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ORIGINAL ARTICLE

Oroxylin a could be a Promising Radiosensitizer for Esophageal Squamous Cell Carcinoma by Inducing G2/M Arrest and Activating Apoptosis

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Abstract To evaluate the radiosensitization of Oroxylin A on esophageal carcinoma cell as well as the optimal scheduling of Oroxylin A and radiotherapy (RT). Cell proliferation was estimated by a CCK8 assay. Radiosensitization was evaluated by a clonogenic survival assay. The progressions of Cell apoptosis and Cell cycle were investigated by flow cytometry. Expressions of survivin and cell cycle regulators were evaluated by Western blot analysis. A dose-dependent cell survival reduction was found in response to radiation with or without Oroxylin A. The apoptosis rates were remarkably dose-dependent higher in combination groups than in either Oroxylin A or radiation alone group. Besides, Oroxylin A could obviously radiosensitize ESCC cells by arresting tumor cells in G2/M phase and regulating cyclin B1 and Cdc 2 protein expression. Oroxylin A could be a promising radiosensitizer for esophageal squamous cell carcinoma by inducing G2/M phase blocking and activating cell apoptosis.

Keywords Oroxylin A · Radiosensitization · ESCC · Apoptosis · G2/M arrest

Introduction

Esophageal carcinoma (EC) is one of the most common malignant tumors in the world, with a wide distribution in the south of Iran and China [1]. It is a very deadly disease, with roughly 480,000 new patients every year, and it is the sixth leading cause of cancer deaths worldwide, the fifth in men and the eighth in women [2]. The survival rate is significantly low, and the average five-year survival rate of one-third of patients is 35.45% [3]. The most common type of EC is esophageal squamous cell carcinoma (ESCC). Nowadays, radiotherapy has been proved to be the effective option for nonsurgical management of ESCC. Ionizing irradiation can induce apoptosis in malignant tumor cell, and then treats patients. Some studies have shown that the overexpression of inhibitor of apoptosis proteins (IAP) could result in the development of apoptosis resistance and carcinogenesis in various human cancers including ESCC [4–6].

Oroxylin A, the major flavonoids of the stem bark, is an active flavonoid of a Chinese traditional medicinal plant, which had been reported at first time as natural flavonoids with potent inhibitory activity against proprotein convertases and endoprotease enzymes. Flavonoids are the active components of bioactive extracts. And it had been proved to be a key role in the process of cancer, also in viral and bacterial infections. Several medicines had been produced either along with this plant or singly using other medicinal herb for the treatment of variety of diseases. And it was previously reported can modulate glycolysis in cancer cells. For example, Oroxylin A can inhibits glycolysis via the Sirtuin 3 (SIRT3)-mediated destabilization of hypoxia-inducible factor 1α (HIF1α) in breast cancer cells, which controls the expression of glycolytic gene [7]. It also be found that there were a wide spectrum of in vitro and in vivo pharmacological activities involving antimicrobial, anti-arthritic, anti-inflammatory, anticancer, anti-ulcer, antidiabetic, hepatoprotective, antioxidant activities and antidiarrheal [8]. These studies above have important significance for the investigation on anticancer effects of oroxylin A, and provide the theoretical basis for the clinical trial and further study of oroxylin A in cancer patients.

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Materials and Methods

Reagents and Antibodies

Oroxylin A was bought from Novartis Pharmaceuticals (Basel, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 100 mM. Mouse antibody against cyclin B1 and Cdc 2 were purchased from Cell Signaling Technology. Mouse antibody against β-actin was obtained from Millipore (Billerica, MA).

Cell Culture

Human esophageal carcinoma cell lines TE13 an ECA109 were provided by Shanghai Institute of Cell Biology (Shanghai, China). The esophageal carcinoma cell lines were maintained in high-glucose dulbecco's modified eagle medium (DMEM) (Millipore, Billerica, MA) supplemented with 10 % fetal bovine serum (Invitrogen, Carlsbad, CA), 100μg/ml streptomycin and 100 U/ml penicillin in an incubator (5 % CO2, 37 °C).

CCK-8 Assay

A CCK8 assay was used to estimate the cell proliferation rate. About 5000 cells/well were seeded into 96-well plates, and then we use different concentrations of Oroxylin A treat with the cells. After 24 and 48 h, each well was added with 10 μl CCK-8 reagent. Plates were then estimated on a microplate reader after 2 h incubation (5 % CO2, 37 °C). The relative cell viability was calculated by normalizing the absorbance of an individual sample to that of the corresponding control.

