Primary impact of Gli1 on radioresistance in esophageal cancer

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Abstract. Radioresistance is the primary cause for the low efficacy of radiotherapy in the treatment of esophageal cancer (EC). Increasing evidence has demonstrated that the Sonic Hedgehog (Shh) signaling pathway may be involved in the pathology of various tumors, including EC. The present study aimed to examine the association between radioresistance in EC and the Sonic Hedgehog pathway, and to determine whether a downstream transcription factor of the Shh pathway, glioma-associated oncogene family zinc finger 1 (Gli1), serves a primary role in radioresistance. The radiation-resistant cell line Eca109R was established by repeated low dose (cumulative dose 60 Gy) irradiation of the human EC cell line Eca109. The level of cell radiosensitivity was determined by colony formation assay, and the localization of Gli1 was detected using immunofluorescence. Western blotting was used to determine the protein expression levels of Gli1, Shh, patched 1 (Ptc1) and smoothened frizzled class receptor (Smo) in the two cell lines. Significantly higher levels of Gli1 were identified in the Eca109R cell line compared with those in Eca109 cells (P<0.05). Additionally, western blotting analysis demonstrated an increased expression level of the Gli1, Shh, Ptc1 and Smo proteins in Eca109R, compared with Eca109 cells (P<0.05). Overexpression of Gli1 in the parental cell line led to decreased levels of radiosensitivity and radiosensitivity of the radioresistant cell line was restored through knockdown of Gli1. The present study demonstrated that Gli1 may be associated with the development of radioresistance in EC.

Introduction

One of the most common types of cancer affecting the digestive system is esophageal cancer (EC). EC is a malignant tumor listed globally as the sixth highest in mortality rate and seventh highest in frequency of occurrence (1-3). Esophageal squamous cell cancer (ESCC) is the predominant type of EC, as nine out of 10 people living with EC in China are diagnosed with this subtype (4). The other subtype is esophageal adenocarcinoma (EA) (5,6). The majority of patients are diagnosed in an advanced stage of the disease due to indiscernible early symptoms and a lack of efficacious screening methods (7,8). Extensive research focused on the elucidation of the pathophysiological mechanisms of EC has been performed (9,10); however, the 5-year survival rate of patients with EC is only 15 to 25% and the recurrence rate is ~38.14%, which results in the continued severity of this disease (11,12). Various treatment options are available for EC, including chemotherapy, radiotherapy, surgery and comprehensive therapy (13). The optimum treatment plan for patients with advanced EC is a combination of surgery and radiotherapy (14,15). However, reduced treatment efficacy is a consequence of the development of radioresistant tumor cells following radiotherapy, which is the major clinical obstacle in the treatment of EC (16). Thus, the establishment of efficacious means of improving tumor cell radiosensitivity is crucial to successful EC therapy.

At present, an increasing number of studies have reported the abnormal activation of the Sonic Hedgehog (Shh) signaling pathway in several types of human cancer, including hepatic, breast, bladder and pancreatic cancers (17-20). The majority of these studies focused on the association between tumor cell radioresistance and the Shh signaling pathway; however, there are a few studies on radiosensitivity in EC and Shh signaling pathway. A previous study established the inhibitory effect of P162, which is a new peptide with anti-cancer mechanism, on glioma-associated oncogene family zinc finger 1 (Gli1), which resulted in noticeable improvements in the level of radiosensitivity in EC cells (21). The present study aimed to establish the association between Shh signaling pathway activation and EC cell radiosensitivity.

The current study examined the contribution of Gli1, a factor in the Shh signaling pathway, to the radiosensitivity...
of EC cells. The results of the present study may form the foundation for enhancing radiotherapy efficacy as part of the treatment regimen for future patients with EC.

Materials and methods

Cell culture. The Eca109 cell line was supplied by Taihe Medical College (Shiyian, China). Cells were maintained in liquid nitrogen and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with fetal bovine serum (10%) and penicillin (100 µg/ml) in a humidified atmosphere at 37°C and 5% CO₂.

