Original Paper

α -Solanine Modulates the Radiosensitivity of Esophageal Cancer Cells by Inducing MicroRNA-138 Expression

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Key Words

Esophageal cancer cells • α -Solanine • miR-138 • Radiosensitivity • Survivin

Abstract

Background: Esophageal cancer (EC) is one of the most common malignant tumors in the world. Due to difficulties with performing the operation, most patients choose to have palliative treatment instead. Radiotherapy is one of the main palliative treatments of EC. However, the clinical efficacy of radiotherapy is not satisfactory. α -Solanine is a bioactive component of steroidal glycoalkaloids which has been demonstrated to exhibit anti-metastasis activity in different cancers. In the present study, we determined the effect of α -solanine on the radiosensitivity of EC cells and preliminarily explored the underlying molecular mechanisms.

Methods: Cell Counting Kit-8 (CCK-8) assay was conducted to found the cytotoxic effect of α -solanine on EC cells. CCK-8 assay and colony-forming survival assays were performed to explore the effect of α -solanine on cell viability and proliferation of EC cells after irradiation. Immunofluorescence and comet assays were used to detect the effect of α -solanine on DNA repair capacity of EC cells after irradiation. The flow cytometry (FCM) and Hoechst/PI staining were conducted to study the effect of α -solanine on apoptosis of EC cells after irradiation.

Results: The cytotoxic effect of α -solanine to EC cells was dose-dependent. The results of CCK-8, colony-forming survival assay, immunofluorescence, comet assay, FCM and Hoechst/PI staining showed that α -solanine could enhance the radiosensitivity of EC cells. α -Solanine could downregulate Survivin expression level by upregulating miR-138 expression in EC cells. Upregulation of miR-138 and knock down Survivin both enhanced the radiosensitivity of EC cells. Moreover, Survivin could restore the effect of α -solanine and miR-138 on radiosensitivity of EC cells. **Conclusions:** α -solanine could enhance the radiosensitivity of esophageal cancer cells by inducing microRNA-138 expression, and probably be an effective radiosensitizer in treating EC.

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Introduction

Esophageal cancer (EC) is the eighth most frequently diagnosed cancer and the sixth most common cause of cancer related death in the world [1, 2]. Most patients with advanced EC are unable to undergo curative resection and have to turn more often to cytotoxic treatments including chemotherapy and radiotherapy [3]. Although substantial progress has been made in chemotherapy, radiotherapy and combination of the two therapies, the rate of local recurrence and distant metastasis remain high [4, 5]. Acquired radioresistance during radiotherapy has been considered as one of the most important reasons for treatment failure [6].

In recent years, the interest in exploring the use of small-molecule anticancer compounds for the prevention or treatment of tumors has increased [7-9]. α -Solanine, a bioactive component of steroidal glycoalkaloids, is mainly found in the potato tuber and the nightshade plant. α -Solanine has been demonstrated to exhibit anti-metastasis activity in different cancers [10-14] and also exert chemoprotective and chemotherapeutic effects in animal models of breast cancer [15]. Therefore, α -solanine may possess a potential effect on the progress and therapy of EC cells.

MicroRNAs (miRNAs), typically 18-25 nucleotides, are a class of small non-coding single-stranded RNAs (ssRNAs) that are highly conserved and endogenously expressed across many species [16-21]. Although the full extent of miRNA biological functions has yet to be elucidated, they have been suggested to act as intrinsic regulators for many cellular processes, including cell invasion, differentiation, proliferation and apoptosis [22-27]. Aberrant expression of miRNAs has been linked to the development and progression of cancer and they have been shown to have prognostic significance for certain tumor types, such as lung and esophageal cancer, neuroblastoma and lymphocytic leukemia [28-31]. Some studies have also found that miRNA can affect the chemosensitivity or radiosensitivity of cancers [32-34]. It has been revealed that miR-138 can affect radiosensitivity in lung cancer cells [35, 36]. We were greatly interested in whether there is a relationship between α -solanine and miR-138 in the progress and radiotherapy of EC. Through bioinformatics analyses, we hypothesized that survivin was a target of miR-138. Survivin protein, a member of the inhibitors of apoptosis family, is implicated in the regulation of cell proliferation and apoptosis. It is overexpressed in malignant cells and can be elevated by both high and very low doses of ionizing radiation, and its role in elevating tumor cell resistance to radiation therapy is well documented [37-39]. In the present study, we determined the effect of α -solanine on the radiosensitivity of EC9706 and KYSE30 cells and preliminarily explored the underlying molecular mechanisms.

