doi.org/10.36721/PJPS.2021.34.3.SP.1289-1295.1

Study of the radiosensitization of docetaxel in human esophageal squamous carcinoma ECA109 cell line

Li He[1]*, Liang Dong[1], Sujing Zhang[2], Yongqian Zhang[2], Zheng Zheng[2], Ying Guo[2], Liping Guo[2] and Changwen Bo[2]*

[1]Department of Oncology, the First Hospital of Hebei Medical University, Hebei, China
[2]Department of Oncology, the First Hospital of Hebei Medical University, Hebei, China

Abstract: The aim of this study was to determine the radio sensitization of docetaxel in human esophageal squamous carcinoma ECA109 cell line by observing the effects of docetaxel in ECA109 cell proliferation, cell cycle distribution, apoptosis rate and related protein expression. Docetaxel inhibits the proliferation in ECA109 cell line in a dose-dependent and time-dependent manner, and 1nM was chosen for radio sensitization according to the inhibition curves. The D0 and SF2 values of ECA109 cells were 3.00Gy and 0.95, respectively, and of docetaxel (1nM) with irradiation group were 2.54Gy and 0.88. G0/G1 decreased ($P<0.05$), G2/M phase saw a spike ($P<0.05$) in the docetaxel with radiation group at 12h, 24h and 48h, while the apoptotic index witnessed a surge at 24h and 48h ($P<0.05$). The docetaxel with radiation group obtained a higher expression of p21 and bax protein than the docetaxel group and the radiation group ($P<0.05$), and a higher ratio of bcl-2/bax than the others ($P<0.05$). Docetaxel could inhibit the proliferation in ECA109 cell line. p21, bax, bcl-2 and other related proteins can regulate cell cycle phase distribution and induce cell apoptosis, thereby increasing the radiosensitivity effect of docetaxel in ECA109 cell line.

Keywords: Esophageal carcinoma, docetaxel, radiosensitization, cell cycle, apoptosis, signal protein.

INTRODUCTION

Esophageal cancer a common malignant tumor with a top eight incidence rate across the world and a top five in China is recorded with a mortality ranking the fourth among all malignant tumors (Chen *et al*., 2014). The 5-year survival rate of early esophageal cancer treated by surgery is 80%-90%. For most middle and advanced esophageal cancers that cannot be treated with surgery nor be surgically removed, radiotherapy is of necessity (Gholami *et al*., 2018). After radiotherapy, the 5-year survival rate is only 10% ~ 15%, and the local uncontrolled and recurrent rate of the tumor was as high as 60%~75% (Grela *et al*., 2018). The improvement of the radiosensitivity of esophageal carcinoma cells to further reduce the recurrence rate, elevate the local control rate and the curative effect has been an important direction of radiobiology research.

Docetaxel is a taxol drug, which has toxic effects on a variety of cells *in vitro*, directly induce cell apoptosis, and exerts an obvious anti-tumor effect on experimental animal tumors (Grèze *et al*., 2019). Clinically, docetaxel has been used as a chemotherapy drug alone or in combination with other chemotherapy drugs to treat tumors such as breast cancer, lung cancer, gastric cancer and esophageal cancer with rosy efficacy and few side effects. Moreover, this drug with its radio sensitization efficacy, has also been used as a radiotherapy sensitization agent in the treatment of lung cancer (Koukourakis *et al*., 1998), breast cancer (Kris and Manegold, 2001), head and neck tumors (Lu and Meng, 2019) and so on.

In this study, combined radiation of docetaxel on esophageal carcinoma ECA109 cell lines was used to preliminarily investigate the radio sensitization effect of docetaxel on ECA109 cells and the related mechanisms.