Clonogenic Survival Assay

We trypsinized cultured cells into single-cell suspension and seeded them at six-well plates in serial densities. Treated cells with DMSO as control and Oroxylin A (10 μM or 50 μM) for 24 h when cell adhesion. Cells were incubated for about 14 days in the condition of 5 % CO2 at 37 °C. After being exposed to various doses of radiation (0, 2, 4, 6, 8 Gy), seeded cells were fixed with methaol and stained with Giemsa for 15 min. The colonies which containing more than 50 cells were counted and the single-hit multi-target model was used to fit survival curves. The survival enhancement ratio (SER) was defined as the ratio of the mean inactivation dose in control cells divided by the mean inactivation dose in the cells treated with Oroxylin A. We carry on each experiment for three times and triplicate record.

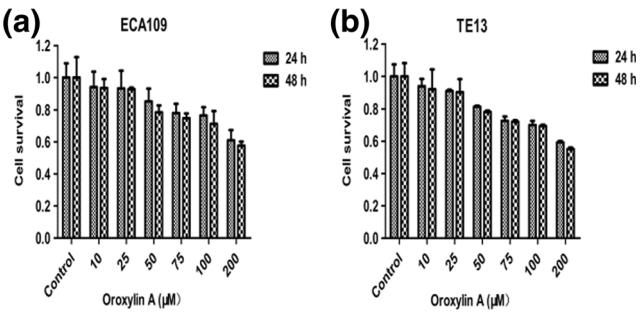

Fig. 1 Oroxylin A Inhibited Growth of ESCC Cells in a Time-and Dose-Dependent Manner (A and B). **a** ECA109 cell lines, **b** TE13 cell lines

Western Blotting Analysis

Cultured cells were collected and homogenized in RIPA lysis buffer followed by being centrifuged at 12,000 rpm at 4 °C for 20 min. The BCA assay was used to evaluated the protein concentrations of the supernatants. Protein concentrations were separated by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, they were blotted onto PVDF membranes. In the next step, this membranes were blocked and probed with primary antibodies at 4 °C in one night followed by incubating with conjugated secondary antibodies at 37 °C for 1 h. Finally, immunoblotted proteins were visualized by ECL reagents and the signals were detected by Chemidoc XRS imaging system (Quantity One Quantitation software, BioRad Laboratories, Hercules, CA, USA). Protein level was normalized to the matching densitometric value of the internal control β-actin.

Cell Cycle Analysis We used 0.25 % trypsin to trypsinize cultured cells which in exponential growth phase to obtain a single-cell suspension. After seeding cells in six-well plates at a density of 20*10^4 cells/well for 24 h, we then treated cells with Oroxylin A in the concentration of 10 and 50 μmol/L in natural circumstance for 24 h. In the next step, cells were irradiated at 6Gy and subsequently trypsinized 24 h later. After washing the trypsinized cells with PBS, we added 70 % Cold ethanol and stored them at −20 °C overnight. Propidium iodide(PI) was used to stained the cells. Finally, we treated them with RNase A (5 μg/mL) for cell cycle analysis by flow cytometry (FACSCalibur).

Apoptosis Assay

Apoptotic cells were evaluated by Annexin V-FITC and PI dual staining. The cells were seeded at six-well plates and treated with Oroxylin A or DMSO (control). The cells were treated with 6Gy X-ray after incubated for 8 h at 37 °C. After 48 h, both attached and detached cells were collected and then washed with cold phosphate buffer saline (PBS). Next, the cells were resuspended in binding buffer followed by being incubated at room temperature for 15 min with PI and Annexin VFITC in the dark. Finally, we used flow cytometry to analyzed the samples at once.