Materials and Methods

Cell culture and reagents

Human EC cell lines (EC9706 and KYSE30) and human esophageal epithelial cell line (Het-1A) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) and incubated at 37 °C, 5 % CO₂. α -Solanine was dissolved in dimethylsulfoxide (DMSO), both of which were purchased from Sigma-Aldrich (St. Louis, MO, USA). After dissolution α -solanine was stored at -20 °C.

CCK-8 assay

Cells in logarithmic phase growth were seeded into a 96-well plate at a density of 1×10^4 cells/well. The next day the cells were treated with increasing concentrations (0, 2, 4, 8, 12 and 16 μ mol/L) of α -solanine for 24 h, and subjected to 0-6 Gy of IR or not. The viability of cells was determined according to Cell Counting Kit-8 manufactures (Dojindo Laboratories, Japan). Cells were cultured at 37°C in 5% for 3 h, after which the optical density was measured at 450 nm. The experiments were performed in triplicate.

Colony-forming survival assay

Clonogenic survival is the ability of cells to maintain clonogenic capacity and to form colonies. Cells were treated with α -solanine (0, 2, 6 $\mu\text{mol/L}$) for 24 h, and subjected to 0, 2, 4, 6, 8 Gy of IR. Cells from each cell line were subdivided into groups according to the concentrations of α -solanine: Blank group that was treated with nothing, α -solanine 0 $\mu\text{mol/L}$ group that was treated with DMSO, α -solanine 2 $\mu\text{mol/L}$ group that was treated with 2 $\mu\text{mol/L}$ α -solanine, α -solanine 6 $\mu\text{mol/L}$ group that was treated with 6 $\mu\text{mol/L}$ α -solanine. Immediately after IR, cells were harvested. 200 cells were then plated into 10 cm dishes containing DMEM supplemented with 10% FBS with 200 cells per dish. The colony formation was visible, which usually occurred in approximately 2 weeks. Then, the colonies formed were fixed with ethanol and stained with crystal violet, and the colonies with >50 cells were scored as surviving colonies. The cloning efficiency was calculated by dividing the average number of colonies per dish by the amount of cells plated.

Immunofluorescence microscopy

Immunofluorescence was performed to observe the expression level of γ -H2AX in the nucleus of cells that treated with α -solanine (0, 2, 6 $\mu\text{mol/L}$) and irradiation. After irradiated with 4 Gy X-ray, cells were fixed with 4 % paraformaldehyde for 15 min at room temperature, permeabilized with 0.1 % Triton X-100. After blocking in PBS containing 5% BSA for 1 h, cells were incubated with anti- γ -H2AX primary antibodies (Santa Cruz, USA) and stained with FITC-conjugated secondary antibodies (Santa Cruz, USA). Then, the nuclear were stained with Hoechst 33342 for 15 min. Immunofluorescence was quantified by counting the mean number of immunostained nuclei per high-power field. Results are presented as the average of at least three counts.

Comet assay

The comet assay was used to measure single strand breaks (SSBs) and DNA repair capacity of the cells treated with α -solanine (0, 2, 6 $\mu\text{mol/L}$) and irradiation (4 Gy). EC9706 and KYSE30 cells were cultured in medium containing different concentrations of α -solanine for 24 h, then the cells were diluted to a concentration of $0.5\text{--}1 \times 10^6$ cells/mL, and layered onto prepared slides coated with 1 % normal melting point agarose. Next, the cells were mixed with low melting point (LMP) agarose gently at a 1:10 ratio; this cell suspension was layered onto the precoated slides and coverslipped immediately. After the agarose solidified, the coverslip was removed and the second layer of 100 mL 1 % LMP was layered onto the slides. Each slide was exposed to 4 Gy X-ray, and then the cells were allowed to undergo repair for 6 h. After 6 h, slides of each cell line were immediately put into cold lysing solution overnight at 4 °C. Following lysis, slides were washed for 5 min and placed into cold electrophoresis buffer to allow the DNA to unwind. Slides were then electrophoresed at 25 V for 40 min, and washed three times and fixed with cold ethanol. The second set of slides for each cell line was handled in the same fashion as the treated cells but with no irradiation. After staining slides with EB, 100 cells were scored per slide using Comet Assay software version 4.11. The extent of DNA damage was measured as the percentage of migrated DNA (% Tail DNA). In particular, “adjusted mean” and “adjusted SD” values were calculated for the irradiation-exposed % Tail DNA of each slide as follows:

Adjusted mean = mean of % Tail DNA(irradiation) / mean % Tail DNA (untreated) at the same time point $\times 100\%$

Adjusted SD = % Tail DNA SD(irradiation) / % Tail DNA SD(untreated) at the same time point $\times 100\%$