MATERIALS AND METHODS

Main regents and instruments

RPMIi-1640 dry powder, methyl thiazolyl tetrazolium (MTT), Sodium dodecyl sulfate (SDS) were purchased from Sigma; docetaxel was bought from SNY, France, Low-molecular-weight protein Marker was purchased from Sino-American Biotechnology co., ltd. Mouse anti-human P21 Monoclonal antibody was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd. Mouse anti-human Bcl-2 monoclonal antibody was purchased from SANTACRUZ, the United States; Mouse anti-human baX monoclonal antibody was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., and fluorescent labeled mouse secondary antibody was purchased from Odyssey Co., Ltd. Flow cytometry was from Beckman Coulter, Germany, and linear accelerator was from Elekta, Sweden. Drug preparation: Dissolve docetaxel in anhydric ethanol under as eptic conditions, prepare the original solution with a concentration of 1 M/mL, store it in refrigerator at 4°C; prepare the corresponding concentration with medium

**Corresponding authors:* e-mail: heli19860611@163.com; bochangwen13@163.com

when using with the solvent concentration lower than 0.1%.

Cell lines and cell irradiation

Esophageal cancer ECA109 was from the Institute of Cancer Prevention and Treatment, Chinese Academy of Medical Sciences. In vitro cell irradiation was carried out with the linac medical accelerator. The dose rate was 200cGy/min, the radiation field area was 20cm×20cm, and the fixed source distance was 100cm. Under the culture bottle or the culture plate 5cm solid water was placed for tissue compensation, with a stand angle of 180° and irradiation was carried out at room temperature.

MTT test

When the cells reached the logarithmic stage, they were digested and counted with 0.25% trypsin. The concentration of cell suspension was adjusted and inoculated on a 96-well plate with 180μl/well and the number of cells per well was 5,000~8,000. The cells were cultured at 37℃ and 5%C O_2 under saturated humidity. After the cells were adherent to the wall, the culture medium containing docetaxel was added with 20μl, to ensure a final concentration of docetaxel of 0nM, 0.1nm, 0.5nm, 1nM, 5nM, 10nM, 50nM and 100nM. MTT 20μl (5mg/mL) was added after the culture for 20h, 44h and 68h, respectively. The remaining culture medium was extracted, and dimethyl sulfoxide 150μl was added. The cells were shaken at a low speed for 10-15min at room temperature to fully dissolve the cell crystals (Mishra *et al*., 2017). The absorbance (OD) value of each hole was measured by ELISA with the measuring wavelength of 490nm and the reference wavelength of 630nm. Each group was set with 6 repeated holes. Tumor cell growth inhibition rate (%) = (1-(OD value of experimental group/control group) ×100%.

Colony formation in soft agar

Esophageal cancer ECA109 cells at logarithmic growth stage were digested with 0.25% trypsin solution and prepared into single-cell suspension. The cells were inoculated in a six-well plate with gradient dilution. The same irradiation dose was used to inoculate 3 parallel culture wells. The number of cells inoculated in the Petri dish of each irradiation dose varied, the number of clones formed in each hole was 50~200 and the cells were evenly distributed by gentle horizontal shaking. After 24h of adherent growth in the incubator, 0, 2, 4, 6 and 8Gy irradiation was carried out at room temperature. After irradiation, the medium was adsorbed and discarded, the medium was added to the irradiation group and the medium containing 1nM docetaxel was added to the docetaxel group. After 48h of culture, the medium was sucked out, carefully soaked in PBS buffer twice, and then the medium was replaced with the one with no docetaxel and was cultured for another 9-12 days. The methanol was fixed for 15min with 1ml methanol; then, the methanol fixing solution was sucked out, and the dye solution was added with 1ml Giemsa at room temperature for 30min.The dye solution was rinsed with distilled water and dried in the air. A sheet of transparent film with a grid was added under the culture plate, and the number of clones greater than 50 cells was counted under an inverted microscope (Sarwar *et al*., 2020). Inoculation efficiency (PE)= (clone number of the control group/inoculated cells number of the control group) × 100%. Survival score (SF) = clone number of the experimental group / (inoculated cells number of the experimental group × PE. The survival score of the docetaxel group was the ratio of the colony formation rate of the docetaxel group to the blank control group. The survival score (SF) of the docetaxel group (0Gy) was taken as the benchmark to correct the SF of the docetaxel with irradiation group to obtain the radiosensitization effect of the drug. The experiment was repeated three times and the average value was taken. The cell survival curve was fitted with a multi-target click model.

