cells exposed to irradiation. The nuclear translocation of glioma-associated oncogene homolog (Gli), expression levels of Shh and Bcl2 were increased in MG63R cells compared to MG63 cells. Emodin pretreatment significantly inhibited cell viability and survival colony formation but increased cell apoptosis of irradiation exposed MG63R cells in a concentration dependent manner. Moreover, emodin pretreatment inhibited Shh and Bcl2 expressions as well as Gli1 nuclear translocation but increased C-caspase-3 expression of irradiation exposed MG63R cells in a concentration dependent manner.

Conclusions: Shh signaling activation was involved in the radioresistance of human OS cells. Emodin impaired the radioresistant capacity of OS cells by inhibiting Shh signaling pathway.

MeSH Keywords: **Emodin • Osteosarcoma • Radiotherapy, Adjuvant**

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Background

Osteosarcoma (OS) is one of the most frequent primary malignant tumor, without optimistic prognosis, found in the bone [1]. In patients diagnosed with OS, the current therapy is a combination of surgical excision, multi-agent neoadjuvant chemotherapy, and adjuvant chemotherapy. These therapies result in an approximately 65% 5-year survival rate in OS patients with localized lesions [2]. Radiotherapy is utilized in OS patients who have inoperable tumors and who have painful bone metastasis to achieve local control [3]. However, similar to several other malignant tumors, OS is also characterized by radioresistance [4]. The exact molecular mechanism of radioresistance is still unclear.

Dysregulation of sonic hedgehog (Shh) signaling has been identified in several human malignant tumors, including gastric cancer, breast cancer, and liver cancer [5–7]. Previous studies reported that overexpression of Shh signaling was correlated with resistance against ionizing radiations [8]. Shh was initially identified as a protein conducting signaling involved in cell proliferation, differentiation, and apoptosis. The downstream nuclear factor glioma-associated oncogene homolog (Gli) is activated by Shh and initiates nuclear translocation to facilitate its target genes regulating cell fate such as Bcl2 [9].

Drugs or agents which could reduce radioresistance of malignant cancer are promising and of therapeutic potential. Emodin, (1,3,8-trihydroxy-6-methylanthraquinone) is a bio-active derivative from rhizome of *Rheum Palmatum L*. Previous studies have shown emodin has potent anti-cancer activity by inhibiting cancer growth, invasion, and metastasis [10,11]. Other studies have indicated a role for emodin in attenuation of radioresistance of several cancers [12]. These results informed our interests in assessing whether emodin could reduce the radioresistance of OS. Moreover, the possible molecular mechanism involving Shh signaling and emodin's therapeutic effect on OS was also investigated.

In this current study, radioresistant OS cell line MG63R was established by repeating irradiation by using MG63 as parental cells, which was in accordance to previous descriptions and our pre-study experiments [13]. Emodin was used to incubate these cells. We hypnotized that: 1) emodin could reduce the radioresistance of OS; and 2) emodin attenuates radioresistance by regulating Shh signaling pathway. We believe that results from this study would be helpful in not only understanding the mechanisms of radioresistance of OS, but also in providing a theoretical basis for potential application of emodin in clinical treatment of OS in the future.

Material and Methods

Cell culture, establishment of MG63R and treatments

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

MG63R cells were generated by repeat low-dose irradiation. MG63 cells in the period of logarithmic phase were irradiated with x-rays at 6 MV using Varian Cx irradiator (Varian Medical System). The cells received a single x-ray irradiation at dosage of 2 Gy for one minute. 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 10–20 passages of the resulted cells were named MG63R and were stable and radioresistant.

In some cases, cultured OS cells were pre-treated with emodin (Sigma-Aldrich) at serial diluted concentrations at 0, 15, 30, 45, and 60 μmol/L.

Cell apoptosis determinations

The apoptosis of cultured OS cells was detected by terminal transferase UTP nick end labeling (TUNEL) assay. Cultured OS cells were fixed by 10% neutral buffered formaldehyde. Slides were treated with proteinase K (20 μmol/L, Sigma-Aldrich). A TUNEL assay was performed by using a TUNEL assay kit (Roche) according to the manufacturer's instructions. An inverted fluorescence microscope was used to observe and capture the fluorescent images. The apoptotic rate was analyzed by comparing positively stained cell numbers to total cell numbers using software Image-Pro Plus (version 5.0).