Flow cytometry assay

Apoptosis was analyzed by flow cytometry assay and Hoechst/PI staining assay. Flow cytometry assays were conducted using FITC Annexin V Apoptosis Detection Kit I (BestBio, Shanghai, China), according to the manufacturer’s instructions. EC9706 and KYSE30 cells were cultured in medium containing different concentrations of α -solanine for 24 h, then each cell lines was divided into two groups: one group was irradiated with 4 Gy X-ray, and the other group was irradiated with 0 Gy X-ray served as the control. Cells from each group were harvested by trypsinization and resuspended at a density of 1×10^6 cells/mL in 1 $\times$ binding buffer. After double staining with FITC Annexin V and propidium iodide (PI), cells were analyzed using an FACScan® flow cytometer (BD Biosciences, USA) equipped with CellQuest software (BD Biosciences, USA).

Hoechst/PI staining assay

Hoechst/PI staining assay was characterized by nuclear condensation /fragmentation revealed by double staining with Hoechst 33342 (Sigma, St Louis, MO, USA) and PI (Sigma) staining, according to the manufacturer's instructions. EC9706 and KYSE30 cells were cultured in medium containing different concentrations of α -solanine for 24 h, then each cell lines was divided into two groups: one group was irradiated with 4 Gy Xray, and the other group was irradiated with 0 Gy Xray served as the control. The cells were harvested by trypsinization and resuspended at a density of 1×10^6 cells/mL in $1 \times$ binding buffer. After double staining with Hoechst 33342 and PI, apoptotic cells were scored under a Zeiss Axioskop Fluorescence Microscope (Carl Zeiss, Thornwood, NY, USA). PI uptake (red fluorescence) and Hoechst uptake (blue fluorescence) was observed and photographed with a fluorescence microscope for image detection. Apoptosis was quantified by averaging cell counts in three to four randomly selected fields per dish. To evaluate the apoptotic cells, the ratio of both stained cells to total nuclei (%) was calculated.

Western blot assay

Total proteins from the transfected cells were extracted using RIPA buffer containing phenylmethanesulfonyl fluoride (PMSF). A BCA protein assay kit (Beyotime, Haimen, China) was used to determine the protein concentrations. 40 μ g proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinyl difluoride (PVDF) membranes. After blocking, the membranes were incubated overnight at 4 °C with diluted (1:1000) primary antibody (polyclonal rabbit anti-Survivin; Santa Cruz, USA). Following extensive washes, the membranes were incubated with diluted (1:5000) horseradish peroxidase conjugated goat anti-rabbit IgG (Santa Cruz). Signals were determined using DAB detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). GAPDH (Santa Cruz, USA) served as the endogenous reference.

RNA extraction and quantitative real-time PCR

RNA was extracted using the Total RNA Kit I (R6834-01; Omega) from the aforementioned cells. To verify mature miRNA expression levels, quantitative real-time PCR (qRT-PCR) was performed using a High-Specificity miR-138 qRT-PCR Detection Kit (Stratagene Corp, La Jolla, CA) in conjunction with an ABI 7500 thermal cycler, according to the manufacturer's recommendations. We used U6 small nuclear RNA (U6 snRNA) as an endogenous control for normalization. For the detection of Survivin mRNA, reverse transcription and quantitative real-time PCR (qRT-PCR) were performed using ABI TaqMan PCR Master Mix (Applied Biosystems). GAPDH was amplified in parallel as an internal control. The corresponding CT values were recorded with ABI 7500 Software, and then the relative expression levels were calculated according to the formula $2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$.

miRNA transfection

For cell transfection, EC9706 and KYSE30 cells were seeded into six-well plates at a density of 5×10^4 cells/well. Once the cells reached approximately 50-80 % confluence, transient transfection was conducted using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacture's instructions. MiR-138 mimics and scrambled oligonucleotide were synthesized by GenePharma Co. Ltd. (Shanghai, China). Cells from each cell line were subdivided into groups as follows: miR-138 mimics group (miR-138) that was transfected with miR-138 mimics, negative control group (NC) that was transfected with scrambled oligonucleotide, the blank group (Blank) that was treated with DMSO, and Survivin siRNA group (Si-Survivin) that was transfected with siRNA of Survivin.

Dual-luciferase assay

The human Survivin 3'untranslated region (3'UTR) fragment containing putative binding sites for miR-138 were amplified by PCR from human genomic DNA. The mutant Survivin 3'UTRs were obtained by overlap extension PCR. The fragments were cloned into a pmirGLO reporter vector (Promega), to generate the recombinant vectors pmirGLO-W-Survivin and pmirGLO-M-Survivin. For the luciferase reporter assay, EC9706 cell line was transiently co-transfected with miRNA (miR-138 agomir or scrambled-miR-138 negative control) and reporter vectors (wild-type reporter vectors or mutant-type reporter vectors), using Lipofectamine™2000. Luciferase activities were measured using a Dual-Luciferase assay kit (Promega) according to manufacturer' s instructions at 48h post-transfection.

