Int. J. Morphol.,
41(6):1712-1719, 2023.

Effect of Survivin Down-Regulation by Egr1-Survivin shRNA Combined with Radiotherapy on Radiosensitivity of Esophageal Squamous Carcinoma

Efecto de la Regulación Negativa de Survivina por shRNA de Egr1-Survivina Combinado con Radioterapia sobre la Radiosensibilidad del Carcinoma Escamoso de Esófago

Yin Lv; Jinrong Zhang; Yanrong Lu; Xin Zhang & Haifeng Wang

LV, Y.; ZHANG, J.; LU, Y.; ZHANG, X. & WANG, H. Effect of survivin down-regulation by Egr1-survivin shRNA combined with radiotherapy on radiosensitivity of esophageal squamous carcinoma. *Int. J. Morphol., 41(6)*:1712-1719, 2023.

SUMMARY: This study is to investigate the effect of survivin down-regulation by Egr1-survivin shRNA combined with radiotherapy on the apoptosis and radiosensitivity of esophageal squamous cell carcinoma ECA109 and KYSE150 cells. ECA109 and KYSE150 cells were transfected with Egr1-survivin shRNA, and then treated with radiotherapy. After 24 h, the mRNA and protein levels of Egr1-survivin were detected by qPCR and Western-Blot. Cell cycle and apoptosis were detected by flow cytometry. Western blot also detected levels of cleavaged Caspase 3 and Caspase 9. YM155 was used as a positive control to inhibit survivin expression. The levels of survivin mRNA and protein in ECA109 and KYSE150 cells treated with Egr1-survivin shRNA combined with radiotherapy were significantly lower than those of the blank control group, the empty vector control group, and, the YM155 + radiotherapy group ($P<0.05$). Meanwhile, after survivin down-regulation, the ratio of G2 to S phase of ECA109 and KYSE150 cells increased significantly, leading to significant G2 and S phase arrest. Additionally, apoptosis of ECA109 and KYSE150 cells increased significantly ($P <0.01$). Further, protein levels of cleavaged Caspase 3 and Caspase 9 significantly increased in Egr1-survivin shRNA combined with radiotherapy group. Egr1-survivin shRNA combined with radiotherapy can down-regulate survivin expression, which further increases the apoptosis, and enhances the radiosensitivity of ECA109 and KYSE150 cells.

KEY WORDS: Esophageal squamous cell carcinoma; Survivin; Gene-radiotherapy; Radiosensitivity.

INTRODUCTION

Esophageal cancer is a common malignant tumor of the digestive system. In 2018, there were 572,200 new cases of esophageal cancer and 506,600 deaths worldwide, ranking 7th in the incidence and 6th in the fatality rate of malignant tumors, respectively (Global Burden of Disease Cancer Collaboration, 2018). China is an area with a high incidence of esophageal cancer. Statistics from the National Cancer Center show that there are 246,000 new cases of esophageal cancer and 188,000 deaths in China, which are the 6th in the incidence and 4th in the mortality of malignant tumors in China (Zeng *et al*., 2015). Esophageal squamous cell carcinoma is the most common pathological subtype of esophageal cancer. In the high incidence area of esophageal cancer in northern China, about 90 % of esophageal cancer is squamous cell carcinoma (Arnold *et al*., 2015). Radiation therapy is the most important treatment for inoperable esophageal cancer (Chiappa *et al*., 2018), but the effect of radiation therapy is not satisfactory. Zhang *et al*. (2015) reported that the 5-year survival rate of esophageal cancer radiotherapy using three-dimensional conformal radiotherapy technology was only 24.5 %. Yang *et al*. (1992) reported that the rate of locally uncontrolled or recurrence of esophageal cancer after radiotherapy was as high as 68.6 %. These indicate that locally uncontrolled or recurrent tumors remain the main cause of radiotherapy failure. In order to further improve the local control rate of radiotherapy for esophageal cancer, it is urgent to determine the molecular mechanism of radiotherapy resistance in esophageal cancer and develop new treatment strategies.

Department of Radiotherapy of the Chest and Abdomen, Affiliated Tumor Hospital of Xinjiang Medical University, Urumqi 830011, Xinjiang Uygur Autonomous Region, China.
Funding. This work was supported by National Natural Science Foundation of China (No. 81760499).

Received: 2023-07-14 Accepted: 2023-09-20

Survivin plays an important role in the progression of esophageal cancer and resistance to radiotherapy, and is a promising therapeutic target (Dizdar *et al*., 2018). Survivin is the smallest member of the inhibitor of apoptosis protein family, which can inhibit apoptosis and regulate the cell cycle (Ambrosini *et al*., 1997). It is highly expressed in embryonic tissues and most malignant tumor tissues, but not in normal mature adult tissues (Garg *et al*., 2016). It is also highly expressed in esophageal cancer tissues (Dizdar *et al*., 2018), and its down regulation can increase the radiosensitivity of esophageal cancers (Zhou *et al*., 2018). Therefore, survivin can be a potential therapeutic target for esophageal cancer.

Egr-1 belongs to the immediate early gene family, and its promoter contains a radiation-sensing component to enable it to be induced by radiation. Taking advantage of this feature, some studies have proposed the gene-radiotherapy theory, in which the promoter sequence of Egr-1 is fused with the target gene, and the expression of the target gene is driven by ionizing radiation (Weichselbaum *et al*., 1994). The results showed that ionizing radiation obviously induced the expression of target genes downstream of the Egr-1 promoter, and its tumor suppressive effect was significantly better than that of radiotherapy and gene therapy alone (Weichselbaum *et al*., 1994).

Herein, we use Egr-1 based promoter to construct Egr1-survivin shRNA to transfect esophageal cancer cells. Then, the cells were treated with radiotherapy. The apoptosis and cell cycle of esophageal cancer cells were analyzed. Our findings demonstrate that survivin down-regulation by Egr1-survivin shRNA combined with radiotherapy increases the radiosensitivity of esophageal cancer cells.