Eca109R cell line establishment. X-ray irradiation of the Eca109 parental cell line was performed during the logarithmic growth phase at an absorbance dose rate of 2 Gy/min using the Varian 2300 linear accelerator (6 MV). On reaching 90% confluence, the cells were digested with trypsin in 0.25% EDTA, reinoculated and treated with repeated irradiation (2 Gy) during the logarithmic growth phase. A cumulative irradiation dose of 60 Gy was achieved by repeating the process 30 times. Subculture of the cells was performed to establish stable morphology, resulting in the formation of the radiation-resistant Eca109R cell line. Following continuous subculturing without irradiation, the other continued to be subcultured. The Eca109R cell line was still resistant to radiation through 50 generations of subculturing without irradiation.

Clone formation assay. A single cell suspension of Eca109 and Eca109R cells was prepared following trypsinization of each cell line in the logarithmic growth phase. Cells were seeded at a range of concentrations (400, 400, 800, 1,000 and 1,200 cells/well) in six-well plates and irradiated with 0, 2, 4, 6 and 8 Gy, with an average dose rate of 200 cGy/min. Following a two-week incubation period, the cells were fixed and 1,200 cells/well) in six-well plates and irradiated with 0, 2, 4, 6 and 8 Gy, with an average dose rate of 200 cGy/min. Following a two-week incubation period, the cells were fixed and 1,200 cells/well) in six-well plates and irradiated with 0, 2, 4, 6 and 8 Gy, with an average dose rate of 200 cGy/min.

Immunofluorescence assay. Immunofluorescence was used to determine Gli1 protein expression. Following fixation of Eca109 and Eca109R cells onto microscope slides with pure methanol at room temperature for 15 min, the cells were blocked for 10 min with 10% goat serum (ZSBG-Bio), followed by incubation with the primary antibody against Gli1 (1:150; cat. no. NBP2-45872; Novus Biologicals) and penicillin (100 µg/ml) in a humidified atmosphere at 37°C and 5% CO₂.

Eca109R cell line establishment. X-ray irradiation of the Eca109 parental cell line was performed during the logarithmic growth phase at an absorbance dose rate of 2 Gy/min using the Varian 2300 linear accelerator (6 MV). On reaching 90% confluence, the cells were digested with trypsin in 0.25% EDTA, reinoculated and treated with repeated irradiation (2 Gy) during the logarithmic growth phase. A cumulative irradiation dose of 60 Gy was achieved by repeating the process 30 times. Subculture of the cells was performed to establish stable morphology, resulting in the formation of the radiation-resistant Eca109R cell line. Following continuous subculturing without irradiation, the other continued to be subcultured. The Eca109R cell line was still resistant to radiation through 50 generations of subculturing without irradiation.

Cell transfection. For Gli1 overexpression, the full-length Gli1 sequence was synthesized by cloning the Gli1 gene into the GV146 vector (Shanghai GeneChem Co., Ltd.) via Xhol and EcoRI sites, and was referred to as pGli1-IRES-EGFP. Empty GV146 vector acted as a negative control (NC) of pGli1-IRES-EGFP and was referred to as pIRES-EGFP. For knockdown of Gli1, short hairpin RNA (shRNA) directed against Gli1 was ligated into the GV102 vector (Shanghai GeneChem Co., Ltd.) and was referred to as sh-Gli1; a non-targeting sequence was ligated into the GV102 vector (Shanghai GeneChem Co., Ltd.) and was referred to as sh-NC. The target sequence was 5'-CCCTCTGTCTACTCACACA-3' and the NC sequence was 5'-TTCTCCGAACGTGTCACTG-3'. Eca109 and Eca109R cells were seeded in 6-well plates at a density of 6x10⁵ cells/well one day before transfection. Briefly, 5 µl Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and 2.5 µg plasmid were diluted into 125 µl Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) and mixed for 5 min at room temperature. Cells were then incubated with this solution, and complete medium was added to the final volume of 2 ml. Cell transfection was performed for 24 h following the manufacturer's protocols prior to further experiments.

Statistical analysis. SPSS software (version 17.0; SPSS, Inc.) was used for statistical analysis. Data are expressed as the mean ± standard deviation. Differences among multiple groups were analyzed by one-way ANOVA with Duncan's multiple range test; the unpaired Student's t-test was used for comparisons between two groups. P<0.05 was considered to indicate a statistically significant difference.
Results

Differences between the Eca109 parental and Eca109R radiation-resistant cell lines. The Eca109R radiation-resistant cell line was established by repeated irradiation of the Eca109 parental cell line with 2 Gy/time (cumulative 60 Gy, 30 times in total). The morphology of the cells was observed using a light microscope (Olympus Corporation). At a higher magnification, Eca109 cells appeared as spherical or irregular polygons with a high degree of nuclear chromosome refractivity, whereas Eca109R cells appeared as irregular shuttle shapes with dense granular cytoplasm and small nuclei (Fig. 1A).