Statistical analysis

Statistical testing was conducted with the assistance of SPSS 17.0 software. All the data is expressed as means $\pm$ standard deviation (SD). One-way ANOVA and LSD tests were used to the analyze data. Results were considered significant when P values were <0.05 .

Results

α -Solanine showed cytotoxic effect in EC9706, KYES30 and Het-1A cells

The results of CCK-8 assay showed the cytotoxic effect of α -solanine on EC9706, KYES30 and Het-1A cells. As illustrated in Fig. 1A, treatment with 16 $\mu\text{mol/L}$ α -solanine could significantly decrease the viability of EC9706, KYES30 and Het-1A cells, and no significant difference existed between the response of Het-1A cells to α -solanine and that of EC9706 and KYES30 cells ($P < 0.05$; Fig. 1B, C, D). Moreover, treatment with α -solanine at no more than 12 $\mu\text{mol/L}$ did not cause obvious cytotoxicity in EC9706, KYES30 and Het-1A cells.

α -Solanine enhanced the effect of irradiation inhibition of proliferation to EC9706, KYSE30 and Het-1A cells

To explore the effect of α -solanine on the radiosensitivity of EC9706, KYSE30 and Het-1A cells, we performed CCK-8 and colony-forming survival assays. Cells were incubated with different concentrations of α -solanine (0, 2, 6 $\mu\text{mol/L}$) for 48 h and exposed to different doses of radiation (0, 2, 4, 6, 8 Gy). The cell viability results are presented in Fig. 2A, 2B and 2C. The cell viability decreased with increased radiation dose. In Het-1A cells, the α -solanine 6 $\mu\text{mol/L}$ group declined more significantly, and the decline has statistical differences with the α -solanine 0 $\mu\text{mol/L}$ and Blank groups after irradiated with 6 Gy and 8 Gy ($P < 0.05$, Fig.

Fig. 1. Cytotoxic effect of α -solanine in EC9706, KYES30 and Het-1A cells. (A) Chemical structure of α -solanine. (B) EC9706 cells line was treated with various concentrations of α -solanine for 24 h and 48 h. Cell viability was determined by CCK-8 assay. Compared with the untreated control, the percentage of cell viability in different concentrations of α -solanine was presented. (C) KYES30 cells line was treated with various concentrations of α -solanine for 24 h and 48 h. (D) Het-1A cells line was treated with various concentrations of α -solanine for 24 h and 48 h. * $P < 0.05$.

Fig. 2. Effects of α -solanine on the radiosensitivity of EC9706, KYSE30 and Het-1A. Cells were incubated with different concentrations of α -solanine (0, 2, 6 μ mol/L) for 48 h and exposed to different doses of radiation (0, 2, 4, 6, 8 Gy). (A, B, C) The results of CCK-8 assay showed that the cell viability decreased with increased radiation dose. In Het-1A cells, the α -solanine 6 μ mol/L group declined more significantly than the α -solanine 0 μ mol/L and Blank groups after irradiated with 6 Gy and 8 Gy. In EC9706 and KYSE30 cells, the α -solanine 2 μ mol/L and 6 μ mol/L groups declined more significantly than the α -solanine 0 μ mol/L and Blank groups at different irradiation doses. (D, E, F) The results of colony-forming survival assay showed that the α -solanine 6 μ mol/L group declined more significantly than the α -solanine 0 μ mol/L and Blank groups after irradiated with 6 Gy and 8 Gy in Het-1A cells. In EC9706 and KYSE30 cells, the α -solanine 2 μ mol/L and 6 μ mol/L groups declined more significantly than the α -solanine 0 μ mol/L and Blank groups at different irradiation doses. * $P < 0.05$.

2 A). In EC9706 and KYSE30 cells, the α -solanine 2 μ mol/L and 6 μ mol/L groups declined more significantly, and the decline has statistical differences with the α -solanine 0 μ mol/L and Blank groups at different irradiation doses ($P < 0.05$, Fig. 2 B, C). The clonogenic survival rate curves are shown in Fig. 2D, 2E and 2F. The surviving fraction decreased with increased radiation dose. In Het-1A cells, the α -solanine 6 μ mol/L group declined more significantly, and the decline has statistical differences with the α -solanine 0 μ mol/L and Blank groups after irradiated with 6 Gy and 8 Gy ($P < 0.05$, Fig. 2 D). In EC9706 and KYSE30 cells, the α -solanine 2 μ mol/L and 6 μ mol/L groups declined more significantly, and the decline has statistical differences with the α -solanine 0 μ mol/L and Blank groups at different irradiation doses ($P < 0.05$, Fig. 2E F).