MATERIAL AND METHOD

Cells and reagents. ECA109 and KYSE150 cells were purchased from China Center for Type Culture Collection (Beijing, China). The pAAV-EGR1 promoter plasmid was synthesized and provided by Viraltherapy Biotechnology Co., Ltd. (Wuhan, China). The pUC57-Survivin shRNA plasmids were from Viraltherapy Biotechnology Co., Ltd. (Wuhan, China). DNA endonucleases of EcoRI, HindIII, and T4 DNA Ligase were purchased from NEB (Ipswich, USA). YM155 was purchased from Selleck. Trizol was purchased from Aidlab (Beijing, China). HiScript Reverse Transcriptase (RNase H) and 50 ¥ ROX Reference Dye 2, as well as SYBR Green Master Mix was purchased from VAZYME (Nanjing, China). RIPA lysate and BCA protein concentration kit were from Beyotime Biotechnology (Beijing, China). Rabbit polyclonal anti-GAPDH antibody was from Goodhere Biological Technology Group (Hangzhou, China). Rabbit polyclonal anti-survivin antibody was from ProteinTech (Wuhan, China). Rabbit polyclonal anti-Caspase3 antibody was from Bioss (Beijing, China). Rabbit mAb anti-Caspase9 antibody was from CST (Beverly, USA). HRP-labeled goat anti-rabbit secondary antibody was purchased from Boster (Wuhan, China). ECL substrate solution was from Thermo (Rockford, USA). The APC / 7-AAD apoptosis kit was from Sungene Biotech (Tianjin, China). PI was from Solarbio (Beijing, China).

Construction and transfection of pAAV-Egr1-Survivin shRNA Plasmid. The pAAV-EGR1 promoter plasmids and the pUC57-Survivin shRNA plasmids were digested with EcoRI and HindIII, respectively. The corresponding fragments were recovered. The Survivin shRNA was ligated into the pAAV-EGR1 promoter plasmid by T4 ligase to construct the pAAV-Egr1-Survivin shRNA Plasmid. The sequence of Survivin-shRNA synthesis sequence is: GAATTCTAGGGATAACAGGGTAATTGTTTGAATGAG GCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTG GAAACACTTGCTGGGATTACTTCTTCAGGTTAACCCAA CAGAAGGCTAAAGAAGGTAATTGCTGTTGACAGTGA GCGCACCGCATCTCTACATTCATAGTGAAGCCACAGATGTAT GAATGTAGAGATGCGGTGTGCCTACTGCCTCGGACTT CAAGGGGCTACTTTAGGAGCAATTATCTTGTTTACTAAAA CTGAATACCTTGCTATCTCTTTGATACATTTTTACAAAG CTGAATTAAAATGGTATAAATTAAATCACTTTTTTCAA TTGGAAGACTAATGCGTTTAAACACGCGGCGAC GTTAAGCTT (386bp). The constructed clones were further verified by enzyme digestion and sequencing.

Cell culture and treatment. ECA109 and KYSE-150 cells were cultured in RPMI-1640 medium + 10 % FBS + 1 % Penicillin-Streptomycin. The cells were divided into the following groups according to the different treatments: (1) blank control group: no treatment + radiotherapy, (2) empty vector control group: transfection of empty vector plasmid + radiotherapy, (3) Egr1-survivin shRNA Group, transfected with pAAV-EGR1 promoter-survivin shRNA plasmid + radiotherapy, (4) YM155 group, YM155 treatment + radiotherapy.

The IC50 value of YM155 on esophageal cancer ECA109 and KYSE150 cells was 5 nM. Thus, the final concentration of YM155 was adjusted to 5 nM. At 48 h after transfection and YM155 treatment, the cells were irradiated. The irradiation method was: 6MV-X-rays were used for vertical irradiation at room temperature, with the dose rate of 200 cGy / min and the irradiation field of 10 cm ¥ 10 cm. The irradiation dose was 4 Gy once. Cells were collected at 24 h after irradiation.

RT-qPCR. Total RNA was extracted from the cells with Trizol and then transcribed into according to the reverse transcription kit instructions. The survivin mRNA level was then detected by RT-qPCR. The RT-qPCR reaction system was: cDNA 4 μl, Forward Primer (10μM) 0.4 μl, Reverse Primer (10μM) 0.4 μl, SYBR Green Master Mix 10 ml, 50 × ROX Reference Dye 2 0.4 ml, ddH_2O 4.8 μl. The reaction conditions were: 50 °C 2 min, 95 °C 10 min; 95 °C 30 s, 60 °C 30 s, 40 cycles. The final data was analyzed with $2^{-\Delta\Delta}$Ct. The primer sequences were: survivin-F: 5'-CCCTT TCTCAAG GACCACCGCATCT-3', and survivin-R: 5'-CTCGTTCTCAGTGGGGCAGTGGAT G-3'. GAPDH-F: 5'-AAGCCGTTCTTGACAAATGG-3', and GAPDH-R: 5'-ATTGG TGT TGGCCTTCAGAC-3'.

Western-Blot. Cells were lysed using RIPA and centrifuged at 12000 rpm for 5 min at 4 °C. The supernatant was collected and the protein concentration was determined by BCA kit. After SPS-PAGE electrophoresis, the sample was transferred to a membrane. The membrane was blocked and washed, and then were incubated with primary antibodies against survivin, cleavaged Caspase 3, cleavaged Caspase 9 and GAPDH and secondary antibodies. Finally, the gray values for protein bands were analyzed by BandScan.