A colony formation assay was performed to characterize the radiosensitivity of Eca109 and Eca109R cells. The cells were seeded into six-well plates at different concentrations (400, 400, 800, 1,000 and 1,200 cells/well) and received irradiation at different doses (0, 2, 4, 6 and 8 Gy). Following irradiation, the survival fraction of the two cell lines was measured. Cell survival curves were generated using the single-hit multi-target mode (Fig. 1B). The results demonstrated that with the radiation dose increasing, cell survival rate gradually decreased and Eca109 cells were more sensitive to radiation than Eca109R cells (Fig. 1C). Radiological measurements of Dq, D0 and N values were higher in Eca109R cells compared with those in Eca109 cells (Table I). Thus, the Eca109R cell line exhibited higher resistance to irradiation compared with the Eca109 cell line.

Different expression of key factors in Shh signaling pathway between the Eca109 parental and Eca109R radiation-resistant cell lines. Gli1 expression and localization were determined through immunofluorescence. The results demonstrated that although Gli1 was present in the nucleus and cytoplasm of Eca109 and Eca109R cells (Fig. 2), and that Gli1 appeared

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Table I. Radiobiological parameters of Eca109 and Eca109R cells.

| Group   | Eca109          | Eca109R         | P-value |
|---------|-----------------|-----------------|---------|
| n       | 1.426±0.395     | 3.310±0.200     | 0.032   |
| D0 (Gy)| 2.142±0.332     | 3.092±0.153     | 0.024   |
| Dq (Gy)| 0.944±0.157     | 3.692±0.101     | 0.041   |
| SF2 (%)| 0.499±0.042     | 0.937±0.013     | 0.027   |

N, value of curve extension to the Y-axis intercept; D0, average lethal dose; Dq, quasi-threshold dose; SF2, cell survival fraction following irradiation with 2 Gy.
abundant in the nucleus and the cytoplasm of Eca109R cells (Fig. 2A) compared with Eca109 cells (Fig. 2B and C).

The expression levels of Gli1, patched 1 (Ptch), smoothened frizzled class receptor (Smo) and Shh were detected by western blotting, and the results revealed that the expression levels of these proteins were significantly higher in Eca109R, compared with Eca109 cells (P<0.05; Fig. 3). Thus, these results demonstrated a significant upregulation of the proteins in the Shh signaling pathway in radiation‑resistant cells.

Decreased Eca109 cell radiosensitivity following Gli1 overexpression. The results of plasmid construction were confirmed by sequencing and through enzyme digestion using both EcoRI and XhoI, generating fragments of 3 and 5 kb in length (Fig. 4). This confirmed the correct insertion of the target fragment and accurate construction of the pGli1-IREs-EGFP plasmid. Gli1 overexpression was established by transfecting the pGli1-IREs-EGFP plasmid into the Eca109 cell line, while the NC (empty plasmid) was transfected as a negative control, and the control group contained untransfected Eca109 cells. Following 48 h of transfection, the cells were identified using fluorescence microscopy. The transfection efficiency reached ~70% (Fig. 5A). Next, the protein expression level of Gli1 was detected by western blotting analysis; the results indicated significantly higher levels of Gli1 expression in cells transfected with pGli1-IREs-EGFP compared with those in the two control groups (both P<0.05; Fig. 5B and C).