FCM test

After ECA109 cells grew to the logarithmic growth stage, they were divided into four groups for culture: The blank control group; the irradiation group; the docetaxel group; the docetaxel with irradiation group. After the cells were adherent to the wall for 24h, corresponding treatments were given respectively. The irradiation dose was 4Gy and the concentration of docetaxel was 1nM. Cells were collected 0h, 12h, 24h and 48h after treatment, fixed with 70% ethanol pre-cooled at -20℃ and stored at -20℃ for later use. The cells fixed with iced ethanol were washed with PBS buffer solution for 3 times and diluted to 1×10^6 cells/ml. Then, 1ml propidium iodide (containing 50mg/mL PI, 1%triton-x100) was added and dyed at 4℃ for 30 minutes. After the cells were washed with PBS again, the DNA cell cycle and apoptosis index (APOI) were analyzed by Multicycle AV software (Scopa *et al*., 2001). The chicken erythrocytes were taken as the internal reference standard.

Western blotting detection of signal protein

ECA109 cells at the logarithmic growth stage were divided into four groups: the blank control group; the irradiation group; the docetaxel group; the docetaxel with irradiation group. The irradiation dose was 4Gy and the concentration of docetaxel was 1nM. After 24h treatment in each group, total cell protein was extracted and was quantified by BCA method. The cells were sampled at 20 microliters per well, followed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 1.5-2 hours; then, they underwent Coomassie brilliant blue staining, and were electro transferred to PVDF membrane. After which, the samples received lichon red staining, distilled water rinsing, and 5% skimmed milk powder was added for 1 hour at 37 ^{o}C shaking table. After sealing, the PVDF membrane was immersed in TTBS antibody

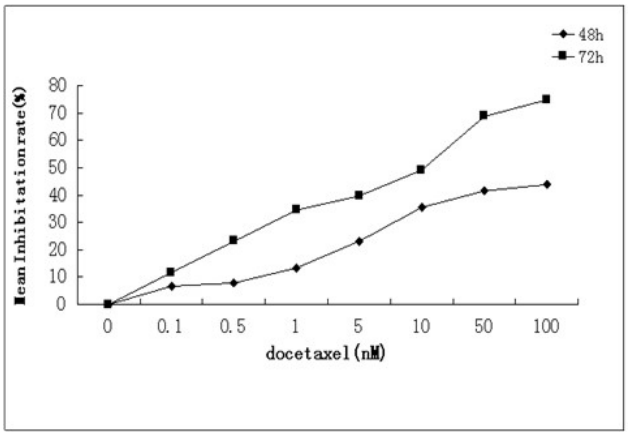

Fig. 1: Effects of different concentration and action time of docetaxel on the growth of ECA109 cells