α -Solanine impairs DNA repair capacity after irradiation in EC9706 and KYSE30 cells

Immunofluorescence was performed to observe the expression level of γ -H2AX in nucleus of different groups after irradiated with 4 Gy X-ray. The merged confocal immunofluorescence microscopy images of EC9706 and KYSE30 showed that γ -H2AX were strongly enhanced in the α -solanine 2 μ mol/L and α -solanine 6 μ mol/L groups compared with α -solanine 0 μ mol/L and Blank groups (Fig. 3A).

DNA strand breaks were measured by comet assay in EC9706 and KYSE30 cells after they were irradiated with 4 Gy X-ray. The comet assay (single cell gel electrophoresis) results are shown in Fig. 4b. The α -solanine 2 μ mol/L and α -solanine 6 μ mol/L groups demonstrated a higher level of strand breaks after irradiation than the α -solanine 0 μ mol/L and Blank groups ($P < 0.05$, Fig. 3B). These results indicate that α -solanine markedly impairs DNA repair capacity after irradiation in EC9706 and KYSE30 cells.

Fig. 3. α -solanine impairs DNA repair capacity after irradiation in EC9706 and KYSE30 cells. (A) Immunofluorescence was performed to observe the expression level of γ -H2AX in nucleus of different groups after irradiation. The merged images of EC9706 and KYSE30 showed that γ -H2AX was strongly enhanced in the α -solanine 2 μ mol/L and α -solanine 6 μ mol/L groups compared with α -solanine 0 μ mol/L and Blank groups. (B) Comet assay was performed to measure strand breaks of different groups after irradiation. Mean % Tail DNA was normalized to the untreated mean % Tail DNA in the respective cell lines. The α -solanine 2 μ mol/L and α -solanine 6 μ mol/L groups demonstrated a higher level of strand breaks after irradiation than the α -solanine 0 μ mol/L and Blank groups. * $P < 0.05$.

α -Solanine enhanced the effect of irradiation-induced apoptosis in EC9706 and KYSE30 cells

We detected the effects of α -Solanine on apoptosis of EC9706 and KYSE30 cells that were treated with irradiation and/or α -Solanine. The FCM results showed that the apoptosis rate was significantly raised after irradiated with 4 Gy X-ray. To the cells irradiated with 4 Gy X-ray, the apoptosis rates of α -Solanine 2 μ mol/L and α -Solanine 6 μ mol/L groups were significantly higher than α -solanine 0 μ mol/L and Blank groups (Fig. 4A, $P < 0.05$). Apoptosis was also evaluated by double staining with Hoechst and PI. The amount of apoptosis cells was significantly raised after irradiated with 4 Gy X-ray. And to the cells irradiated with 4 Gy X-ray, the amount of apoptosis cells of α -Solanine 2 μ mol/L and α -Solanine 6 μ mol/L groups were significantly higher than α -solanine 0 μ mol/L and Blank groups (Fig. 4B).

Induction of apoptosis was further confirmed by the expression of apoptosis related proteins using western blot. Compared to α -solanine 0 μ mol/L and Blank groups, the protein levels of cleaved caspase-3 and cleaved caspase-9 were enhanced in α -Solanine 2 μ mol/L and α -Solanine 6 μ mol/L groups cells after irradiated with 4 Gy X-ray, while Survivin decreased (Fig. 4C). These results inferred that α -Solanine enhanced the effect of irradiation-induced apoptosis in EC9706 and KYSE30 cells, which due to the increasing expression of cleaved caspase-3 and cleaved caspase-9 and decreasing expression of Survivin.

Fig. 4. α -Solanine enhanced the effect of irradiation-induced apoptosis in EC9706 and KYSE30 cells. (A) Flow cytometric analysis showed the apoptosis rates of EC9706 and KYSE30 cells. Compared to α -solanine 0 μ mol/L and Blank groups, the apoptosis rates were higher in α -Solanine 2 μ mol/L and α -Solanine 6 μ mol/L groups cells after irradiated with 4 Gy X-ray. (B) Results from Hoechst 33342 staining assay showed that the amount of apoptosis cells were higher in α -Solanine 2 μ mol/L and α -Solanine 6 μ mol/L groups cells than after α -solanine 0 μ mol/L and Blank groups cells that irradiated with 4 Gy X-ray. (C) Protein expression was measured by Western blot assay. Compared to α -solanine 0 μ mol/L and Blank groups, the protein levels of cleaved caspase-3 and cleaved caspase-9 were enhanced in α -Solanine 2 μ mol/L and α -Solanine 6 μ mol/L groups cells after irradiated with 4 Gy X-ray, while Survivin decreased * P <0.05.