Transfection of Eca109 cells with the pGli1-IREs-EGFP plasmid established the association between overexpression of Gli1 and the radiosensitivity of the cells. The three cell types were seeded into 6‑well plates and then irradiated at 0, 2, 4, 6 and 8 Gy, with a mean dose rate of 200 cGy/min, followed by 2 weeks of culture. A clone formation assay was performed to measure the survival fraction of each experimental group, and the single‑hit multi‑target mode was used to establish cell survival curves (Fig. 5D). The results demonstrated that
with the radiation dose increasing, cell survival rate gradually decreased and pGli1-IRES-EGFP-transfected Eca109 cells were more resistant to radiation. The two control groups exhibited significantly lower D0 and Dq values compared with the pGli1-iRES-EGFP-transfected Eca109 cells (P<0.05; Table II). The SF<sub>2</sub> in pGli1-iRES-EGFP-transfected Eca109 cells was 0.760±0.033, whereas those in the nC and control groups were 0.532±0.062 and 0.350±0.029, respectively (Table II). These findings demonstrated a reduction in radiation sensitivity in Eca109 cells following Gli1 overexpression (Table II). Therefore, the transfection of Eca109 cells with the pGli1-IRES-EGFP established an association between overexpression of Gli1 and the radiosensitivity of the cells.

Increased Eca109R cell radiosensitivity may be attributed to Gli1 knockdown. Reduced Gli1 expression was established by transfecting the Gli1-silencing plasmid into the Eca109R cell line. The non-targeted Gli1 plasmid was transfected into the Eca109R cell line to create the nC, and the control group contained untransfected Eca109R cells. The transfection efficiency of the Gli1-silencing plasmid in Eca109R cells was analyzed using fluorescence microscopy after 48 h. Green fluorescence confirmed successful transfection, and the transfection efficiency reached ~70% (Fig. 6A). Gli1 expression levels were significantly lower in sh-Gli1-transfected Eca109R cells compared with the nC and control groups, as determined by western blotting (P<0.05; Fig. 6B and C). These results indicated the effective generation of a Gli1-silenced Eca109R cell line.

The cells were harvested, seeded into six-well plates, irradiated and cultured over two weeks, followed by staining and counting. SF<sub>2</sub> was measured, and the single-hit multi-target model was used to establish cell survival curves (Fig. 6D). The results indicated that with the radiation dose increasing, cell survival rate gradually decreased and Gli1-silenced Eca109R cells were more sensitive to radiation. The Gli1-silenced Eca109R cells exhibited significantly lower levels of the radiation-associated biological measurements (Dq, D0 and N values) compared with the two control groups (P<0.05; Table III). The SF<sub>2</sub> in the sh-Gli1 plasmid-transfected Eca109R cell line was 0.446±0.010, whereas in the nC and the control groups it was 0.867±0.070 and 0.916±0.128, respectively (Table III). These results indicated that the radiosensitivity of Eca109R Gli1-silenced cell line was significantly enhanced and that the radioresistance was weakened.

### Discussion

EC frequently occurs as a primary tumor in the upper gastrointestinal tract, and its malignant nature is harmful to humans (22). A primary form of treatment for EC is radiation therapy (15,23). However, reduced radiosensitivity develops with gradual increases in the irradiation dose and frequency, leading to the development of radiotherapy-resistant tumors (24). The recurrence and metastasis of tumors have been widely attributed to radiotherapy resistance (25,26), and the options available to individuals suffering from inoperable forms of EC are further reduced by the development of radioresistance (27,28).
Figure 5. Gli1 overexpression decreases the sensitivity of Eca109 cells to radiation. (A) Eca109 cells were transfected with NC and pGli1-IRES-EGFP plasmids. Magnification, x10. (B and C) Expression levels of Gli1 protein in Eca109 cells after transfection. (D) Survival curves of control, NC and pGli1-IRES-EGFP-transfected Eca109 cells. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01 vs. negative control. Gli1, glioma-associated oncogene family zinc finger 1; NC, negative control.

Figure 6. Silencing of Gli1 increased the sensitivity of Eca109R cells to radiation (x10 magnification). (A) Eca109R cells were transfected with NC and Gli1-silencing plasmids. (B and C) Expression levels of Gli1 protein in Eca109R cells following transfection. (D) Survival curves of control, NC and sh-Gli1 Eca109R cells. Data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. negative control. Gli1, glioma-associated oncogene family zinc finger 1; sh, short hairpin (RNA); NC, negative control.
To enhance the efficacy radiotherapy, it is crucial to research radioresistance mechanisms. Various studies have been performed on the mechanisms of radioresistance, including DNA damage and repair (29), cell proliferation and apoptosis (30), abnormal expression of microRNA and lncRNA (31-34) and disruption of the cell cycle (30) and altered expression of Shh signaling pathway (35). Xie et al (35) demonstrated that Raf kinase inhibitory protein reduction enhances radioresistance by activating the Shh signaling pathway. The present study also tried to explore whether radioresistance was associated with Shh signaling pathway activation.

