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Analysis of esophageal cancer cell lines exposed to X-ray based on radiosensitivity influence by tumor necrosis factor-α

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Abstract. Assess the effects of tumor necrosis factor-α (TNF-α) in enhancing the radiosensitivity of esophageal cancer cell line *in vitro*. Three esophageal cancer cell line cells were exposed to X-ray with or without TNF-α treatment. MTT assay was used to evaluate the cell growth curve, and flow cytometry was performed to assess the cell apoptosis. The radiosensitizing effects of TNF-α were detected by cell colony formation assay. Western blotting was applied to observe the expression of NF-κB and caspase-3 protein in the exposed cells. Our results indicated that cellular inhibition rate increased over time, the strongest is combined group ($P < 0.05$). Western blotting showed that the decline expression of NF-κB protein was stated between only rhTNF-α and only X-ray radiation group and the maximum degree was manifested in combined group. Caspase-3 protein content expression just works opposite. Three kinds of cells in the NF-κB protein were similar without rhTNF-α. Then SEG1 NF-κB protein content was reduced more than other two kinds. We concluded that the cells treated with TNF-α showed significantly suppressed cell proliferation, increasing the cell apoptosis, and caspase-3 protein expression after X-ray exposure. TNF-α can enhance the radiosensitivity of esophageal cancer to enhancing the effect of the former.

Keywords: Tumor necrosis factor-α, radiation-sensitizing agents, rhTNF-α, esophageal cancer

1. Introduction

Esophageal cancer is the 8th most common cancer in the world, which accounts for more than 80% of total cases and deaths in the developing countries [1]. According to the National Cancer Institute, in the United States, there will be approximately 18000 new cases and 16000 deaths in 2013 [2]. Esophageal cancer is considered as a serious malignancy with respect to prognosis and mortality rate. Squamous cell carcinoma (SCC) is the most common type of esophageal cancer in China; meanwhile, adenocarcinoma has become the leading cause of esophageal cancer in the western countries [3]. Locally advanced or metastasis have already been confirmed when patients were diagnosed. Surgery, chemotherapy and radiotherapy are the standards for the treatment, but patients do not get more effects from them and have a low sensitivity, thus the usage of appropriate radiation-sensitizing agent is particularly important. Tumor necrosis factor-α(TNF-α) is a kind of cytokines, which can modulate many inflammation and

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immune response and is so far recognized as the strongest cytokines with antitumor activity. TNF-α can make cancerous tissue necrosis, which is closely related to the occurrence and development of various diseases [4]. The mechanism may have correlation with caspase-3, NF-κB and MAPK, etc [5]. Many researches have proved that TNF-α plays a role in other therapy or combination therapy methods besides the function in inhibition of tumor cells and cytotoxicity [6–13]. Radiotherapy is a important method to treat malignant tumor, though limited in efficacy because of the low sensitivity to radiotherapy. So radiosensitivity is gradually becoming the hot spot of attention. Veeraraghavan [14] found TNF-α can be used as a Radiation-sensitizing agent to reinforce the sensitivity of radiotherapy of tumor cells, which is related to the NF-κB signal path. NF-κB is a kind of transcription factors, playing a vital role in modulating various genes involved in cell viability and immune responses as well as inflammatory. It is composed of five distinct but structurally related subunits.p65 is the active part during these five subunits and plays the central role. In unstimulated cells, NF-κB is sequestered in the cytoplasm by the inhibitor of NF-κB family (IκB) via their ankyrin repeat domains. Upon stimulation, such as radiation or antitumor agents, the IκB kinase (IKK) complex is activated and subsequent IκB degraded by the ubiquitin-proteasome pathway. Then, NF-κB releases and translocates into the nucleus to stimulate the expression of its target genes via combining with corresponding DNA binding sites [15–18]. Lots researches confirmed that NF-κB is connected with radiation resistance and anti-apoptosis effect of cells [19–20]. Through the regulation of genes, NF-κB regulates immune and inflammatory response. It is also involved in cell differentiation and apoptosis [21], playing a significant role in cell radiation resistance. Studies show that inhibitors of NF-κB would strengthen the radiosensitivity of cervical cancer, lung cancer, etc. It can be used as a relate protein connected to radiosensitivity [22, 23].