α -Solanine induced the upregulation of miR-138 and downregulation of Survivin expression in EC9706 and KYSE30 cells

qRT-PCR and Western blotting assays were used to detect the changes of miR-138 and Survivin expression in EC9706 and KYSE30 cells treated with different concentrations of α -solanine (0, 2, 4, 8, 12, 16 μ mol/L). Relative expressions of miR-138 in EC9706 and KYSE30

Fig. 5. Effects of α -solanine on the expression of miR-138 and Survivin in EC9706 and KYSE30 cells. (A) Results of qRT-PCR showed that relative expression of miR-138 in EC9706 and KYSE30 cells treated with different concentrations of α -solanine had an obvious upregulation. (B) Results of qRT-PCR showed that relative expression of Survivin mRNA in EC9706 and KYSE30 cells treated with different concentrations of α -solanine had an obvious downregulation. (C) Results of Western blotting showed expression of Survivin in EC9706 and KYSE30 cells treated with different concentrations of α -solanine had an obvious downregulation. * $P < 0.05$.

Fig. 6. Survivin was identified as a target of miR-138 in EC9706 cell. (A) The putative miR-138 binding sequences for the Survivin 3' UTR. (B) Western blot analysis of Survivin expression in transfected cells. Survivin expression was down-regulated in EC9706 cell following transfection with the miR-138 mimics. GAPDH was used as a reference. (C) Luciferase activity determined 48 h after transfection. Co-transfection with miR-138 significantly suppressed the luciferase activity of the reporter containing the wild-type 3' UTR of Survivin ($P < 0.05$). pmirGLO-W-Survivin, wild-type pmirGLO-Survivin; pmirGLO-M-Survivin, mutant pmirGLO-Survivin. * $P < 0.05$.

cells treated with α -solanine (2, 4, 8, 12, 16 μ mol/L) had an obvious upregulation than that of 0 μ mol/L α -solanine ($P < 0.05$, Fig. 5A). The results of qRT-PCR revealed that Survivin expression was downregulated in EC9706 and KYSE30 cells treated with α -solanine (2, 4, 8, 12, 16 μ mol/L) than that of 0 μ mol/L α -solanine ($P < 0.05$, Fig. 5B), which showed an inverse tendency compared with miR-138 expression. Results of Western blotting showed an obvious downregulation of Survivin expression ($P < 0.05$, Fig. 5C). These results demonstrate that α -solanine can induce the upregulation of miR-138 and downregulation of Survivin expression in EC9706 and KYSE30 cells.

miR-138 targets Survivin via binding to its 3'UTR

Bioinformatic analyses using TargetScan and miRanda predicted that the 3' UTR of Survivin contain binding sites for miR-138 (Fig. 6A). Subsequent Western blot analysis indeed

Fig. 7. Effects of upregulation of miR-138 and knock down Survivin to the radiosensitivity of EC9706 and KYSE30 cells. (A) After transfection, qRT-PCR results showed that miR-138 expression was significantly increased in the miR-138 group compared with the Blank, NC and si-Survivin groups. (B) After transfection, Western blotting results showed that Survivin expression was significantly decreased in the miR-138 and si-Survivin groups compared with the Blank and NC groups. (C, D) CCK-8 assay revealed that viability of EC9706 and KYSE30 cells was significantly inhibited with different dose of radiation in the miR-138 and si-Survivin groups compared with Blank and NC groups. (E, F) Colony-forming survival assay revealed that the survival fraction of EC9706 and KYSE30 cells was significantly reduced with every dose of radiation (0, 2, 4, 6, 8 Gy) in the miR-138 and si-Survivin groups compared with the Blank and NC groups. miR-138: cells transfected with miR-138 mimics; NC: cells transfected with scrambled miR-138 negative control; Blank: cells with DMSO; si-Survivin: cells transfected with siRNA-Survivin. * $P < 0.05$.

showed that Survivin expression was down-regulated in EC9706 cell following transfection with the miR-138 mimics (Fig. 6B). To verify whether Survivin is direct targets of miR-138, we used a Dual-Luciferase reporter system containing either wild-type (pmirGLO-W-Survivin) or mutant (pmirGLO-M-Survivin) 3' UTR of Survivin, respectively. Co-transfection with miR-138 significantly suppressed the luciferase activity of the reporter containing the wild-type 3' UTR of Survivin ($P < 0.05$, Fig. 6C). These results indicate that miR-138 negatively regulates Survivin expression by directly binding to putative binding sites in the 3' UTR.