Colony formation assay

The radiosensitivity of MG63 cells and MG63R cells was assessed by colony formation assay. This assay was carried out by using OptiCell culture chambers according to previous descriptions [14]. Cell culture medium containing 400–600 cells at a volume of 10 mL was added to each chamber. Then 0, 2, 4, and 6 Gy irradiation was delivered to each chamber at depth of 0 cm. The cells were cultured for seven days until survival colonies were visualized. Colony consisting of over 50 cells was identified as a survival colony. Survival fractions were calculated.

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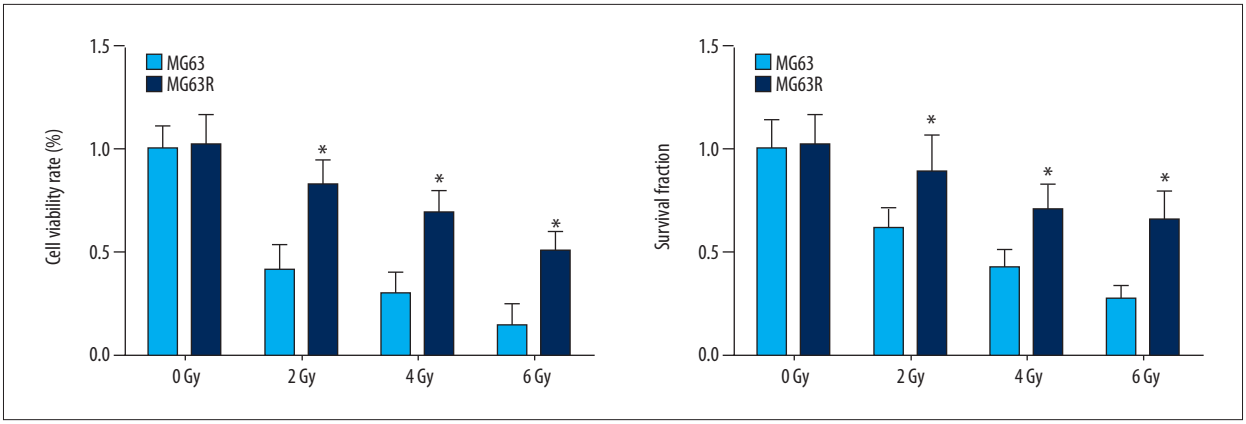

Figure 1. Columns on the upper panel of this figure indicated the cell viability determined by CCK-8 assay. Columns on the lower panel indicated the survival fractions determined by colony formation assay. The white and black columns indicated cell viability of MG63 and MG63R cells, respectively; * differences were significant when compared with MG63 ($p<0.05$).

Cell viability assessment

The cell viability was assessed by Cell Counting Kit-8 (CCK-8) assay kit (Beyotime) per manufacturer's instructions. Cultured cells were seeded into 96-well plates and were incubated with CCK-8 reagent at 25°C for two hours. A plate reader was used to determine the absorbance at 450 nm (A450). Viability was determined relative to the control group measurement.

Western blotting

The whole cell extracts from cultured OS cells were collected by using a cell lysis buffer system (Santa Cruz). Nuclear protein and cytoplasmic protein were prepared with Nuclear Extraction Reagents (Beyotime) and Cytoplasmic Extraction Reagents (Beyotime). Using a BCA protein assay kit (Pierce), the concentrations of protein were determined. Then 30 μg of protein was loaded and then separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Specific antibodies against Gli1 (Cell Signaling Tech, 1: 500), histone H3 (Santa Cruz, 1: 1,000), Shh (Cell Signaling Tech, 1: 500), caspase-3 (Abcam, 1: 500), cleaved caspase-3 (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 hours. Anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Tech) and anti-mouse IgG HRP-linked secondary antibody (Cell Signaling Tech) were used to incubate the membranes at room temperature for two hours. The immunoblots were visualized on x-ray films with Super Signal West Pico chemiluminescence reagent (Pierce).

Statistical analysis

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

Results

MG63R cells showed stronger radioresistant capacity than MG63 c ells

The MG63 cells and MG63R cells received irradiation of 0, 2, 4, and 6 Gy. As shown 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. Also shown in Figure 1, results of CCK-8 assay indicated that the cell viabilities of MG63R cells were significantly higher than MG63 cells receiving irradiation at dosages of 2, 4, and 6 Gy.