Most of the available reports demonstrated the effect of TNF-α in combination with radiation in lung cancer, laryngeal cancer, tongue carcinoma, sarcoma and lymphoma cancer cells and the mechanism of its enhanced antitumor activity in recent years [6–13], however, the effectiveness has not been reported in esophagus cancer in China and abroad. This study is to investigate the radiosensitivity efficacy of TNF-α in combination with irradiation against human esophagus cancer *in vitro*, TE13 (poorly differentiated squamous cell carcinomas), KYSE140 (moderately differentiated squamous cell carcinomas) and SEG1 (adenocarcinoma).

2. Materials and methods

2.1. Cell culture and irradiation

Human esophagus Cancer cells TE13, KYSE-140 and SEG-1 were obtained from Shanghai valley research industrial (http://guyan201301.cn.china.cn). These cell lines were cultured in 60-mm culture dish (Corning, USA) and maintained in RPMI-1640 medium supplemented with 100 U/ml of penicillin, 100 ug/ml of streptomycin, and 10% fetal bovine serum. In all experiments, reagents were purchased from Gibco. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. TNF-α (0.5 million IU/bottle) was purchased from Shanghai weike biopharmaceutical Co. Ltd.

After reaching confluence, they were subcultured using 1 × sterile phosphatebuffered saline (PBS) and 1 × trypsin. For experimental stimulation, cells were cultured in 96-well plates at a density of 5×10^4 cells/well. Reagents for cell culture were obtained from Gibco and materials from Thermo Fisher Scientific (Shanghai, China).

2.2. Radiation treatment

Esophageal cells during exponential growth phase were treated with X irradiation with a dose rate of 300 MU/min (VARIAN 23EX.USA). Appropriate output dose was delivered by 6 MV X-ray under

radiation field of 40×40 and 100 mm of source skin distance. Before irradiation, the medium was replaced with fresh one which contains TNF-α with different concentration. The dishes were then returned to the incubator for further culture, and the cells were harvested at indicated time.

2.3. MTT assay

Three cells lines were seeded on 96-well plates (Corning Costar) at 5×10^4 cells/well in RPMI-1640 medium containing 10% FBS. Twenty-four hours later, the cells were treated with its different concentrations of TNF-α for another 24 h. Viable cells were counted by the MTT assay. Briefly, cells were incubated with 50 μl of 0.2% MTT for 4 h. Following MTT incubation, cells were lysed in 150 μl of DMSO and the absorbance was read at 490 nm with a automatic microplate reader. The experiments were run in triplicate. The cells during exponential growth phase were divided into four groups: control, TNF-α, radiation and TNF-α in combination with radiation group. The cells were harvested and treated with MTT assay after 12, 24, 36, 48, 72 h.

2.4. Annexin-V/propidium iodide staining

Cells were washed twice with cold PBS and resuspended in 500 μl binding buffer at a concentration of 5×10^5/ml.Then, 5 μl Annexin V-FITC (keyGEN BioTECH) and 5 μl Presidium iodide were added to these cells. After incubation at room temperature for 10 min, each sample was kept on ice before counting the stained cells by flow cytometry. The mean fluorescence intensity of Annexin V-FITC/PI was determined by flow cytometry using the excitation wavelengths is 488 nm, emission wavelengths is 530 nm(BD Calibur, USA). Then, the apoptosis index (AI) was calculated as the mean fluorescence intensity.

2.5. Cell colony formation assay

The radiosensitizing effects of TNF-α combined with X-ray were detected by cell colony formation assay. The sensitization concentration of TNF-α was set according to the preliminary test that can inhibit 50% cell growth. Cultured esophageal cancer cells were randomly assigned to three groups: control, radiation alone, TNF-α and radiation combined groups, each group was seeded on 60-mm culture dishes apart, following which the cells were subjected to 0, 2, 4, 6, and 8 Gy X-ray irradiation. After 3 hours, the medium was exchanged for fresh medium, and cells were maintained to grow for two weeks to allow for the formation of colonies and then stained with crystal violet (Sigma Chemical Co). The number of colonies with more than 50 cells was counted using the microscope. Experiments were repeated at least three times, and the data were expressed as the mean ± SEM.