Upregulation of miR-138 and knock down Survivin both enhanced the radiosensitivity of EC9706 and KYSE30 cells

MiR-138 mimics, a scrambled miR-138 negative control or siRNA-Survivin was transfected into EC9706 and KYSE30 cells, respectively. qRT-PCR results showed that miR-138 expression of miR-138 group cells (cells transfected with miR-138 mimics) was significantly increased compared with the Blank (cells treated with DMSO), NC (cells transfected with scrambled miR-138 negative control) and si-Survivin groups (cells transfected with siRNA-

Fig. 8. Expression of Survivin restored the effect of α -solanine and miR-138 on the radiosensitivity of EC9706 cells. (A) Western blotting detected the expression of Survivin after transfection with miR-138 mimics, si-Survivin or treated with 4 μ mol/L α -solanine respectively, and simultaneously or separately transfected with vector containing Survivin but lacking the 3'UTR sequence (pcDNA3.1-Survivin). (B) FCM assay detected the radiosensitivity of EC9706 cells exposed to 4 Gy radiation after transfection with miR-138 mimics, si-Survivin or treated with 4 μ mol/L α -solanine respectively, and simultaneously or separately transfected with pcDNA3.1-Survivin. (C) Cell viability assay detected the radiosensitivity of EC9706 cells exposed to 4 Gy radiation after transfection with miR-138 mimics, si-Survivin or treated with 4 μ mol/L α -solanine respectively, and simultaneously or separately transfected with pcDNA3.1-Survivin. (D) Colony-forming survival assay detected the radiosensitivity of EC9706 cells exposed to 4 Gy radiation after transfection with miR-138 mimics, si-Survivin or treated with 4 μ mol/L α -solanine respectively, and simultaneously or separately transfected with pcDNA3.1-Survivin. * $P < 0.05$.

Survivin)($P < 0.05$, Fig. 7A). Western blotting results showed Survivin expression was significantly decreased in the miR-138 and si-Survivin groups compared with the Blank and NC groups ($P < 0.05$, Fig. 7B). Then we conducted CCK-8 and colony-forming survival assays to study the effect of miR-138 and Survivin to the radiosensitivity of EC9706 and KYSE30 cells. CCK-8 assay revealed that the viability of EC9706 and KYSE30 cells was significantly inhibited with different dose of radiation in the miR-138 and si-Survivin groups compared with Blank and NC groups ($P < 0.05$, Fig. 7C, D), and the inhibitory action became gradually obvious along with increased dose of radiation (2, 4, 6, 8 Gy). Colony-forming survival assay revealed that the survival fraction of EC9706 and KYSE30 cells was significantly reduced with every dose of radiation (0, 2, 4, 6, 8 Gy) in the miR-138 and si-Survivin groups compared with the Blank and NC groups ($P < 0.05$, Fig. 7E, F). These results indicate that upregulation of miR-138 and knock down Survivin both enhanced the radiosensitivity of EC9706 and KYSE30 cells.

Expression of Survivin restored the effect of α -solanine and miR-138 on the radiosensitivity of EC9706 cells

To explore the relationship between α -solanine, miR-138, and Survivin in EC9706 cell, we performed Western blotting to detect Survivin expression in cells after being simultaneously or separately exposed with α -solanine, miR-138 mimics, and the vector containing Survivin but lacking the 3'UTR sequence (pcDNA3.1-Survivin). The results showed that expression of Survivin was significantly decreased after transfection with

miR-138 mimics, si-Survivin or treated with 4 μ mol/L α -solanine respectively but was significantly increased after simultaneously or separately transfection with pcDNA3.1-Survivin ($P < 0.05$, Fig. 8A). The FCM assay result showed that after 4 Gy radiation, the apoptotic cells were significantly increased after transfection with miR-138 mimics, si-Survivin or treated with 4 μ mol/L α -solanine respectively, but was significantly decreased after being simultaneously or separately transfected with pcDNA3.1-Survivin ($P < 0.05$, Fig. 8B). The cell viability assay showed that the cell viability of EC9706 cell treated with 4 Gy radiation was significantly decreased after transfection with miR-138 mimics, si-Survivin or treated with 4 μ mol/L α -solanine respectively, but was significantly increased after being simultaneously or separately transfected with pcDNA3.1-Survivin ($P < 0.05$, Fig. 6C). Colony-forming survival assay showed that the survival fraction of EC9706 cell exposed to 4 Gy radiation was significantly decreased after transfection with miR-138 mimics, si-Survivin or treated with 4 μ mol/L α -solanine, respectively, but was significantly increased after being simultaneously or separately transfected with pcDNA3.1-Survivin ($P < 0.05$, Fig. 6D). These results further suggest that expression of Survivin restores the effect of α -solanine and miR-138 on the radiosensitivity of EC9706 cells.