FACS. After 24 h of culture, cells were collected. For cell cycle detection, cells were fixed with 80 % ethanol for 4 h at 4 °C. Then, cells were washed twice with pre-chilled PBS, and stained with 400 μl PI (50 mg/mL) at 4 °C in the dark for 30 min. For cell apoptosis detection, cells were washed with 1 mL of Binding Buffer and then stained with 5 μl 7-AAD and 5 μl APC-Annexin V in the dark for 10 min. The cell cycle and cell apoptosis were determined by FACS.

Statistical analysis. Was performed using Spss17.0 statistical software. Data are expressed as mean ± standard deviation (Mean ± SD). The two-group comparison was analyzed by t test. One-way analysis of variance was used for comparison among multiple groups. A P value <0.05 was considered statistically significant.

RESULTS

Survivin expression is knocked down by Egr1-survivin shRNA and radiation. RT-qPCR was used to detect levels of survivin mRNA. As shown in Figures 1A and 1B, survivin mRNA in ECA-109 cells and KYSE150 cells after Egr1-survivin shRNA + radiation treatment was significantly lower than that in the blank control group, the empty vector control group, and YM155 group (in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.000 , *vs* empty vector control P =0.000, *vs* YM155 P =0.013 in KYSE150 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000, *vs* YM155 P =0.01). Similarly, the survivin mRNA in ECA-109 cells and KYSE150 cells after YM155 + radiation treatment was significantly lower than that in the blank control group and the empty vector control group (In ECA-109 cells, YM155 *vs* blank control P =0.003, *vs*

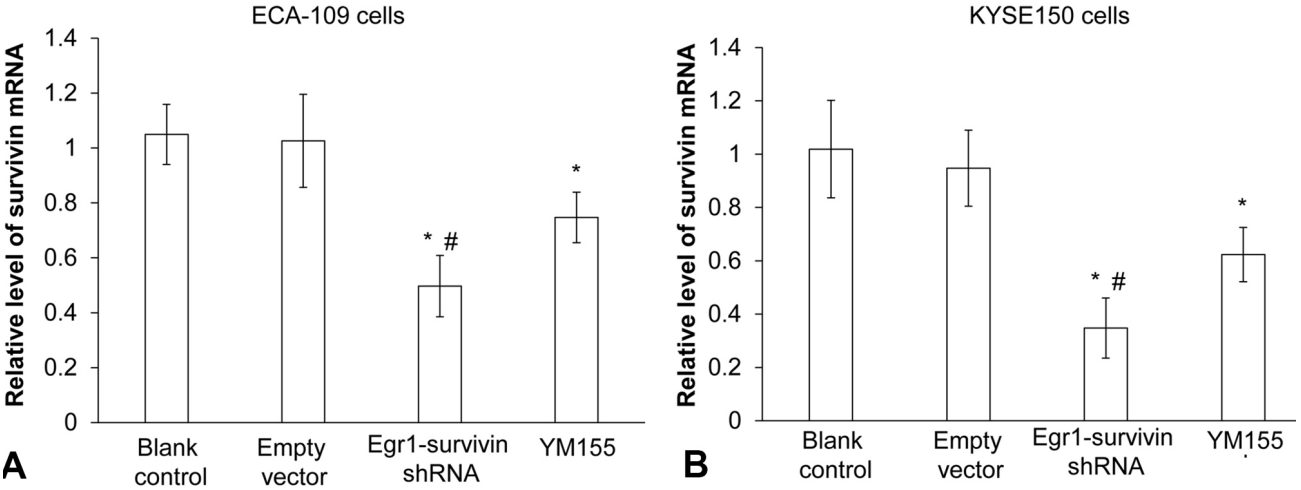

Fig. 1. Analysis of survivin mRNA levels. Survivin mRNA levels in ECA-109 cells (A) and KYSE150 cells (B) were detected with RT-qPCR. Compared with blank control or empty vector control, *P<0.05. Compared with YM155, #P<0.05. In ECA-109 cells, Egr1-survivin shRNA *vs* blank control, P=0.000; Egr1-survivin shRNA *vs* empty vector control, P =0.000; Egr1-survivin shRNA vs YM155, P =0.013; YM155 vs blank control, P =0.003; YM155 vs empty vector control, P =0.006. In KYSE150 cells, Egr1-survivin shRNA *vs* blank control P =0.000; Egr1-survivin shRNA *vs* empty vector control, P =0.000; Egr1-survivin shRNA *vs* YM155, P =0.01; YM155 *vs* blank control P =0.000; YM155 *vs* empty vector control, P =0.000.

empty vector control P =0.006. In KYSE150 cells, YM155 *vs* blank control P =0.000, *vs* empty vector control P =0.000). The above results indicate that Egr1-survivin shRNA activated shRNA expression after radiation induction, thereby inhibiting survivin levels.

Down-regulation of survivin protein increases Caspase 3 and Casepase 9 levels. Western blot was performed to analyze protein expression of survivin, cleavaged Caspase 3 and cleavaged Caspase 9. The test results showed that the expression of survivin protein in ECA-109 cells of Egr1-survivin shRNA + radiotherapy group was significantly lower than that of the blank control group and empty vector control group (Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000) (Fig. 2A). There was no significant different between Egr1-survivin shRNA group and YM155 + radiotherapy (P =0.102). However, in KYSE150 cells, survivin protein level in the Egr1-survivin shRNA + radiotherapy group was significantly lower than that in the blank control group, the empty vector control group, and YM155 group (Egr1-survivin shRNA *vs* blank control, P =0.000; *vs* empty vector control, P =0.000; vs YM155, P =0.01) (Fig. 2B). Survivin protein in ECA-109 cells and KYSE150 cells after YM155 + radiation treatment was significantly lower than that in the blank control group and the empty vector control group (in ECA-109 cells, YM155 *vs* blank control, P =0.001; vs empty vector control, P =0.000; in KYSE150 cells, YM155 *vs* blank control, P =0.001; vs empty vector control, P =0.001). The above results suggest that Egr1-survivin shRNA significantly reduced the expression of survivin protein after inducement by radiotherapy.