2.6. Western blot analysis

Untreated, TNF-α and/or radiation treated TE 13,KYSE 140 and SEG 1 cells were used for the detection of caspase 3 and p65 NF-κB expression. After received their respective treatment, the cells were harvested and the whole cell lysates from control and treated cells were prepared with RIPA buffer (Thermo Fisher Scientific). The samples were boiled and quantified with Bradford (Nanjing Jiancheng Bioengineering Institute). Cell lysates containing 50 μg protein/sample and equal amounts of proteins were separated on 12% SDS- polyacrylamide gel electrophoresis and subsequent transferred to a PVDF membrane. Membranes were blocked in TBST containing 0.1% Tween-20, then were treated with specific anti-NF-κB mouse monoclonal antibody (1 : 200) or caspase-3 (1 : 400) at 4°C overnight, and then incubated with the horseradish peroxidase-conjugated secondary antibody (1 : 5000) for 2 h. The bands were detected using ECL chemiluminescent detection reagents (BI). Anti-actin (1 : 400)

monoclonal antibody was used for normalization of western blot analysis. All the antibodies were gotten from Santa Cruz Biotechnology.

2.7. Statistics methods

All data are expressed as mean ± SD. One-sample *t*-test was used to statistically analyze the significance in the expression ratios of irradiated versus controlled or TNF-α samples. A p value less than 0.05 were considered significant. One-hit multitarget model was adopted. Radiosensitization ratio (SER) was defined as D_0 of the radiation group/D_0 of the sensitization Group.

3. Results

3.1. Effect of TNF-α on tumor growth inhibition

The growth inhibitory effect of TNF-α in combination with radiation on three kind's esophageal cancers was analyzed by the MTT assay. Just as showed in Fig. 1, the trend of the rate of TE 13 cell line is gradually raising following the increase concentration of TNF-α, which was performed by our pre-experiment. The same phenomena were observed between KYSE 140 and SEG 1 cell lines. Then we can calculate the concentration of suppressed 50% cell growth through the usage of SPSS software. The concentrations of TNF-α that suppressed 50% cell growth (IC50) of TE 13, KYSE 140 and SEG 1 are 400IU/m, 400IU/ml, and 1200IU/ml respectively. As shown in Fig. 2, TNF-α have no obvious inhibitory effect, the same as irradiation under 200cGy. After added TNF-α with the concentration of its IC50, TE-13 cells exhibited an increased suppression rate in a time-dependent manner. Similar phenomenon was observed after TNF-α treatment in KYSE 140 and SEG 1, the differences are statistically significant ($P < 0.05$).

3.2. Effect of TNF-α on cell apoptosis

To determine whether the radiosensitivity of TNF-α among these cells was correlated with the differences of apoptosis induction, cells were exposed to radiation (2 Gy) with or without TNF-α, and then cell apoptosis was detected by flow cytometry analysis as performed in Fig. 3 of TE 13. The apoptosis induction effects of TNF-α or radiation alone was moderately upregulated, and the apoptosis induction rates seemed to have undergone a synergistic increase in TE-13 cells after the combination

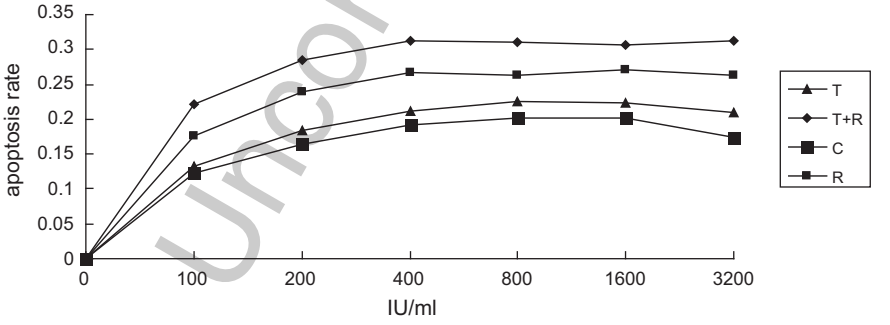

Fig. 1. Apoptosis rate of TE 13 cell line under different concentration of TNF-α under the 2Gy radiation. As it is showed, we can state that the apoptosis rate of TE 13 is increasing with the larger condensation of TNF-α. But the tread became smooth from 400 IU/ml. C" = control group, "T" = TNF-α treated only group, "R" = radiation only group and "T+R" = TNF-α in combination with radiation group.