The Sonic Hedgehog signaling pathway consists of Shh ligands, the transmembrane proteins Ptch and Smo, and the downstream Gli transcription factors (Gli1, Gli2 and Gli3) (36). Abnormal activation of the Shh signaling pathway is reliably detected through the expression of Gli1 (37,38). An earlier study established the activation of the Shh signaling pathway during tissue repair and an absence of this signaling in normally-functioning adult tissues and organs (39). Furthermore, a previous study suggested an association between Shh signaling pathway activation and the development of resistance in a range of human cancer types, including EC (40).

In normal tissues, Smo protein activity is inhibited by Ptch (41). However, when Shh associates with Ptc, the Gli1 protein enters the nucleus to activate the transcription of the downstream target genes (42). Increased Gli1 expression levels were demonstrated in EC tissues and adjacent tissues compared with normal tissues (43), and Gli1 has been detected in the nuclei of a number of tumor-cell types. A recent study by Huang et al (44) demonstrated that Hh signaling pathway activation is activated in Hela-RR and Siha-RR, which was also demonstrated in the present study. Furthermore, the expression of Shh, Ptc and Smo has been detected in 34 ESCC cell lines, and Gli1 was highly expressed in 31 EC cell lines (45). In addition, silencing of Gli1 expression was achieved through specific inhibitors of Smo, which led to the inhibition of fission, recurrence and metastasis in ESCC (45). Gli1 transcription efficacy is positively associated with its expression, which can be used to efficiently detect abnormal activation of the Shh signaling pathway (46). The results of the current study revealed higher Gli1 protein expression levels in Eca109R cells, compared with Eca109 cells. Furthermore, Gli1 in radiation-resistant cells was aggregated around the nucleus, as determined by immunofluorescence. These results suggested an association between radioresistance in EC and the Shh signaling pathway.

The radiation-resistant cell line Eca109R was generated through low-level X-ray irradiation of the human EC cell line Eca109. Colony formation assays demonstrated higher measurements of the radiation-related biological parameters (D0, Dq and N) in Eca109R, compared with Eca109 cells, which indicated increased levels of resistance in the Eca109R cell line compared with the parental cells. Furthermore, the expression of Gli1, Ptc, Shh and Smo was confirmed by western blotting in Eca109 and Eca109R cells; all of the tested proteins exhibited significantly higher expression levels in Eca109R cells compared with Eca109 cells, and immunofluorescence displayed Gli1 protein aggregation around the nucleus. A previous study reported that Shh signaling pathway activation is associated with the development of esophageal squamous cell carcinoma (ESCC) (47). High expression of Shh signaling pathway-related genes is present in ESCC, and patients with high Gli1 expression in ESCC are not sensitive to radiation therapy (47), which was the case in the present study. A Gli1 overexpression plasmid was constructed and subsequently transfected into Eca109 cells, and analyses confirmed that Gli1 protein expression was increased. In addition, the clone formation assay showed that radiosensitivity was decreased in Gli1-overexpressing Eca109 cells compared with untransfected cells. Furthermore, the Eca109R cell line was transfected with a Gli-1 silencing plasmid. These cells exhibited significantly lower Gli1 expression levels and higher levels of radiosensitivity compared with the control groups. A previous study reported that Hh signaling pathway can influence the radiation response in some patient-derived murine xenograft (PDX) model of esophageal adenocarcinoma, and that inhibition of this pathway could increase the radiation efficacy (48). In conclusion, these findings demonstrated an association between Gli1 and radioresistance in EC.

The mechanism underlying radioresistance, which contributes to the pathogenicity of EC, is a complex process; further research into how the Shh signaling pathway impacts radioresistance is required. The present study described novel research that may be used as a foundation for future studies into the processes regulating Shh signaling pathway activation-induced radioresistance.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

QW, HLi, QL and HLo designed the experiments. FY, JY, YH and JL performed the experiments and analyzed all data. FY and JY drafted the manuscript and all authors approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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