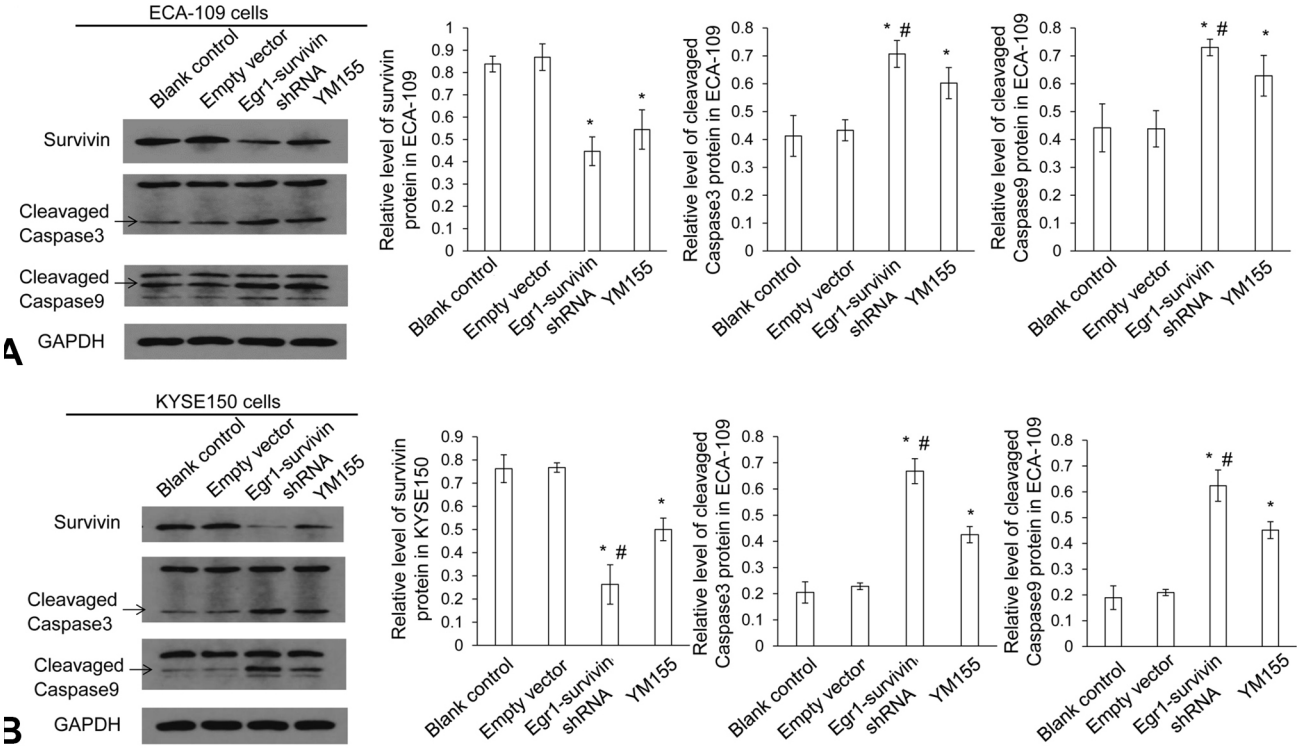

Fig. 2. Analysis of survivin, Caspase 3, and Caspase 9 protein levels. The protein levels of survivin, cleavaged Caspase 3, and cleavaged Caspase 9 in ECA-109 cells (A) and KYSE150 cells (B) were detected with Western blot. Representative and quantitative Western blot results were shown on the left and right panel, respectively. Compared with blank control or empty vector control, *P<0.05. Compared with YM155, #P<0.05. For surviving: in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.000; *vs* empty vector control P =0.000; YM155 *vs* blank control, P =0.001; *vs* empty vector control, P =0.000. In KYSE150 cells, Egr1-survivin shRNA vs blank control P =0.000; vs empty vector control, P =0.000; *vs* YM155 P =0.01; YM155 *vs* blank control, P =0.001; *vs* empty vector control, P =0.001. For, cleavaged caspase3: in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000, *vs* YM155 P =0.045; in KYSE150 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000, *vs* YM155 P =0.000. For the cleavaged caspase9: in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.01, *vs* empty vector control P =0.01, *vs* YM155 P =0.099; in KYSE150 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000, *vs* YM155 P =0.001.

The cleavaged caspase3 and caspase9 of Egr1-survivin shRNA + radiation group cells increased significantly than the other three groups in both ECA-109 cells and KYSE150 cells (Figs. 2A and 2B) (For the cleavaged caspase3: in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.000, vs empty vector control P =0.000, *vs* YM155 P =0.045; in KYSE150 cells, Egr1-survivin shRNA vs blank control P =0.000, *vs* empty vector control P =0.000, *vs* YM155 P =0.000. For the cleavaged caspase9: in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.01, *vs* empty vector control P =0.01, vs YM155 P =0.099; in KYSE150 cells, Egr1-survivin shRNA vs blank control P =0.000, *vs* empty vector control P =0.000, *vs* YM155 P =0.001). This indicates that after down-regulation of survivin protein, the apoptosis of cells may increase.