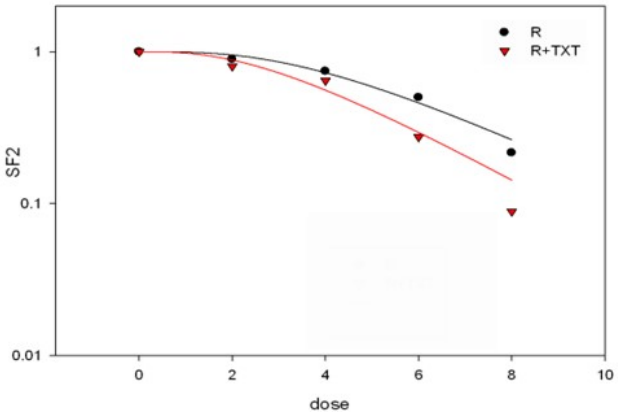

Fig. 2: Effect of 1nM docetaxel on the radiosensitivity of ECA109 cells

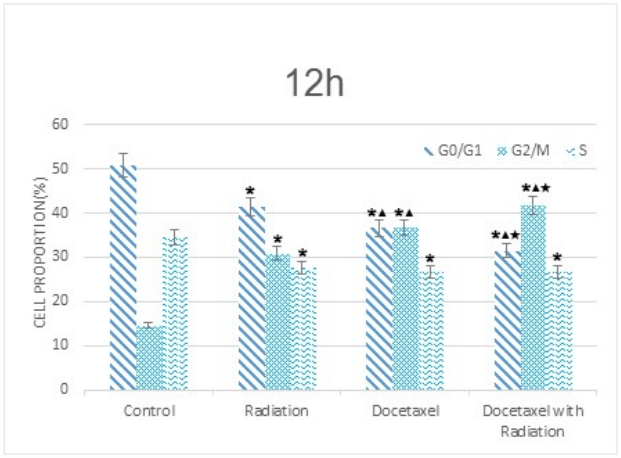

Fig. 3: ECA109 cell cycle distribution after 4GyX irradiation with 1nM docetaxel for 12h

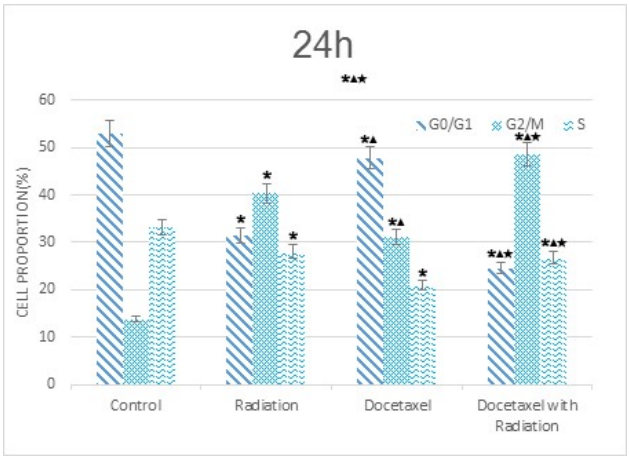

Fig. 4: Cell cycle distribution of ECA109 after irradiation with 1nM docetaxel combined with 4GyX for 24h

diluents prepared with p21 (1:300), bax (1:100), bcl-2 (1:100) and GAPDH (1:300), and the solution was kept overnight at 4°C. The next day, the fluorescence-labeled rats were incubated in a shaker at 37°C for 1 hour, and the two-color infrared laser imaging system scanned and measured the average optical density (OD value). GAPDH was used as the internal standard (Sponseller *et al*., 2014). The ratio of each optical density value to the internal standard was the final result.

STATISTICAL ANALYSIS

In the study, the data were processed with SPSS21.0 software. The measurement data were analyzed with one-way analysis of variance. $P<0.05$ was considered as statistically significant.

RESULTS

Inhibitory effect of docetaxel on the proliferation of Esophageal Carcinoma ECA109 cells

After treatment with ECA109 cells at different concentrations of docetaxel (0, 0.1, 0.5, 1, 5, 10, 50 and 100nM) for 24h, compared with the control group, the OD value of the other groups saw no obvious changes ($P>0.05$). After treatment for 48h and 72h, the OD value of each group decreased drastically compared with the control group ($P<0.05$) in a concentration and time dependent manner. According to the growth inhibition curve (fig. 1), IC 12.5 at 48h, i.e. 1nM, was selected as the subsequent sensitization experimental concentration of ECA109 cells.