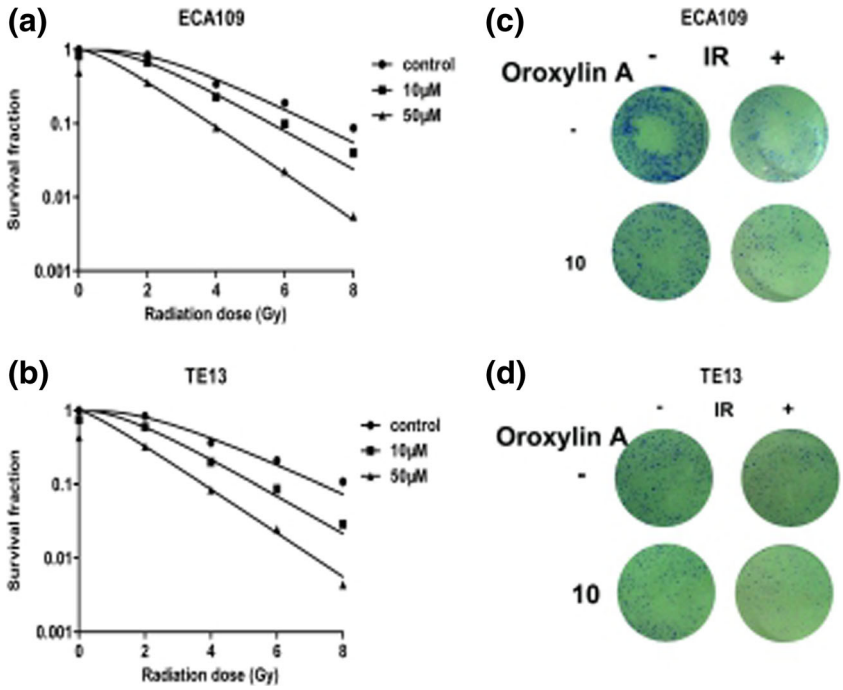

Fig. 2 Clonogenic survival assay showing that Oroxylin A sensitized ESCC cells to IR significantly in normoxia environment and there was a dose-dependent reduction (A and B). **a** ECA109 cell lines, **b** TE13 cell lines

Results

In the first part of our experiment, the CCK8 assay was performed to assess cell viability. It suggested that Oroxylin A inhibited the growth of ECA109 and TE13 cell lines in a time and dose dependent way (Fig. 1). At 24 h, the results of CCK-8 analysis showed that the 50 % inhibitive concentration (IC50) for ESCC cells ECA109 and TE13 were 333.79 μM and 285.79 μM, respectively. Thus, in the following experiments, we used 10 μM and 50 μM as the safe Oroxylin A concentration.

A clonogenic assay was performed to investigate the radiosensitization of Oroxylin A in ESCC cells. The cells were treated with Oroxylin A at the concentration of 10 μM and 50 μM for 24 h. Then we used the doses of 0-8Gy to irradiate this cells and assessed the colon formation efficiency. 14 days later, the colonies were counted and the survival curves were constructed. There was a dose dependent reduction in cell survival with or without Oroxylin A (Fig. 2). The data of SF were fitted into the formula of single hit multi target model: $SF = 1-(1-e^{-D/D0})^n$. Without Oroxylin A, the survival fraction at 2 Gy (SF2) was 0.816 for ECA109 cells, and 0.807 for TE13 cells, respectively. When we treated them with Oroxylin A in different concentration of 10uM and 50uM, SF2 of ECA109 cells decreased to 0.664 and 0.359 of which decreased to 0.592 and 0.326 in TE13 cells, respectively. The sensitizing enhancement ratio (SER_{D0}) was 1.137 and 1.364 for ECA109, 1.246 and 1.446 for TE13 cells (Tables 1 and 2) in concentration of 10uM and 50uM, respectively. These results showed that the Oroxylin A had a significant radiosensitization effect for ESCC cells.

Cell cycle distribution was evaluated to determine the mechanism associated with Oroxylin A induced radiosensitivity of ESCC cells. We evaluated the relative proportion of each phase such as G0/G1 phase, S phase and G2/M phase. The ESCC cells were treated with Oroxylin A for 24 h. The G2/M phase cell count increased in combination (RT+ Oroxylin A) groups (36.77 % and 41.85 % for ECA109 and 38.75 % and 42.46 % for TE13), while the proportion of cells in G0/G1 phase was decreasd, when they were compared with the control (RT) or Oroxylin A alone group, especially in the high dose group. These results implied that Oroxylin A caused ESCC cells to accumulate in G2/M phase post-irradiation in a dose-and time-dependent manner (Fig. 3).