Down-regulation of survivin protein arrests cell cycle at G2 and S phases. To detect cell cycle distribution, flow cytometry was performed. As shown in Figure 3, the cycle distribution of ECA-109 cells and KYSE-150 cells changed obviously. Statistically, the proportion of G2 and S phases of these two cells in Egr1-Survivin shRNA group was significantly higher than in blank control and empty vector groups (G2 phase in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000; in KYSE150 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000. S phase in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000; in KYSE150 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000) (Figs. 3A and 3B). At the same time, compared with the YM155 group, the G2 phase of the Egr1-Survivin shRNA group was significantly increased (in ECA-109 cells, P =0.000; in KYSE150 cells, P =0.001). In addition, compared with the YM155 group, the Egr1-Survivin shRNA group had significantly decreased proportion of S phase in ECA-109 cells (P =0.000), whereas significantly increased S phase proportion in KYSE-150 cells (P =0.000). The above results suggest that the cells are arrested at G2 and S phases after treatment with Egr1-survivin shRNA combined with radiotherapy.

Down-regulation of survivin protein increases cell apoptosis. To determine cell apoptosis, flow cytometry was conducted. The apoptosis rates in the Egr1-survivin shRNA group and YM155 group were significantly higher than those in the blank control group and the empty vector control group in both ECA-109 cells (Fig. 4A) (Egr1-survivin shRNA vs blank control P =0.000, vs empty vector control P =0.000), and KYSE-150 cells (Fig. 4B) (Egr1-survivin shRNA vs blank control P =0.000, vs empty vector control P =0.000). In addition, compared with the YM155 group, the Egr1-survivin shRNA group of ECA-109 cells and KYSE-150 cells also had a significantly increased apoptosis rate (in ECA-109 cells, P =0.000; in KYSE150 cells, P =0.000). The apoptosis rate in ECA-109 cells and KYSE150 cells after YM155 + radiation treatment was significantly lower than that in the blank control group and the empty vector control

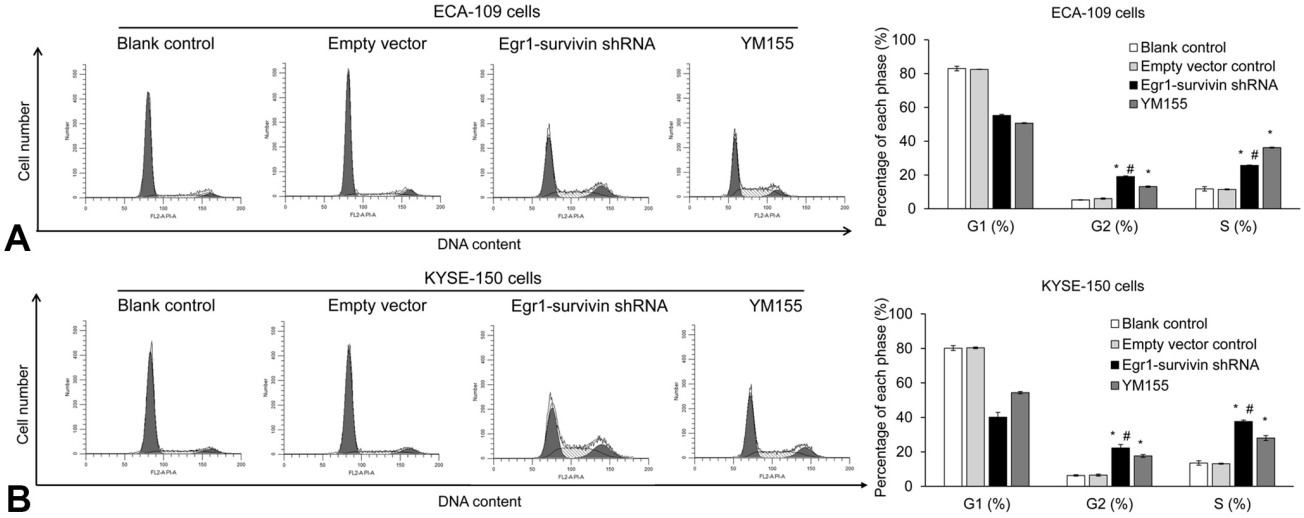

Fig. 3. Analysis of cell cycle distribution. The cell cycle distribution of each treatment group of ECA-109 cells (A) and KYSE150 cells (B) were analyzed with flow cytometry. Representative and quantitative flow cytometry were shown on the left and right panel, respectively. Compared with blank control or empty vector control, *P<0.05. Compared with YM155, #P<0.05. G2 phase in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000; in KYSE150 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000. S phase in ECA-109 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000; in KYSE150 cells, Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000.

group (in ECA-109 cells, YM155 vs blank control P =0.000, vs empty vector control P =0.000. in KYSE150 cells, YM155 vs blank control P =0.000, vs empty vector control P =0.000).The results indicate that Egr1-survivin shRNA combined with radiotherapy can increase the apoptosis of esophageal cancer cells, thus increasing their radiosensitivity.