Shh signaling was activated in MG63R cells

The results are shown in Figure 2. Compared with MG63 cells, expression levels of Shh and Bcl2 were increased in MG63R cells. Moreover, the nuclear translocation of Gli1 was also dramatically increased in MG63R cells compared to MG63 cells. The cleavage of caspase-3 was inhibited in MG63R cells. As a result, the irradiation-induced cell apoptosis was dramatically lowered in MG63R cells compared to MG63 cells under the same dosages of irradiation.

Emodin attenuated radioresistance of MG63R cells

As shown in Figure 3, the survival fractions as well as cell viability were significantly decreased in MG63R cells treated with emodin in a concentration-dependent manner. Moreover, emodin pretreatment significantly increased the irradiation-induced cell apoptosis in a concentration-dependent manner.

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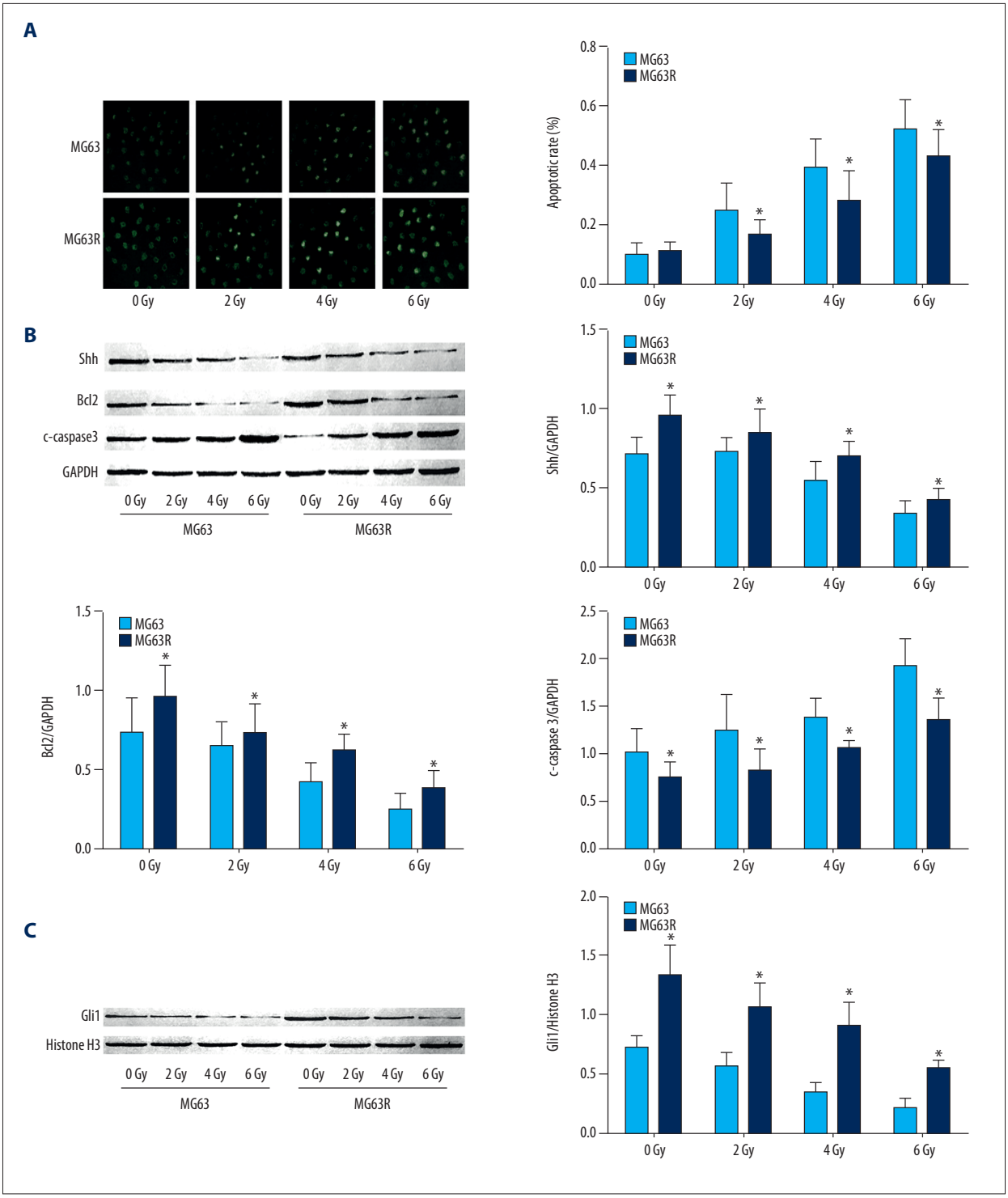