To further explore the radiosensitizing mechanism of Oroxylin A, we used flow cytometry to evaluate the relationship

Table 1 The radiosensitization activity of oroxylin A in ECA109 Cells

	D0	Dq	SF2	SER
Control	1.871	2.607	0.816	
Oroxylin A 10 μM	1.646	1.86	0.664	1.137
Oroxylin A 50 μM	1.372	0.712	0.359	1.364

Table 2 The radiosensitization activity of oroxylin A in TE13 Cells

	D0	Dq	SF2	SER
Control	2.108	2.552	0.807	
Oroxylin A 10 μM	1.691	1.513	0.592	1.246
Oroxylin A 50 μM	1.458	0.433	0.326	1.446

between the radiosentization and cell apoptosis. The results showed the differences between combined treatment groups and radiation alone group were statistically significant (both $P < 0.001$ for ECA109, and $P = 0.003$ and $P = 0.007$ for TE13). The apoptosis rate in 50 μM Oroxylin A group was markedly higher than 10 μM group (15.94 % and 19.37 % for ECA109, 18.49 % and 21.69 % for TE13 cells) (Fig. 4). The results indicated that Oroxylin A and radiation have a synergetic action on the induction of cell apoptosis and the effects were dose dependent.

Further more, we investigated the molecular mechanism of Oroxylin A inhibiting the cell cycle by examining the expression of cyclin B1 and Cdc 2 both in the ECA109 and TE13 cells. Western blotting analysis showed that the Oroxylin A decreased the expression of cyclin B1 and Cdc 2 in a dose dependent pattern (Fig. 5).

Discussion

Although irradiation technology and treatment strategies have been significantly improved today, radiation treatment of advanced esophageal outcome is still unsatisfactory, with a 5-year survival rate of 20–30 % and local control rate of 45% [9]. Therefore, exploring radiosensitization agents to overcome radiation resistance is very importment.

Oroxylin A, a natural mono-flavonoid extracted from Scutellariae radix, is a promising treatment of a variety of cancer therapeutic agents. It has been reported that oroxylin A inhibited the proliferation of malignant glioma cells and induced autophagy in a dose-and time-dependent manner [10]. Wei L et al. [11] found that Oroxylin A sensitized anoikis, which underlies distinct glucose-deprivation-like mechanisms that involved c-Src and HK II.

In the present study, we found that ESCC cell viability and proliferation could be decreased by incubating Oroxylin A in a dose dependent pattern. Besides, the promoted apoptosis also be observed with Oroxylin A, especially with the combination treatment of radiation. Furthermore, Our study provides evidence that the Oroxylin A increased radioactivity of the ESCC through the induction of G2/M arrest.

As we all know, the response of cells to radiation is concerned with their cell cycle phase. The G2/M phase cells were the most sensitive to radiation, whereas during the latter part