Table 1
Apoptosis rate of TE13, KYSE140 and SEG1

group	apoptosis rate(%)		
	TE 13	KYSE 140	SEG 1
control	0.02 ± 0.01	0.07 ± 0.29	0.42 ± 0.05
TNF-α only	1.40 ± 0.15	5.80 ± 0.50	3.33 ± 0.45
X-ray only	4.01 ± 0.44	6.78 ± 0.91	4.73 ± 0.53
TNF-α+ X-ray	7.94 ± 0.31	13.4 ± 1.32	12.18 ± 0.41

Table 2
Cell dose effect curve parameter table

	group	D_0	N	D_q	SF2
TE 13	radiation	3.06 ± 0.06	4.34 ± 0.19	4.49 ± 0.23	0.96 ± 0.14
	combination	2.61 ± 0.27	3.62 ± 0.06	3.36 ± 0.18	0.90 ± 0.15
KYSE 140	radiation	2.52 ± 0.18	3.24 ± 0.17	2.96 ± 0.19	0.92 ± 0.15
	combination	2.04 ± 0.19	4.15 ± 0.27	2.90 ± 0.35	0.86 ± 0.07
SEG 1	radiation	3.41 ± 0.16	4.27 ± 0.11	4.95 ± 0.25	0.97 ± 0.18
	combination	2.84 ± 0.25	3.7 ± 0.33	3.72 ± 0.37	0.92 ± 0.28

treatment as shown in Table 1. A better effect can be seen all of three cell lines. Similar results were also observed in the KYSE 140 and SEG 1.

3.3. Colony formation assay

This study was fitted to the one-hit multitarget model according to the research conditions. In the model, SF was calculated as follows: $SF = 1\text{-}(1\text{-}e^{-KD})^N$. $K = 1/D_0$, $N = D_q/D_0$. D_0 represented the mean lethal dose, D_q is quasi-threshold dose. D_q can stands for the cell's ability to repair after sublethal damage in the former papers. Cells exert higher radiation resist with larger value of D_0. The data shows that, comparing to the control cells, TNF-α combined with irradiation groups exhibited more sensitive among all three esophageal cancer cell lines. Furthermore, the parameters of radiotherapy sensitization, naming D_0, N, D_q, SF2, declined more than the irradiation-alone group. The value of $SERD_0$ is 1.17, 1.24, and 1.20, respectively standing for TE 13, KYSE 140, and SEG 1 (Table 2).

This table showed the radio sensitive of three cell lines after the treatment of TNF-α combined with radiation. D_0 represented the mean lethal dose, D_q is quasi-threshold dose. N and SF will be calculated by D_q and D_0.

3.4. Expression of caspase-3 and nuclear NF-κB protein

To identify the mechanisms behind the apoptosis inducing activities of TNF-α combined with radiation treatment, the expression of caspases 3 was examined by western blotting. As shown in Fig. 2, the expression of caspases 3 protein was increased in TNF-α combined with irradiation group compared with the other three groups in TE 13. Contrary to caspases 3, NF-κB shows a notably declined trend. The same result was observed in KYSE 140 and SEG 1 cell lines as illuminated in Figs. 3 and 4. We observed no sensible changes in NF-κB and caspase 3 expressions between control and TNF-α only group in all kinds of cell lines. The expressions are different among these esophageal cancer cell