Discussion

EC is one of the most common malignant tumors in the world. There are approximately 30 million people that die of esophageal cancer worldwide each year. China is one of the countries with a high incidence of esophageal cancer, and there are approximately 15 million people that die of EC each year [40-42]. Due to difficulties with performing the operation, most patients choose to have palliative treatment instead. Radiotherapy is one of the main palliative treatments of EC. However, the clinical efficacy of radiotherapy is not satisfactory, it has a 5-year survival rate of only 10 to 30% and the local tumor uncontrolled rate and recurrence rate reach up to 60 to 80% [43, 44]. Therefore, improving the efficacy of radiotherapy is currently the focus of researchers. Over the last few years, the interest in exploring the use of small-molecule anticancer compounds for the prevention or treatment of tumors has increased. α -Solanine has been well studied for its impact on antitumor properties. It was found to have proliferation-inhibiting and apoptosis-promoting effect on multiple cancer cells, such as clone, liver, melanoma cancer cells, by decreasing the expression of MMP-2/9, extracellular inducer of matrix metalloproteinase (EMMPRIN), CD44, eNOS, E-cadherin, vascular endothelial growth factor (VEGF) and suppressing phosphorylation of Akt, mTOR and Stat3. But the efficacy and the associated molecular mechanisms of α -solanine against esophageal cancer have not yet been evaluated.

In our study, we conducted CCK-8 assay and found the cytotoxic effect of α -solanine on EC9706 and KYSE30 cells, and the cytotoxic effect of α -solanine was dose-dependent. 16 μ mol/L α -solanine could cause obvious cytotoxicity in EC9706, KYSE30 and Het-1A cells, so concentrations of α -solanine we used in other experiences were under this toxic dose. CCK-8 assay and colony-forming survival assays were performed to explore the effect of α -solanine on the proliferation of EC9706 and KYSE30 cells after irradiation, and the results revealed that the appropriate concentration of α -solanine could enhance the effect of irradiation inhibition of proliferation to EC9706 and KYSE30 cells. The results of immunofluorescence and comet assays indicated that α -solanine markedly impairs DNA repair capacity after irradiation in EC9706 and KYSE30 cells. The FCM and Hoechst/PI staining results showed that α -Solanine enhanced the effect of irradiation-induced apoptosis in EC9706 and KYSE30 cells. These data demonstrate that α -solanine could enhance the radiosensitivity of esophageal cancer cells.

Various miRNAs are reported to affect cancer development at multiple stages and cell radiosensitivity by regulating target gene expression [45]. For example, miR-499 enhances the cisplatin sensitivity of esophageal carcinoma cell lines by targeting DNA polymerase β [34]. Moreover, the role of miR-138 was reported not only to increase the sensitivity of A549/DDP cells to cisplatin but also to increase its radiosensitivity [35]. Here, we found

that α -Solanine could induce the upregulation of miR-138 and downregulation of Survivin expression in EC9706 and KYSE30 cells, and Survivin is the target of miR-138. That is, α -Solanine could downregulate Survivin expression level by upregulating miR-138 expression in EC9706 and KYSE30 cells. Survivin protein, a member of the inhibitors of apoptosis family, is implicated in the regulation of cell proliferation and apoptosis. Its expression has been extensively studied in various cancers making it a promising biomarker and, increasingly, target for therapy [46, 47]. For example, in urothelial carcinoma of the bladder, Survivin has been shown to predict cancer recurrence and survival after radical cystectomy [48, 49]. It is obvious that miR-138 increasing is not the only effect of α -solanine on EC cells, the other effects need to be further evaluated. Our further results of CCK-8 and colony-forming survival assays showed that upregulation of miR-138 and knock down Survivin both enhanced the radiosensitivity of EC9706 and KYSE30 cells. Moreover, Survivin could restore the effect of α -solanine and miR-138 on radiosensitivity of EC9706 and KYSE30 cells. Based on the results, we suggest that α -solanine could modulate the radiosensitivity of esophageal cancer cells partly by inducing miR-138 expression, and the α -solanine/miR-138/Survivin probably could be potential effective radiosensitizers in treating EC.

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Disclosure Statement

The authors have declared that no competing interest exists.

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