The radiotherapy sensitization effect of docetaxel on ESOPHAGEAL cancer ECA109

The clone formation experiment showed that the inoculation efficiency of esophageal carcinoma ECA109 cells was 82.7%. The D_0 and SF_2 values of ECA109 cells were 3.00Gy and 0.95 respectively, and the D q value was 2.55Gy. The D_0 and SF_2 values in the docetaxel (1nM) and irradiation group were 2.54Gy and 0.88 respectively, and the D q value was 2.19G y. The results suggest that docetaxel exerts an evident radio sensitization effect on ESOPHAGEAL carcinoma ECA109 cells (see fig. 2 and table 1).

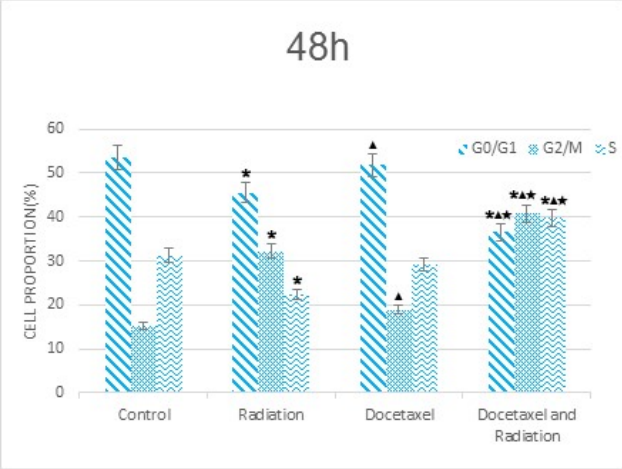

Fig. 5: ECA109 cell cycle distribution after irradiation with 1nM docetaxel combined with 4GyX for 48h

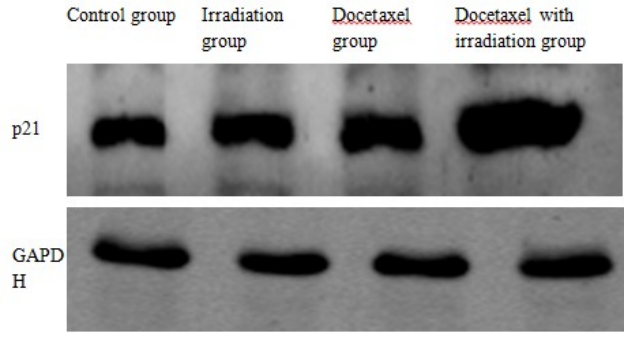

Fig. 6: p21 protein expression in ECA109 cells 24h after 1nM docetaxel action

Cell cycle distribution

At 12h, 24h and 48h after treatment, the proportions of G_0/G_1 phase in the irradiation group, the docetaxel group and the docetaxel with irradiation group all recorded a slump when compared with the blank control group ($P<0.05$, see fig. 3 and table 2). The ratio of G_2/M phase obtained a surge ($P<0.05$, see fig. 4 and table 3). The proportion of cells in stage S showed a downturn ($P<0.05$, see fig. 5 and table 4). The ratio of G_2/M phase in the docetaxel with irradiation group was significantly higher than that in the irradiation group and the docetaxel group ($P<0.05$), suggesting that docetaxel can redistribute the cell cycle after acting on cells.

The docetaxel group presented a lower proportion of G_0/G_1 phase in the at 12h ($P<0.05$) yet a higher result than the irradiation group at 24h ($P<0.05$), indicating an early decline and fast recovery of the proportion of G_0/G_1 phase after drug intervention. At 12h, 24h and 48h, the proportion of G_0/G_1 phase in the docetaxel with irradiation group was significantly lower than that in the docetaxel group and irradiation group ($P<0.05$), indicating an early decrease and slow recovery of the proportion of cells in G0/G1 phase after the combination of drug and irradiation.