Figure 2. (**A**) Captured images on the left show the TUNEL staining of cultured MG63 and MG63R cells exposed to serial dosages of irradiations. Columns on the right indicate the apoptotic rate of these cells. (**B**) Image on the upper part shows the immunoblots of Shh, Bcl2, and cleaved caspase-3 (C-caspase-3) and GAPDH. Columns on the lower panel indicate the relative expression levels of Shh, Bcl2 and C-caspase-3 (normalized to GAPDH) in MG63 cells (white columns) and MG63R cells (black columns), respectively. (**C**) Image on the upper part shows the immunoblots of Gli1 and histone H3. Columns on the lower panel indicate the relative expression levels of Gli1 (normalized to histone H3) in MG63 cells (white columns) and MG63R cells (black columns), respectively; * differences were significant when compared with MG63 cells ($p<0.05$).

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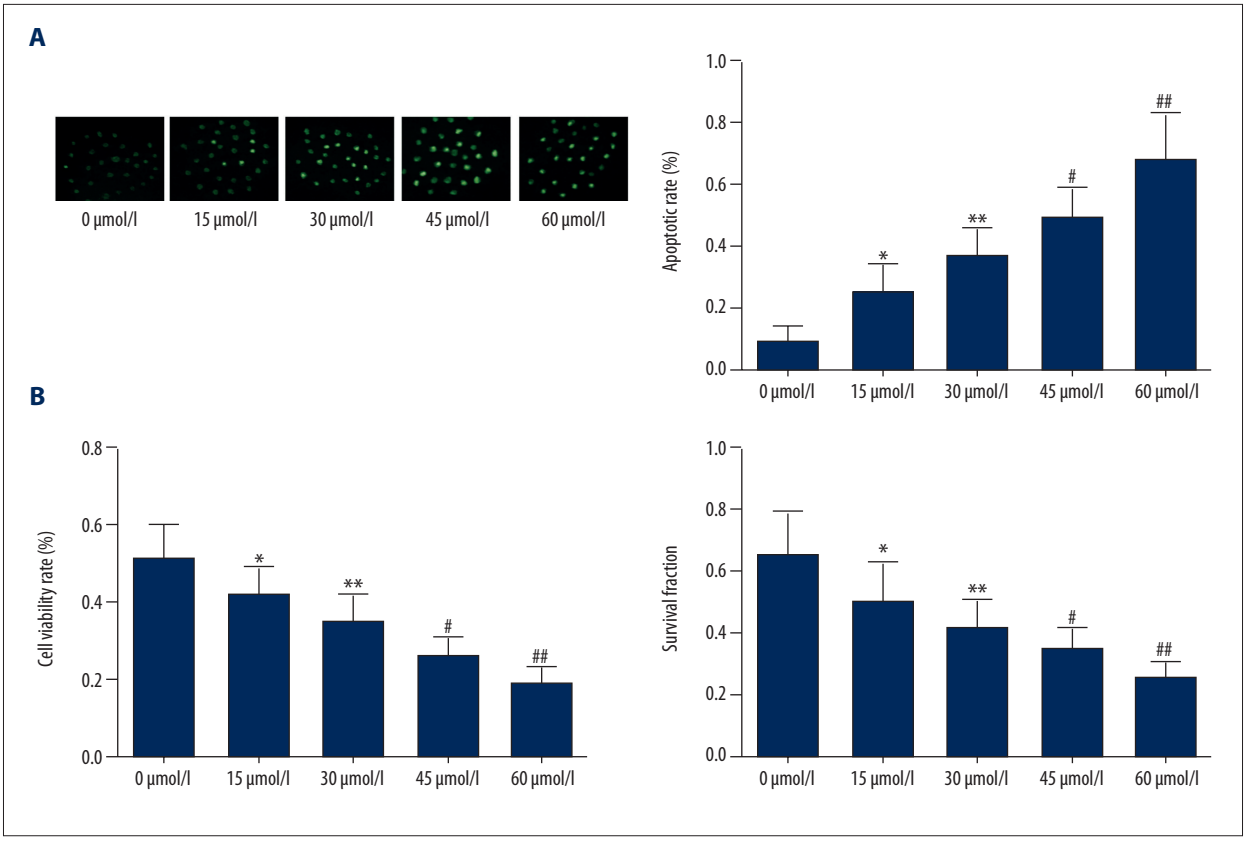