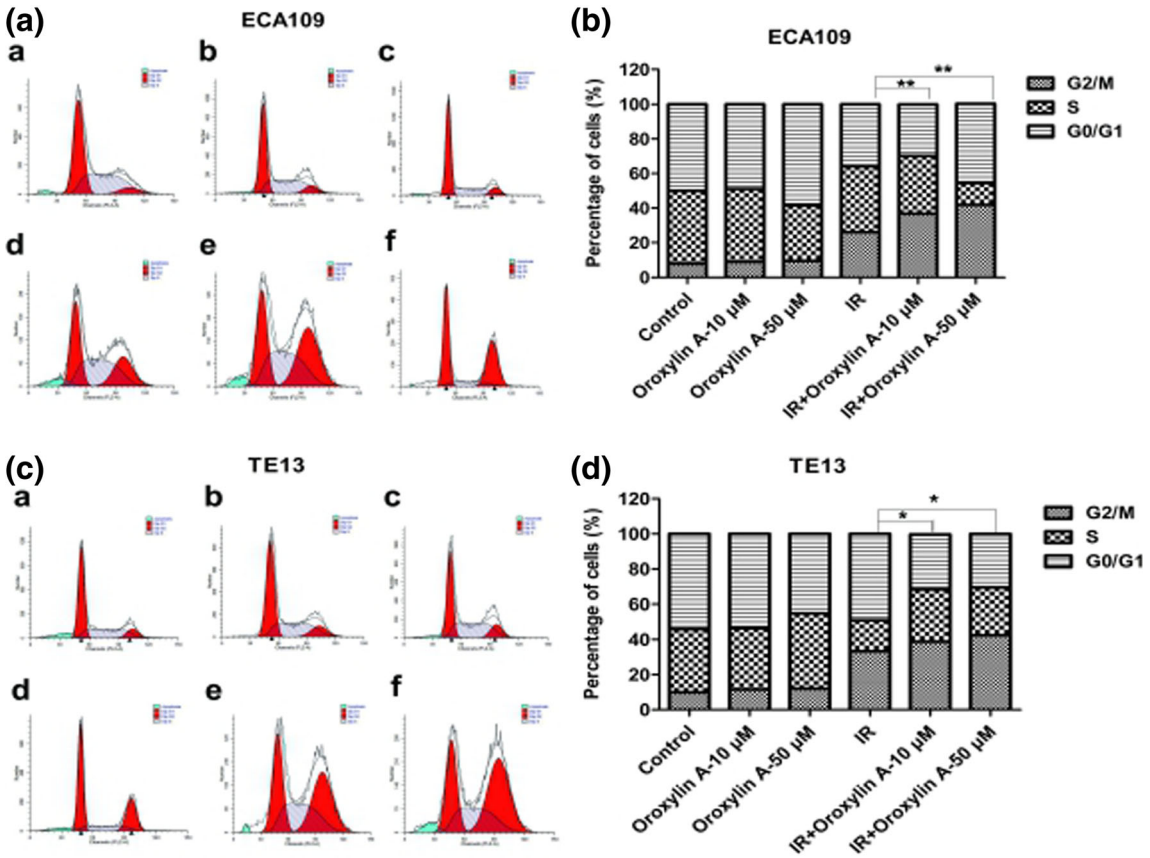

Fig. 3 Flow Cytometric Analysis Showing that Oroxylin A Radiosensitize ESCC Cells through Inducing G2/M Checkpoint. A: a: control; b: Oroxylin A 10 μM; c: Oroxylin A 50 μM; d: IR e: IR+ Oroxylin A 10 μM; f: IR+ Oroxylin A 50 μM. B: Compared with the control or Oroxylin A group, an accumulation of ECA cells in the G2/M phase was noted in combination treatment groups, cupled with a decrease of the G0/G1 proportion, especially in the high dose group. A and B: ECA109 cell lines, C and D: TE13 cell lines