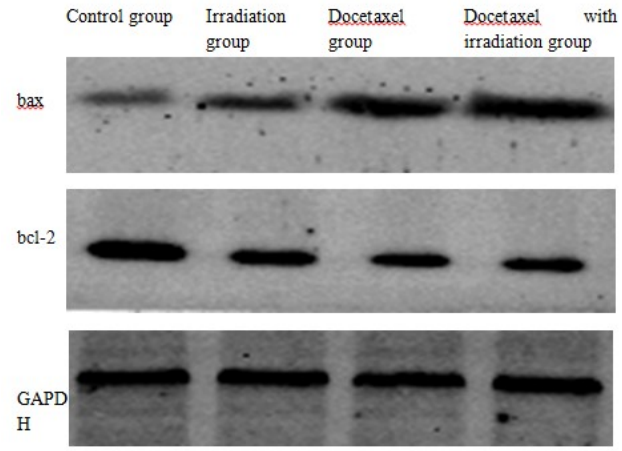

Fig. 7: Expression of bax and bcl-2 proteins in ECA109 cells 24h after 1nM docetaxel action

Higher ratio of G_2/M phase in the docetaxel group at 12h than the irradiation group was observed ($P<0.05$), and it was lower at 24h than that in the irradiation group ($P<0.05$), indicating a rapid rise and early emergence of ratio of G_2/M phase cells after the single drug treatment, and it began to decline at 24h. At 12h, 24h and 48h, the proportion of G_2/M phase in the docetaxel with irradiation group was significantly higher than that in the docetaxel group and irradiation group ($P<0.05$), and the proportion of cells in G_2/M phase increased faster, appeared earlier and maintained for a longer time.

Detection of apoptosis index

Table 5 shows that after docetaxel treated ECA109 cells for 24h and 48h, apoptosis index of both the docetaxel group and the docetaxel with irradiation group increased ($P<0.05$), and the docetaxel with irradiation group observed a higher apoptosis index than that of the drug and irradiation group ($P < 0.05$).

The expression of signal proteins p21, bax, and bcl-2

In all the groups, 24h after treatment of ECA109 cells, p21 protein expression in the irradiation group, drug group and drug plus irradiation group all garnered higher results, as compared to the control group ($P<0.05$), with a superior the expression level in the docetaxel with irradiation group than that in the docetaxel group and irradiation group ($P<0.05$, see fig. 7 and table 6). Similar results were observed in terms of the Bax protein expression in the irradiation group, the docetaxel group and the docetaxel with irradiation group in comparison with the control group ($P<0.05$) and its expression tapered off gradually from the docetaxel with irradiation group, to the docetaxel group, and to the irradiation group in sequence. ($P<0.05$). The bcl-2 protein expression between the treatment groups saw no apparent difference ($P>0.05$). Compared with the control group, the bcl-2/ Bax ratio witnessed a gradual rising trend from the irradiation group, to the docetaxel group, and to the docetaxel with

Table 1: Parameters of cell survival curve fitted by multi-target click model of 1nm docetaxel's radio sensitization in ECA109 cells

Group	D_0 (Gy)	N	Dq (Gy)	SF_2
Eca109	3.00	4.27	2.55	0.95
Eca109+docetaxel	2.54	3.54	2.19	0.88

Table 2: Ratio of G0/G1 phase in ECA109 cells irradiated with 1nm docetaxel combined with 4GyX ray

Group	0h	12h	24h	48h
Control group	50.27±2.39	50.88±0.98	52.94±1.34	53.55±1.83
Irradiation group	51.28±1.90	41.34±2.09*	31.53±1.82*	45.43±1.56*
Docetaxel group	51.45±2.33	36.63±1.26*▲	47.91±1.85*▲	51.85±1.99▲
Docetaxel with irradiation group	49.33±3.50	31.45±1.36*▲★	24.61±1.41*▲★	36.52±2.35*▲★

Table 3: G2/M ratio of ECA109 cells after 4Gy X-ray irradiation with 1nM docetaxel