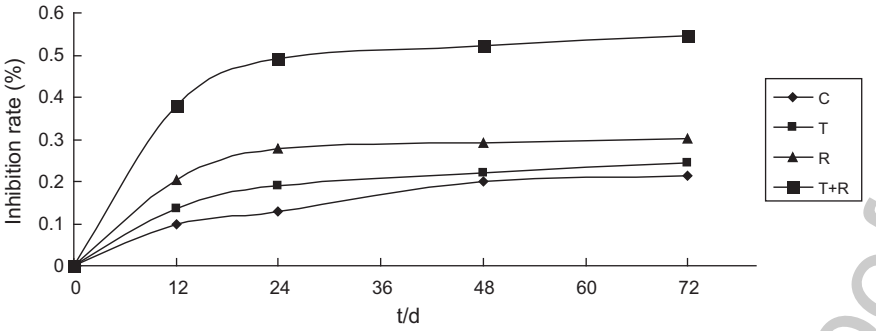

Fig. 2. Inhibition rate of TE 13 cell. This graph shows the inhibition rate of TE13, poorly differentiated squamous cell carcinomas of esophagus cancer. "C" = control group, "T" = TNF-α treated only group, "R" = radiation only group and "T+R" = TNF-α in combination with radiation group.

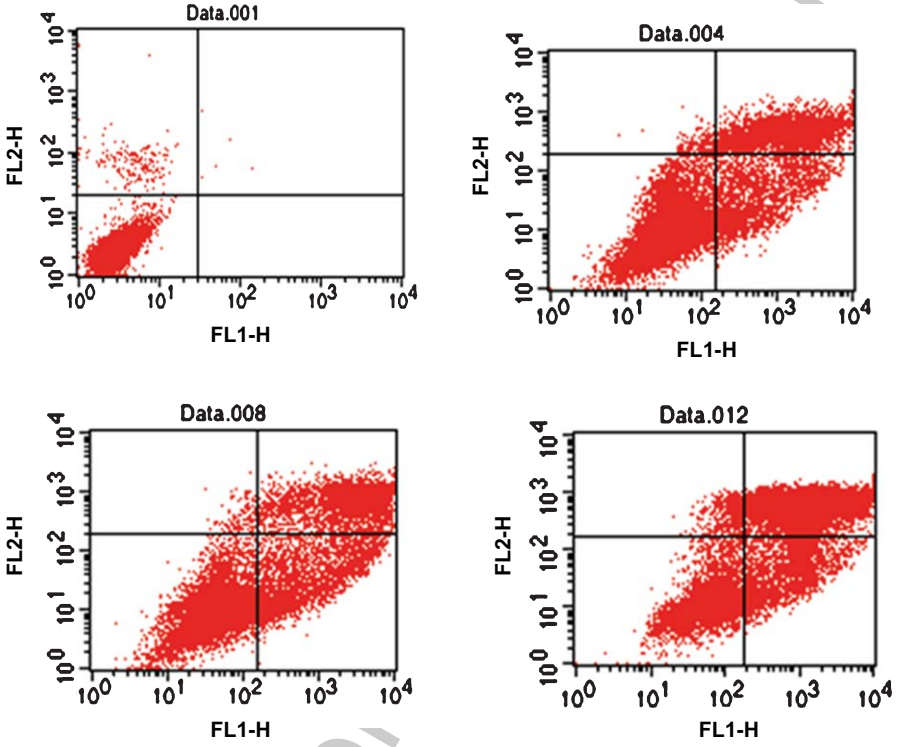

Fig. 3. Cell apoptosis of TE 13 cell line tested by flow cytometry analysis. (a) apoptosis rate of control group; (b) TE 13 cell lines treated only by TNF-α; (c) treatment only by radiation; and (d) the combined treatment of TNF-α and radiation on TE 13.

lines. SEG 1 cells showed relatively strong expression of NF-κB compared to the others, but have a weaker content than other two cell lines after the combined treatment (Fig. 4). However, the increase of radiosensitivity induced by TNF-α in all three cells was observed in esophageal cancer (Fig. 4).