Figure 3. (**A**) The left section of this figure shows the captured images of TUNEL staining of cultured MG63R cells treated with serial concentrations of emodin. These cells were exposed to irradiation of 6 Gy. Columns on the right indicate the apoptotic percentage of these cells. (**B**) Columns indicate the cell viability determined by CCK-8 assay. (**C**) Columns indicate the survival fractions determined by colony formation assay; * differences were significant when compared with 0 μmol/L (p<0.05); ** differences were significant when compared with 15 μmol/L (p<0.05); # differences were significant when compared with 30 μmol/L (p<0.05); ## differences were significant when compared with 45 μmol/L (p<0.05).

Emodin suppressed Shh signaling activation in MG63R cells

The results are shown in Figure 4. The expression levels of Shh and Bcl2 were suppressed in MG63R cells pretreated with emodin in a concentration-dependent manner. Moreover, emodin treatment also significantly inhibited the nuclear translocation of Gli1 in MG63R cells. Correspondingly, the cleavage of caspase-3 was increased in emodin-incubated MG63R cells.

Discussion

Incorporation of radiotherapy has been proven to increase the efficacy of several agents, such as ifosfamide and cisplatin, in OS [15]. However, radioresistance of OS impairs the efficacy of radiotherapy. In the current study, by using repeat low-dose x-ray irradiations, we established a radioresistant OS cell line MG63R originated from MG63 cells. The MG63R cells showed potent radioresistance to irradiation-induced cell apoptosis. Evidences from survival colony formation and CCK-8 assay indicate that MG63R cells showed stronger viability compared to MG63 cells.

Shh signaling is composed of Shh ligand, transmembrane receptor complex (Ptch and Smo), and the downstream nuclear transcription factor Gli. It was reported that Shh signaling was critical for regulating cell growth, differentiation, adhesion, and apoptosis [16]. Previous studies reported that the Shh signaling was activated in many human malignant tumors, including liver cancer, gastric cancer, and breast cancer [17]. The activation of Shh signaling was also considered positively correlated with increased chemoresistance and radioresistance in several human malignant tumors [18,19]. In this study, we found that compared with the parent MG63 cells, expression levels of Shh and nuclear translocation of Gli1 were upregulated, indicating that Shh signaling was dramatically activated in MG63R cells. The expression of Gli1's target gene Bcl2 [20] was increased and thus the cleavage of caspase-3 was attenuated. As a result, cell viability was increased, and irradiation-induced cell apoptosis was reduced in MG63R cells.