Group	Group	0h	12h	24h	48h
Control group	Control group	17.27±1.78	14.63±1.26	13.82±0.71	15.20±1.38
Irradiation group	Irradiation group	15.21±1.21	30.91±2.82*	40.42±1.92*	32.23±0.77*
Docetaxel group	Docetaxel group	15.43±1.65	36.72±3.09*▲	31.13±0.80*▲	18.89±1.92▲
Docetaxel with irradiation group	Docetaxel with irradiation group	16.55±1.03	41.82±3.00*▲★	48.53±1.66*▲★	40.73±2.72*▲★

Table 4: S ratio of 1nM docetaxel combined with 4GyX ray irradiation on ECA109 cells

Group	0h	12h	24h	48h
Control group	32.97±3.54	34.49±1.36	33.24±2.02	31.25±1.75
Irradiation group	33.51±2.34	27.75±1.43*	28.05±2.34*	22.34±1.12*
Docetaxel group	33.12±1.73	26.65±1.27*	20.96±1.84*	29.26±2.02
Docetaxel with irradiation group	34.12±3.37	26.73±1.35*	26.86±1.92*▲★	39.75±2.55*▲★

*P <0.05, vs the control group; ▲P <0.05, vs the irradiation group; ★P <0.05, vs the docetaxel group

irradiation group in sequence (P<0.05).

DISCUSSION

Docetaxel, a taxoid anti-tumor drug, which can inhibit microtubule depolymerization and lead to the arrest of tumor cell division cycle G_2/M phase (Mishra *et al*., 2017), which is the most radiosensitive phase of cells. Consequently, as a selective radiosensitizer of cell cycle, docetaxel has achieved positive results in the treatment of lung cancer, breast cancer and head and neck tumors (Lu *et al*., 2019; Sarwar *et al*., 2020; Scopa *et al*., 2001). Studies have shown that docetaxel is a new member of the paclitaxel family. The elimination of TAMs by inhibiting the colony stimulating factor-1 receptor has become a promising tumor treatment strategy. BLZ945 is a colony stimulating factor-1 receptor inhibitor with anti-tumor effects. The combination of docetaxel and BLZ945 can substantially inhibit tumor growth, diminish the abundance of TAMs, increase CD8+ T cell infiltration, and prevent lung metastasis.

MTT assay was used to detect the inhibitory effect of different concentrations of docetaxel on the proliferation of esophageal carcinoma ECA109 cells, and radiosensitization experiment was conducted with low cytotoxicity concentration. The concentration of docetaxel was designed according to IC value (Mishra *et al*., 2017; Sponseller *et al*., 2014), and IC 12.5(1nM) was selected as the subsequent sensitization experiment concentration.

Clone formation experiment showed a notably lower D_0, SF in the docetaxel with irradiation group compared with the irradiation group; and the multiple targets click model was used in fitting curve, which in the docetaxel with irradiation group was obviously down regulated and the shoulder zone was narrowed down, especially within the scope of 4~8Gy. These indicated that docetaxel can inhibit the fatal damage of cell after irradiation so as to increase radiation sensitivity.

Flow cytometry detection results showed that under normal conditions, most cells were in G_0/G_1 phase, followed by S phase, and G_2/M phase had the least proportion. After the treatment of docetaxel alone, the cell cycle significantly changed; specifically, the G_2/M phase ratio increased and the G_0/G_1 phase ratio decreased and the change was the most obvious at 12h and then

Table 5: Effects of 1nM docetaxel combined with 4Gy X-ray irradiation on apoptosis of ECA109 cells at different time

Group	0h	12h	24h	48h
Control group	0.82±0.18	0.81±0.08	0.88±0.05	0.91±0.08
Irradiation group	0.85±0.11	0.75±0.09	0.9±0.04	0.82±0.07
Docetaxel group	0.83±0.12	0.92±0.04	5.03±1.01*	8.47±1.57*
Docetaxel with irradiation group	0.92±0.15	1.02±0.05	8.08±2.05*★	12.97±1.98*★