4. Discussion

Many scholars used the TNF-α as radiotherapy sensitization agent in basic research and proved that it can induce tumor cell apoptosis, and enhance its sensitivity to radiotherapy through various

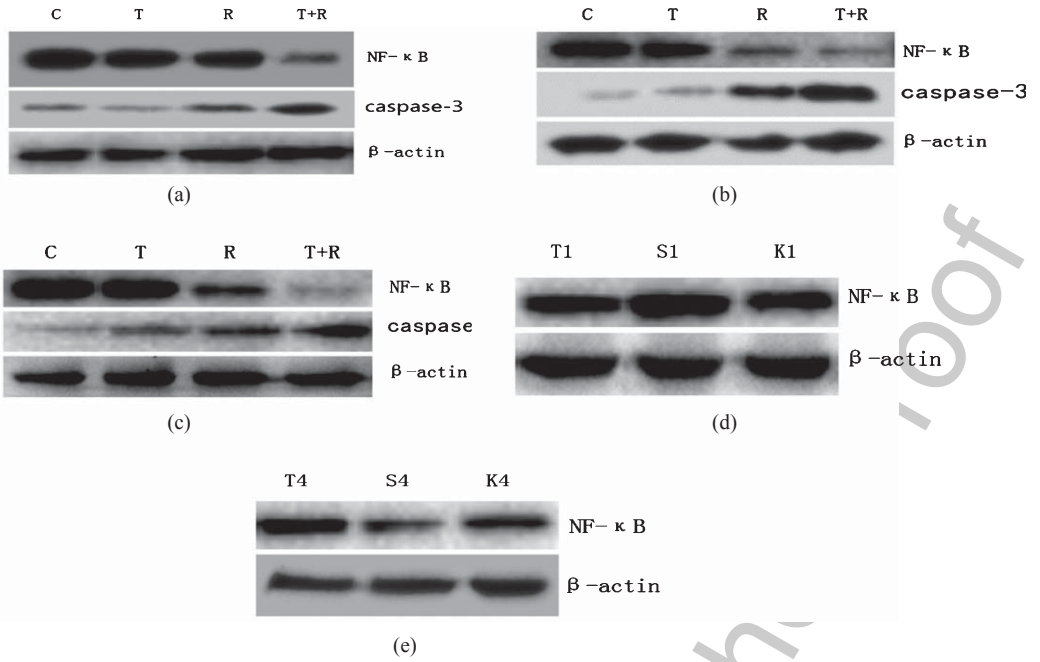

Fig. 4. Showed the expression of corresponding proteins before or after special treatment in three kinds of esophagus cancer. (a) The two cases of proteins' expressed by TE 13 cell lines; (b) KYSE 140, and (c) SEG 1. C: control; T: TNF-α treatment only; R: X-ray treatment only; T+R: TNF-α combined with radiation treatment. (d) and (e) The protein expression of NF-κB in all three cell lines before an after the combined treatment. T1 stands for TE 13 control, T4 represents TE 13 combined treatment group, K1 is on behalf of KYSE 140 control, K4 expresses KYSE 140 combined treatment unit, S1 denote SEG 1 control and S4 signifies SEG 1 combined treatment form.

channels. The NF-κB protein content is related to radiation resistance of cancer cells. Caspases, which belongs to a family of cysteine proteases, are the key proteins that modulate the apoptotic response. These enzymes occur in a latent form in the cytoplasm and are activated late in the apoptotic process. Caspase 3 is a vital mediator of apoptosis that is activated by an initiator caspase.

This study indicated that apoptosis-related protein, caspase 3,increased compared to Separate TNF-α or X-ray treatment group, which declared the application of TNF-α can induce the apoptosis of human esophageal cancer cells, TE13, KYSE 140, and SEG 1 make more apparent trend in the expression of caspase 3. The NF-κB protein involved in radiation sensitivity expression is also different among four discriminatory treatment groups. Its density is significantly lower than the control or TNF-α or X-ray treatment group, suggesting that TNF-α has a significant function on radiotherapy sensitization in poorly differentiated esophageal cancer cell, TE13. Meanwhile, the expression of caspase 3 protein increases correspondingly. The study of moderately differentiated, KYSE 140 and adenocarcinoma, SEG 1, drew the same conclusion with TE 13: the NF-κB protein content was significantly reduced, contrary to caspase 3. Then, we found that NF-κB protein content is higher in adenocarcinoma than squamous cell carcinoma(SCC) before radiation via comparing three cell lines with the treatment of TNF-α and X-ray, which explained why SEG 1 has a higher radiation resistance. The expression of NF-κB protein notably decreased and was lower than the other two after adding TNF-α (1200IU/ml), evaluating that the effects of radiation sensitization is superior than the others. This result is in accord with clone formation assay.