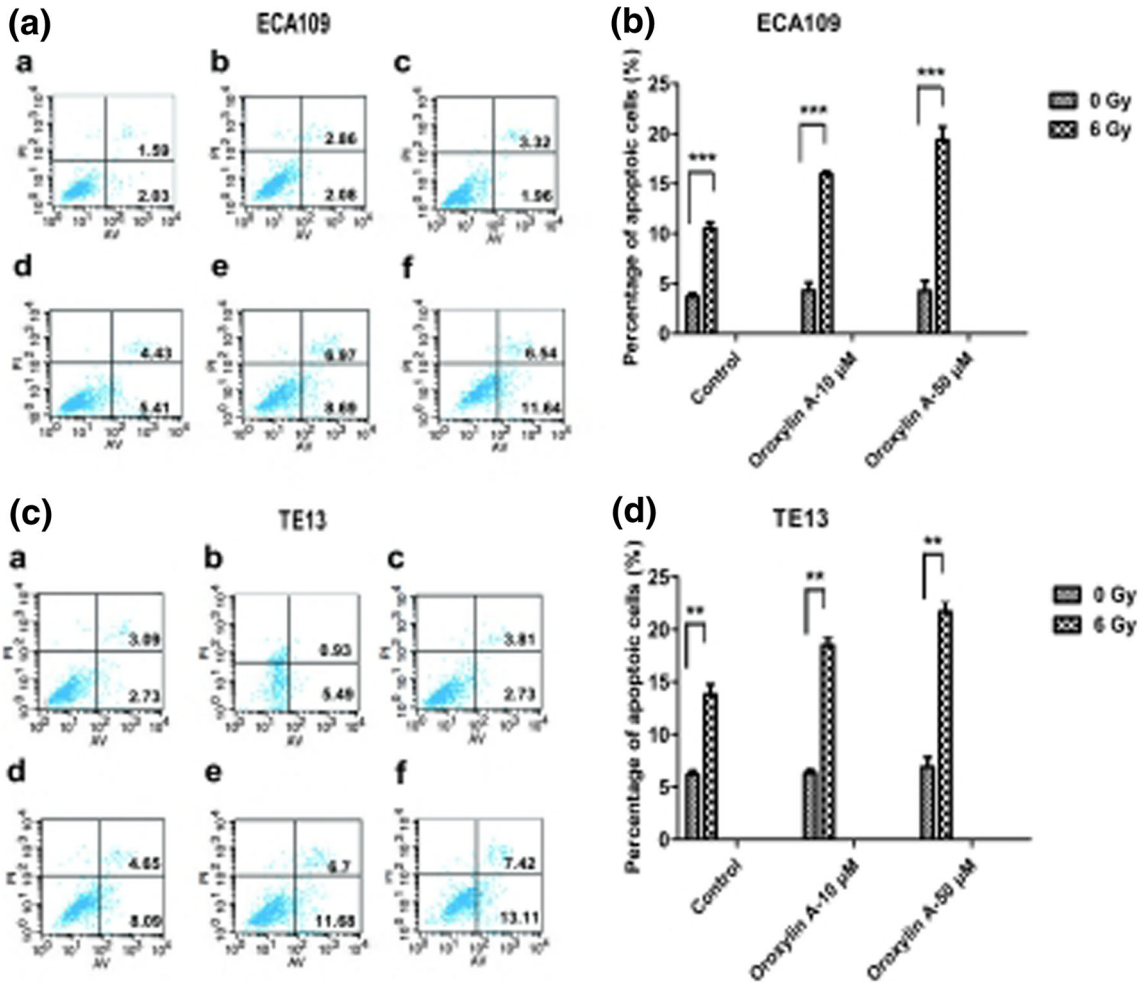

Fig. 4 Flow Cytometric Analysis Showing that Oroxylin A Induced Apoptosis of ESCC Cells. A and C: a: control; b: Oroxylin A 10 μM; c: Oroxylin A 50 μM; d: IR e: IR+ Oroxylin A 10 μM; f: IR+ Oroxylin A 50 μM. B and D: The apoptosis rate were at a great extent higher in combination treatment groups than in irradiation or Oroxylin A group alone and the effects were dose dependent. A and B: ECA109 cell lines, C and D: TE13 cell lines

of the S phase cells were the most radiation tolerance [12].. Cell cycle consists of four distinct sequential phases (G0/G1, S, G2, and M), and it can regulate cellular proliferation [13]. Cell division cycle protein 2 (Cdc2) or cyclin B leads the entry into M phase and is also a key regulator in cell cycle progression by binding to cyclin kinases and causing phosphorylation. It has become clear that cells are blocked in the phase of G2/M during the course of DNA damage and the cells are more susceptible to the toxic effects of radiation in the G2/M phase [14]. Increased induction of G2 / M arrest and cell death may be a useful strategy for cancer treatment [15]. Accumulating evidence indicates that many radiation sensitive agents such as taxol [16] and oxamate [17] play a key role by inducing G2/M arrest through down-regulating expression of cyclin B1 and Cdc 2 in many malignant tumors. In our study, we found a marked decrease of cyclin B1 and Cdc 2 expression in the combined treatment groups. Our study could also provide evidence that the Oroxylin A increased radioactivity of the ESCC cells by affecting the cell cycle and the expression of cell cycle related proteins.

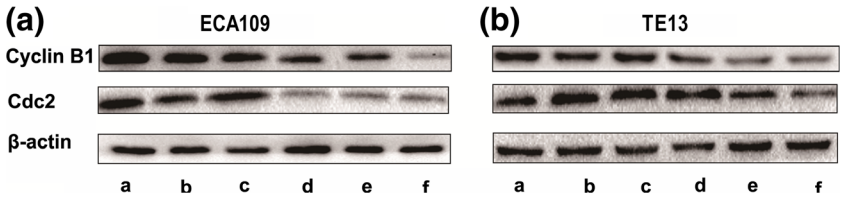

Fig. 5 Western blot analysis revealed that the expression of cyclinB and Cdc2 proteins decreased in Oroxylin A treatment cells in a dose-dependent manner. **a** ECA109 cell lines, **b** TE13 cell lines

Therefore, the cell apoptosis rate in the combination group of Oroxylin A and RT was significantly higher than that in either irradiation or Oroxylin A alone group and the effect was dose dependent. In addition, the Oroxylin A improved the sensitivity of radiotherapy by increasing the cell apoptosis rate and regulating the expression of apoptosis related proteins.

Conclusions

In conclusion, the Oroxylin A can promote the sensitivity of esophageal squamous cell carcinoma to X-ray irradiation by accumulating cells in G2/M phase and increasing cell apoptosis. The Oroxylin A may be a promising effective radiosensitizer for esophageal squamous cell carcinoma. We still need further researchs to explore the mechanism of the radiosensitization of Oroxylin A and animal experiments are also needed development further.

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