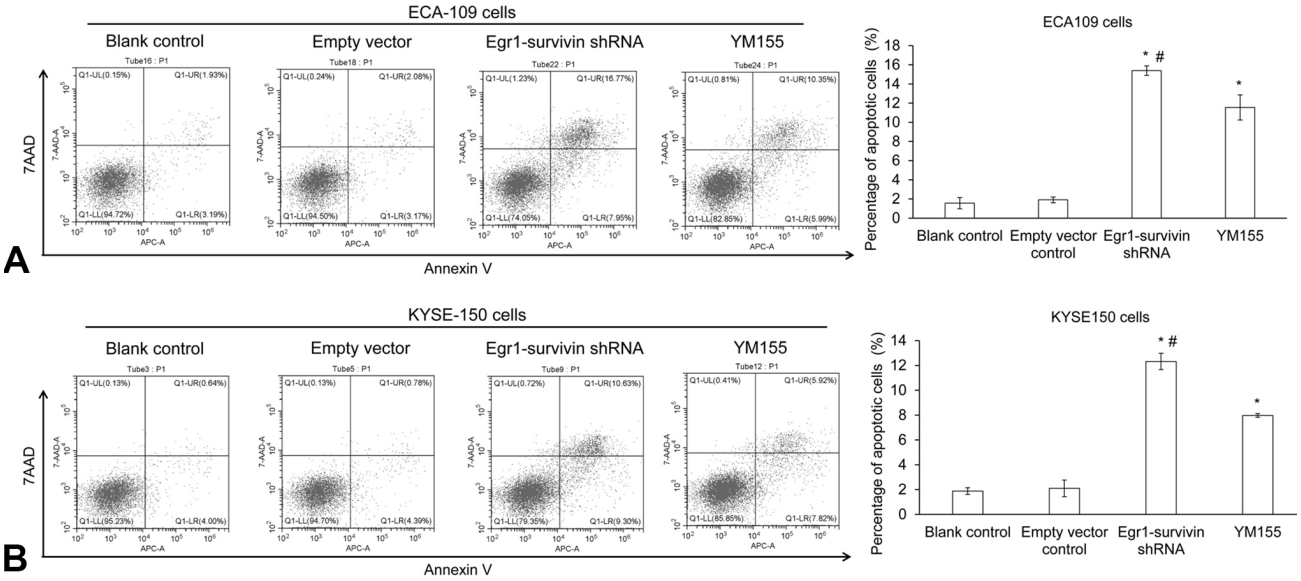

Fig. 4. Analysis of apoptosis. The apoptosis of each treatment group of ECA-109 cells (A) and KYSE150 cells (B) were analyzed with flow cytometry. Representative and quantitative flow cytometry were shown on the left and right panel, respectively. Compared with blank control or empty vector control, *P<0.05. Compared with YM155, #P<0.05. ECA-109 cells: Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000; KYSE150 cells: Egr1-survivin shRNA *vs* blank control P =0.000, *vs* empty vector control P =0.000. In ECA-109 cells, YM155 *vs* blank control P =0.000, *vs* empty vector control P =0.000. In KYSE150 cells, YM155 *vs* blank control P =0.000, *vs* empty vector control P =0.000

DISCUSSION

Esophageal cancer is a common digestive system tumor in China. Its incidence ranks the 6th among common malignant tumors (Zheng *et al*., 2019). Radiotherapy has always been one of the main treatment methods for esophageal cancer, but its curative effect is unsatisfactory. Zhu *et al*. (2005) analyzed the radiotherapy effects of 500 patients with advanced esophageal cancer, and they found that the 1-, 3-, and 5-year survival rates of the patients were 71.1 %, 32.6 %, and 20.8 %, respectively. Even with the rigorous statistical design of the NEOCRTEC5010 study, the results showed that for potentially resectable thoracic esophageal squamous cell carcinoma, the partial complete remission rate in the preoperative concurrent chemoradiotherapy group was 43.2 %, and the overall survival was 100.1 months. Although the efficacy has been significantly improved (Yang *et al*., 2018), compared with other common malignant tumors, the survival time of radiotherapy for esophageal cancer is still not satisfactory. Therefore, there is an urgent need to explore new therapeutic targets and methods for esophageal cancer.

Survivin is closely related to the occurrence and progression of esophageal cancer. Dabrowski *et al*. (2004) showed that the expression of survivin was significantly increased in esophageal squamous cell carcinoma specimens, with a positive rate of 83.33 %. Moreover, survivin overexpression is significantly associated with poor prognosis in patients (Dizdar *et al*., 2018). The Egr1 gene promoter contains a special structure of the CArG box / CC (A + T-rich) GG region, which leads to the increased expression of the Egr1 gene induced by radiation. Based on this, we constructed Egr1-survivin shRNA. After transfection of Egr1-survivin shRNA, esophageal squamous cell carcinoma ECA-109 cells and KYSE-150 cells were irradiated with X-rays. The results showed that Egr1-survivin shRNA combined with radiation therapy down-regulated survivin expression and further affected the viability of ECA-109 cells and KYSE-150 cells.

Down-regulating survivin expression by RNA interference (RNAi) is a commonly used method to inhibit

survivin in tumor cells. Huang *et al*. (2017) reported that transfection of survivin shRNA in non-small cell lung cancer could reduce the expression of survivin mRNA and protein, and that significant tumor growth inhibition was observed in animal models. In addition, Hao *et al*. (2019) reported that knockdown survivin via shRNA inhibited cancer cell proliferation and promoted apoptosis in both A431 cell and *in vivo* xenograft tumor mouse model. In this study, by transfection of survivin shRNA, our results showed that the levels of survivin mRNA and protein in ECA-109 cells and KYSE-150 cells were significantly down-regulated after induction of radiotherapy, and tumor cell apoptosis was promoted. These results are consistent with previous studies (Yang *et al*., 2010; Huang *et al*., 2017; Zhou *et al*., 2018; Hao *et al*., 2019), indicating that survivin expression down-regulation can promote cancer cell apoptosis.

YM155 is a small molecule drug that targets survivin (Nakahara *et al*., 2007). Iwasa *et al*. (2008) showed that YM155 treatment increased the sensitivity of human non-small cell lung cancer cells to gamma rays and inhibited the growth of non-small cell lung cancer xenografts in nude mice. Liu *et al*. (2018) found that YM155 inhibited the up-regulation of survivin caused by radiotherapy and further increased the sensitivity of cancer cells to radiotherapy. Consistently, our results showed that after YM155 and radiotherapy treatment, the levels of survivin mRNA and protein in ECA-109 cells and KYSE-150 cells were significantly down-regulated, however, apoptosis was significantly increased. Although there are many kinds of reagents targeting survivin, such as RNAi and YM155, they all have certain shortcomings and have not been applied to clinical practice (Li *et al*., 2019). Therefore, we hope to explore new ideas for targeted survivin therapy through the concept of gene-radiotherapy.