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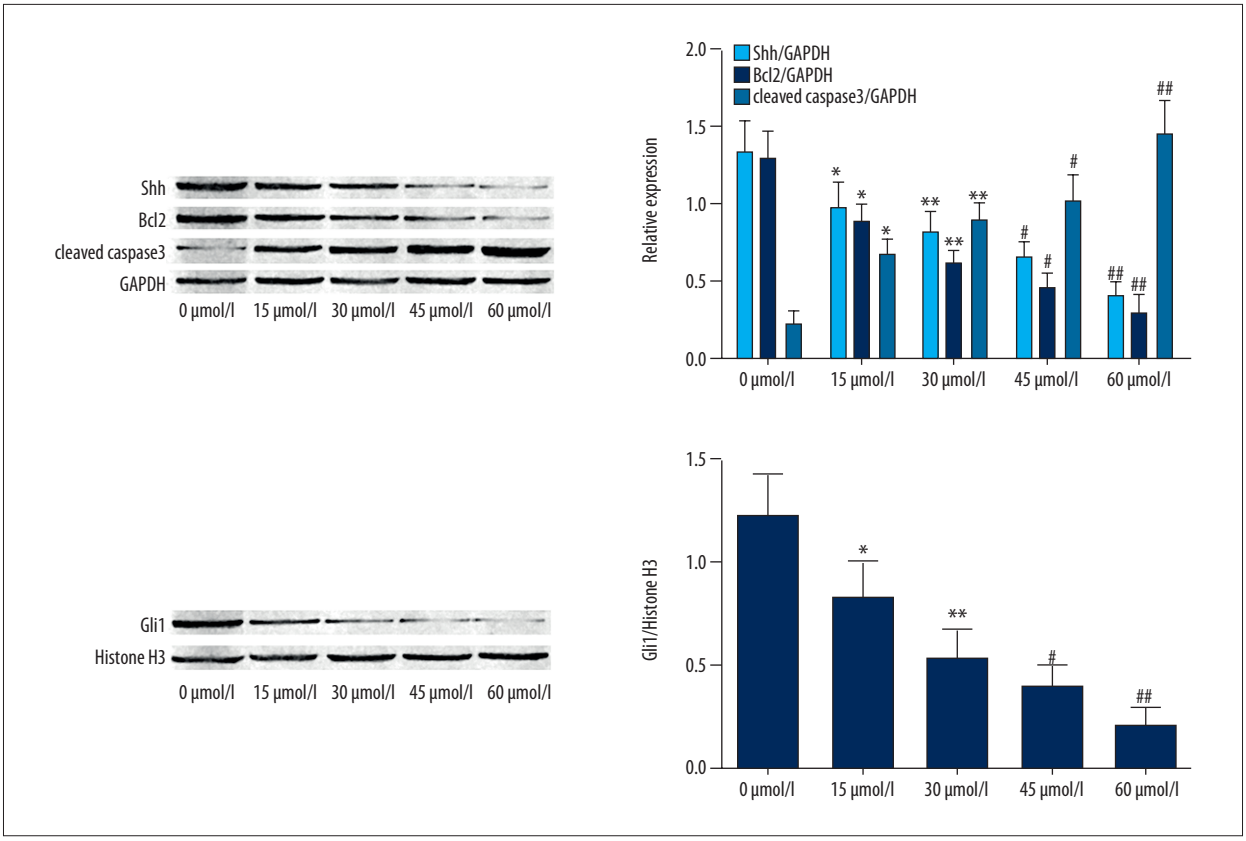

Figure 4. The left section of the upper panel shows the images of immunoblots of Shh, Bcl2, C-caspase-3 and GAPDH in irradiation-exposed MG63R cells treated with emodin at serial concentrations (0, 15, 30, 45, and 60 μmol/L). Columns on the right indicate the relative expression levels of Shh, Bcl2, and C-caspase (normalized to GAPDH). The left section of the lower panel shows the images of immunoblots of Gli1 and histone H3 in irradiation-exposed MG63R cells treated with emodin at serial concentrations (0, 15, 30, 45 and 60 μmol/L). Columns on the right indicate the relative expression levels of Gli1 (normalized to histone H3); * differences were significant when compared with 0 μmol/L (p<0.05); ** differences were significant when compared with 15 μmol/L (p<0.05); # differences were significant when compared with 30 μmol/L (p<0.05); ## differences were significant when compared with 45 μmol/L (p<0.05).

It has been reported that emodin has a broad spectrum of biological activities including anti-bacterial, anti-inflammatory, and anti-cancer activities [21–23]. It has been shown that emodin inhibits cancer cell growth by inducing apoptosis via activating the caspase cascade [24]. In addition, emodin attenuated the radioresistance of several cell lines such as HepG2 liver cancer cells [12]. In the current study, we found that emodin attenuated the radioresistance of MG63R cells. Evidences from survival colony formation and CCK-8 assay indicated that emodin treatment repressed cell viability and of MG63R cells. The irradiation-induced apoptosis was dramatically increased in emodin-treated MG63R cells. Our further investigation showed that the expression levels of Shh and nuclear translocation of Gli1 were reduced by emodin treatment. As a result, the expression of Bcl2 was downregulated and the cleavage of caspase-3 was upregulated, which triggered cell apoptosis.

Conclusions

Data in this study suggested that MG63R cells showed stronger radioresistance compared to the parent cell line MG63. The activation of Shh signaling was involved in the radioresistance of MG63R cells. Emodin treatment attenuated the radioresistance of MG63R cells by deactivating Shh signaling. These results not only broadened our understanding of the possible mechanisms of radioresistance of OS, but also provided a theoretical basis for potential clinical utility of emodin or emodin-contained compounds.

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