*P <0.05, vs the control group; ★P <0.05, vs the docetaxel group

Table 6: p21, bax and bcl-2 protein expressions of 1nM docetaxel combined with 4Gy X ray irradiation in ECA109 cells for 24h (x±s, n=3)

Group	bax	bcl-2	bcl-2/bax
Control group	0.38±0.04	0.94±0.13	2.47±0.22
Irradiation group	0.69±0.08*	0.71±0.06*	1.06±0.14*
Docetaxel group	0.94±0.07*▲	0.75±0.08*	0.80±0.05*▲★
Docetaxel with irradiation group	1.22±0.15*▲★	0.68±0.15*	0.56±0.07*▲★

*P <0.05, vs the control group; ▲P <0.05, vs the irradiation group; ★P <0.05, vs the docetaxel group

gradually recovered until 48h, during which there was no significant difference compared with the blank control group. After the cells were exposed to 4Gy alone, the G_2/M phase ratio increased and the G_0/G_1 phase ratio decreased, with a slower change and recovery than that of the docetaxel group, and the cells still did not return to normal after 48 hours. At 12h, 24h and 48h, the proportions of G_0/G_1 and G_2/M in the docetaxel group and irradiation group were significantly lower and higher than those in the docetaxel with irradiation group. The apoptotic index of both the docetaxel group and the docetaxel with irradiation group augmented obviously at 24h and 48h, and showed a gradually increasing trend along with the time; and indexes of the docetaxel with irradiation group was significantly higher than the docetaxel group. These results showed that the drug combined with irradiation had a synergistic effect on the G_2/M phase arrest of cells and notably elevate the apoptosis rate. Studies have shown that the possibly mechanism of the synergistic effect of docetaxel and tamoxifen is that the extension of circulation time in the body and the evidently elevation of apoptosis rate (Toscano *et al*., 2019).

Western blotting results showed that the expression of cyclin associated protein p21 increased in all treatment groups, with a greatest level of increase in the docetaxel with irradiation group. Studies have confirmed that the expression of downstream gene p21 is induced by p53-dependent and p53-independent pathways when cells encounter stress, while increased expression of P21 can lead to cell cycle arrest, thereby inhibiting tumor cell proliferation and increasing apoptosis (Toscano *et al*., 2019).

Bcl-2 expression enhanced antagonistic apoptosis induced by radiation, while bax expression increased tumor radiosensitivity. Changes in the protein levels of bcl-2 and bax can regulate the response of cells to apoptosis stimulation after radiation, which indirectly reflects the radiosensitivity of tumors. The ratio of bcl-2/bax is decisive in the judgement of the changes in apoptosis (Tsavaris *et al*., 2002). The experiment showed that although there was no significant difference in bcl-2 expression between the treatment groups, the bcl-2/bax value of the docetaxel with irradiation group was significantly lower than that of the docetaxel group and the irradiation group and the decreased ratio of bcl-2/bax protein expression indicated the enhanced radiosensitivity of tumor cells. ScoPa *et al*. (Zhou *et al*., 2019) studied the relationship between the expression ratio of bcl-2/bax in rectal cancer tissues and the clinical efficacy of radiotherapy for this tumor. The results showed that tumor tissues with a high bcl-2/bax ratio were resistant to radiotherapy and had poor efficacy. However, tumor tissues with a low bcl-2/bax ratio have a higher response to radiotherapy, which indicates a better curative effect. These results suggest that radiotherapy sensitization of docetaxel may affect the changes of cell cycle and apoptosis after cell irradiation through p21 and bcl-2/bax pathways, which also provides the possibility of molecular therapy for esophageal cancer (Zhu *et al*., 2016).

CONCLUSION

In conclusion, docetaxel can significantly increase the radiosensitivity of esophageal carcinoma ECA109 cells, which may be affected by p21, bax, bcl-2 and other related proteins to regulate cell cycle and increase apoptosis.

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