Using radiation-induced properties of promoters, gene-radiotherapy uses ionizing radiation to drive the expression of a gene of interest, thereby combining radiation therapy with gene therapy. Wang *et al*. (2012) constructed an Egr1 / TRAIL adenovirus expression vector, which promoted apoptosis of cervical cancer cells after radiation induction. Hu *et al*. (2019) used radiation to induce ras-related C3 botulinum toxin substrate 2 overexpression, which significantly increased the sensitivity of malignant melanoma cells to radiotherapy. The results of this study showed that after Egr1-survivin shRNA + radiation treatment, the expression levels of survivin mRNA and protein in ECA-109 cells and KYSE-150 cells were significantly down-regulated. However, cleaved Caspase3 and Caspase9, which are associated with apoptosis, increased significantly, indicating that inhibition of survivin promoted tumor cell apoptosis. In addition, FACS results showed that after Egr1-survivin shRNA treatment, esophageal squamous cell carcinoma cells had significant G2 and S phase arrests. Consistent results have also been reported in Hela cells (Wang *et al*., 2012), breast cancer cells (Hu *et al*., 2019), and uveal melanoma (Zhou *et al*., 2010).

CONCLUSION

We conclude that Egr1-Survivin shRNA transfection combined with radiotherapy increases the radiosensitivity of esophageal squamous cell carcinoma and may be a promising treatment for esophageal cancer.

LV, Y.; ZHANG, J.; LU, Y.; ZHANG, X. & WANG, H. Efecto de la regulación negativa de survivina por shRNA de Egr1-survivina combinado con radioterapia sobre la radiosensibilidad del carcinoma escamoso de esófago. *I nt. J. Morphol., 41(6)*:1712-1719, 2023.

RESUMEN: Este estudio tuvo como objetivo investigar el efecto de la regulación negativa de survivina por el shRNA de Egr1-survivina combinado con radioterapia sobre la apoptosis y la radiosensibilidad del carcinoma de células escamosas de esófago Células ECA109 y KYSE150. Las células ECA109 y KYSE150 se transfectaron con shRNA de survivina Egr1 y luego se trataron con radioterapia. Después de 24 h, los niveles de ARNm y proteína de Egr1-survivina se detectaron mediante qPCR y Western-Blot. El ciclo celular y la apoptosis se detectaron mediante citometría de flujo. La transferencia Western también detectó niveles de Caspasa 3 y Caspasa 9 escindidas. Se usó YM155 como control positivo para inhibir la expresión de survivina. Los niveles de ARNm y proteína de survivina en células ECA109 y KYSE150 tratadas con shRNA de survivina Egr1 combinado con radioterapia fueron significativamente más bajos que los del grupo control en blanco, el grupo control de vector vacío y el grupo de radioterapia YM155 + ($P < 0,05$). Mientras tanto, después de la regulación negativa de survivina, la proporción entre las fases G2 y S de las células ECA109 y KYSE150 aumentó significativamente, lo que llevó a una detención significativa de las fases G2 y S. Además, la apoptosis de las células ECA109 y KYSE150 aumentó significativamente ($P < 0,01$). Además, los niveles de proteína de Caspasa 3 y Caspasa 9 escindidas aumentaron significativamente en el shRNA de Egr1-survivina combinado con el grupo de radioterapia. El shRNA de survivina de Egr1 combinado con radioterapia puede regular negativamente la expresión de survivina, lo que aumenta aún más la apoptosis y mejora la radiosensibilidad de las células ECA109 y KYSE150.

PALABRAS CLAVE: Carcinoma de células escamosas de esófago; Survivina; Radioterapia genética; Radiosensibilidad.

REFERENCES

Ambrosini, G.; Adida, C. & Altieri, D. C. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med., 3(8)*:917-21, 1997.

Arnold, M.; Soerjomataram, I.; Ferlay, J. & Forman, D. Global incidence of oesophageal cancer by histological subtype in 2012. *Gut, 64(3)*:381-7, 2015.

Chiappa, A.; Andreoni, B.; Dionigi, R.; Spaggiari, L.; Foschi, D.; Polvani, G.; Orecchia, R.; Fazio, N.; Pravettoni, G.; Cossu, M. L.; *et al*. A rationale multidisciplinary approach for treatment of esophageal and gastroesophageal junction cancer: Accurate review of management and perspectives. *Crit. Rev. Oncol. Hematol., 132*:161-8, 2018.

Dabrowski, A.; Filip, A.; Zgodzinski, W.; Dabrowska, M.; Polanska, D.; Wójcik, M.; Zinkiewicz, K. & Wallner, G. Assessment of prognostic significance of cytoplasmic survivin expression in advanced oesophageal cancer. *Folia Histochem. Cytobiol., 42(3)*:169-72, 2004.

Dizdar, L.; Jünemann, L. M.; Werner, T. A.; Verde, P. E.; Baldus, S. E.; Stoecklein, N. H.; Knoefel, W. T. & Krieg, A. Clinicopathological and functional implications of the inhibitor of apoptosis proteins survivin and XIAP in esophageal cancer. *Oncol. Lett., 15(3)*:3779-89, 2018.

Garg, H.; Suri, P.; Gupta, J. C.; Talwar, G. P. & Dubey, S. Survivin: a unique target for tumor therapy. *Cancer Cell Int., 16*:49, 2016.

Global Burden of Disease Cancer Collaboration; Fitzmaurice, C.; Akinyemiju, T. F.; Al Lami, F. H.; Alam, T.; Alizadeh-Navaei, R.; Allen, C.; Alsharif, U.; Alvis-Guzman, N.; Amini, E.; *et al*. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2016: a systematic analysis for the Global Burden of Disease Study. *JAMA Oncol., 4(11)*:1553-68, 2018.

Hao, Y.; Bai, X.; Liu, X.; Kang, S.; Zhang, X.; Liu, C. & Li, Z. Downregulation of survivin by adenovirus-mediated shRNA promotes apoptosis in skin cancer cells. *Onco Targets Ther., 12*:2921-30, 2019.