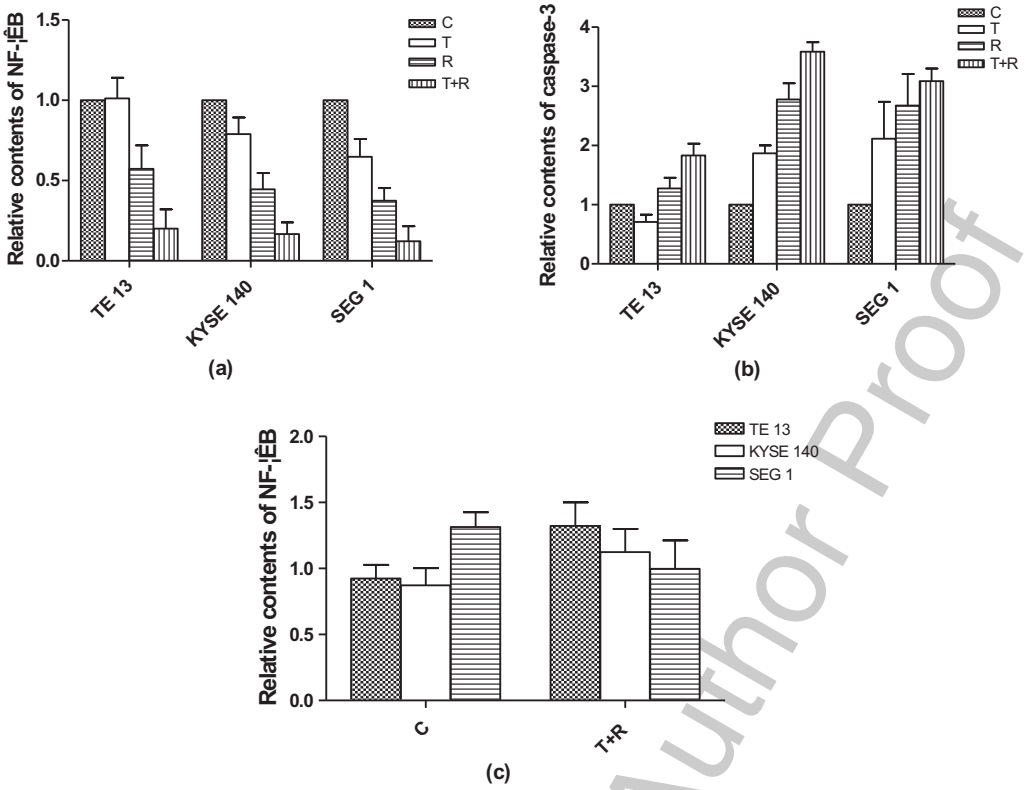

Fig. 5. Influence of expression of protein Caspase 3 and NF-KB induced by the combined application of TNF-α and X-ray in three esophageal cancer cell. (a) NF-KB proteins' expressed by three esophageal cell lines; (b) the proteins of caspase-3 among three cell lines; and (c) the contents of NF-KB protein in all cell lines. "C": control group; "T" : TNF-α treatment only group; "R": X-ray treatment only group; "T+R": TNF-α combined with radiation treatment.

5. Conclusion

We conclude that the therapy of TNF-α combined with X-ray can increase the radiosensitivity of esophageal carcinoma, especially in adenocarcinoma, SEG 1. Not only can TNF-α be used in lung cancer, throat cancer, tongue carcinoma, sarcoma and lymphoma [3–8], but it can also enhance the sensitivity to radiation in esophageal carcinoma, increasing caspase-3 protein expression level. Given our experimental data, these findings may be useful with regards to potential applications of TNF-α in esophageal carcinoma patients undergoing radiotherapy.

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