Hu, W.; Zhu, L.; Pei, W.; Pan, S.; Guo, Z.; Wu, A.; Pei, H.; Nie, J.; Li, B.; Furusawa, Y.; *et al*. Overexpression of Ras-related C3 botulinum toxin substrate 2 radiosensitizes melanoma cells *in vitro* and *in vivo*. *Oxid. Med. Cell. Longev., 2019*:5254798, 2019.

Huang, Q.; Zeng, Y.; Lin, H.; Zhang, H. & Yang, D. Transfection with Livin and Survivin shRNA inhibits the growth and proliferation of non-small cell lung cancer cells. *Mol. Med. Rep., 16(5)*:7086-91, 2017.

Iwasa, T.; Okamoto, I.; Suzuki, M.; Nakahara, T.; Yamanaka, K.; Hatashita, E.; Yamada, Y.; Fukuoka, M.; Ono, K. & Nakagawa, K. Radiosensitizing effect of YM155, a novel small-molecule survivin suppressant, in non-small cell lung cancer cell lines. *Clin. Cancer Res., 14(20)*:6496-504, 2008.

Li, F.; Aljahdali, I. & Ling, X. Cancer therapeutics using survivin BIRC5 as a target: what can we do after over two decades of study? *J. Exp. Clin. Cancer Res., 38(1)*:368, 2019.

Liu, X.; Zhao, Y.; Zhang, W.; Gao, Y.; Huo, M.; Liu, M.; Xiao, Z.; Liang, S.; Xu, N. & Zhu, H. Inhibition of survivin enhances radiosensitivity of esophageal cancer cells by switching radiation-induced senescence to apoptosis. *Onco Targets Ther., 11*:3087-100, 2018.

Nakahara, T.; Kita, A.; Yamanaka, K.; Mori, M.; Amino, N.; Takeuchi, M.; Tominaga, F.; Hatakeyama, S.; Kinoyama, I.; Matsuhisa, A.; *et al*. YM155, a novel small-molecule survivin suppressant, induces regression of established human hormone-refractory prostate tumor xenografts. *Cancer Res., 67(17)*:8014-21, 2007.

Wang, H.; Song, X.; Zhang, H.; Zhang, J.; Shen, X.; Zhou, Y.; Fan, X.; Dai, L.; Qian, G.; Hoffman, A. R.; *et al*. Potentiation of tumor radiotherapy by a radiation-inducible oncolytic and oncoapoptotic adenovirus in cervical cancer xenografts. *Int. J. Cancer, 130(2)*:443-53, 2012.

Weichselbaum, R. R.; Hallahan, D. E.; Beckett, M. A.; Mauceri, H. J.; Lee, H.; Sukhatme, V. P. & Kufe, D. W. Gene therapy targeted by radiation preferentially radiosensitizes tumor cells. *Cancer Res., 54(16)*:4266-9, 1994.

Yang, C. T.; Li, J. M.; Weng, H. H.; Li, Y. C.; Chen, H. C. & Chen, M. F. Adenovirus-mediated transfer of siRNA against survivin enhances the radiosensitivity of human non-small cell lung cancer cells. *Cancer Gene Ther., 17(2)*:120-30, 2010.

Yang, H.; Liu, H.; Chen, Y.; Zhu, C.; Fang, W.; Yu, Z.; Mao, W.; Xiang, J.; Han, Y.; Chen, Z.; *et al*. Neoadjuvant chemoradiotherapy followed by surgery versus surgery alone for locally advanced squamous cell carcinoma of the esophagus (NEOCRTEC5010): a phase iii multicenter, randomized, open-label clinical trial. *J. Clin. Oncol., 36(27)*:2796-803, 2018.

Yang, M.; Wu, Z. & Wang, J. 10-year curative effect of radiotherapy for 1160 cases of esophageal cancer. *Chin. J. Radiat. Oncol., 1(3)*:174-6, 1992.

Zeng, H.; Zheng, R.; Guo, Y.; Zhang, S.; Zou, X.; Wang, N.; Zhang, L.; Tang, J.; Chen, J.; Wei, K.; *et al*. Cancer survival in China, 2003-2005: a population-based study. *Int. J. Cancer, 136(8)*:1921-30, 2015.

Zhang, A.; Kong, J. & Liu, L. Analysis of curative effect and prognosis of 3349 conformal radiotherapy for esophageal cancer. *Sichuan Med. J., 36(6)*:774-8, 2015.

Zheng, R.; Sun, K. & Zhang, S. Analysis of the prevalence of malignant tumors in China in 2015. *Chin. J. Oncol., 41 (1)*:19-28, 2019.

Zhou, C.; Zhang, L. & Xu, P. Growth inhibition and chemo-radiosensitization of esophageal squamous cell carcinoma by survivin-shRNA lentivirus transfection. *Oncol. Lett., 16(4)*:4813-20, 2018.

Zhou, Y.; Song, X.; Jia, R.; Wang, H.; Dai, L.; Xu, X.; Gu, P.; Ge, S. & Fan, X. Radiation-inducible human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene therapy: a novel treatment for radioresistant uveal melanoma. *Pigment Cell Melanoma Res., 23(5)*:661-74, 2010.

Zhu, S.; Li, R. & Wang, Y. Multivariate analysis of simple radiotherapy for 500 cases of advanced esophageal cancer. *Chin. J. Radiat. Oncol., 14(4)*:352-8, 2005.

Corresponding author:
Haifeng Wang
Department of Radiotherapy of the Chest and Abdomen
Affiliated Tumor Hospital of Xinjiang Medical University
No. 789 East Suzhou Street
Urumqi 830011
Xinjiang Uygur Autonomous Region
CHINA

E-mail: gadfly7200@163.com