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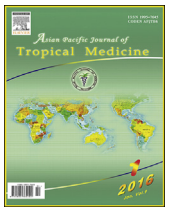

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Effect of recombinant human endostatin onradiotherapy for esophagus cancer

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ABSTRACT

Objective: To investigate the effect of radiotherapy plus recombinant human endostatin (RH-endostatin) on esophageal cancer and its mechanism.

Methods: A total of 50 nudemice were equally randomized into control group, radiotherapy group, and combined therapy group I, II, and III after inoculating with Eca109 cell suspension (1×10^7 cells/mL). On the day of grouping, control group and radiotherapy group were injected normal saline, while radiotherapy group and 3 combined therapy groups received radiotherapy; besides, combined therapy group I, II, and III was injected RH-endostatin of 2.5, 5, 10 mg/kg respectively. After 3-week therapy, the tumors of each group were collected and microvessel density and VEGF expression in tumors were determined. *In vitro*, Eca109 cells were divided into control group, radiotherapy group, and combined therapy group. Forty-eight hours after treatment, cell cycle distribution and apoptosis rate were detected, and the activity of VEGF signal paths was semiquantitatively analyzed.

Results: Since the 6th day of treatment, the relative tumor proliferation rate of combined therapy group II was lower than radiotherapy group ($P < 0.05$) and $\leq$40% since the 15th day. Average microvessel density and EGFR expression in combined therapy group II were lower than radiotherapy group ($P < 0.05$). *In vitro*, the cell percentage in S and G_2/M phase of combined therapy group cells was lower than that in radiotherapy group cells, while the apoptosis rate and the expression of VEGF, AKT, p-AKT, ERK1/2 and p-ERK1/2 in combined group were higher than that in radiotherapy group ($P < 0.05$).

Conclusions: RH-endostatin promotes the efficacy of radiotherapy on esophageal cancer, which may be partly realized by inhibiting the activity of VEGF related signal paths.

1. Introduction

Esophageal cancer is the most common malignant tumor in the digestive system, with characteristics including high incidence, strong invasion activity and poor prognosis [1]. Although operation is still the standard therapy for local esophageal cancer, the cure rates following surgery alone are poor with three-year and five-year survival rates ranging from 6% to 35% [2,3].

As research continues, people learn the important role of tumor neovascularization in tumor growth and metastasis. In 1971, Folkman [4] proposed that the growth of tumor relied on new vessels, because cancer cells stopped growing due to lack of oxygen and nutrition when they were more than 2 mm away from vessels. According to the theory of Angiogenic Switch [5], the growth of tumor vessels is reached together by a series of complicated mechanisms of the promoting factors and inhibitors in which vascular endothelial growth factor (VEGF), basic fibroblast growth factor, epidermal growth factor, and tumor necrosis factor α are included. Thereinto, VEGF is the most specific and strongest promoting factor. Studies showed that VEGF was highly expressed in esophageal cancer, and its high expression was related to high grade, poor prognosis and poor radiosensitivity and chemosensitivity [6,7].

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Anti-angiogenesis drugs are being used in experimental and clinical studies through inhibiting neovascularization and improving hypoxia, *etc*. Whether these drugs could enhance the radiosensitivity in the combination therapy is a hotspot. Recombinant human endostatin (RH-endostatin) is an anti-angiogenesis drug without obvious cytotoxicity and tolerance. Moreover, it can also inhibit tumor angiogenesis in a multi-target way [8,9]. In a clinical study on non-small cell lung cancer (NSCLC), 486 NSCLC patients in progressive stage were randomized into chemotherapy group and chemotherapy plus RH-endostatin group (combined therapy group) and the overall survival rate of two groups was respectively 19.5% and 35.4%, which indicated that the combined therapy could significantly improve the survival of NSCLC patients [10]. Itasaka studied the combined effect of RH-endostatin and radiotherapy on cancer cell A431 and found that RH-endostatin could prevent tumor vascular reconstruction in mice legs after radiation, thus enhancing the efficacy of radiotherapy [11].

Currently, there are few reports on the effect of RH-endostatin in esophageal cancer. In the study we established xenograft model of esophageal cancer in nudemice and adopted the combined therapy of radiotherapy and with RH-endostatin, in order to validate the efficacy of this combined therapy.

2. Materials and methods

2.1. Experimental animals and cell line

A total of 50 male Balb/c nudemice (aging 4–5 weeks and weighing 18–20 g) were fed in separate cages in SPF laminar flow room. Human esophageal cancer cell line Eca109, were cultured in the RPMI 1640 medium containing 10% fetal bovine serum. This study was approved by the Ethics Committee of No.153 Hospital of Liberation Army, and the procedures of the experiments were complied with the Declaration of Helsinki.

2.2. Assays in vitro

Eca109 cells incubated in 6-well plate were adjusted to logarithmic phase and divided into control group, radiotherapy group, and combined therapy group, with 3 parallel wells for each group. The radiotherapy group and combined therapy group were both radiated by linear accelerator with dose of 2 Gy at room temperature; before the radiation, the combined therapy group cells additionally received the treatment of 20 μg/mL RH-endostatin. After the radiation, all groups were further cultured for 48 h, and then cells were harvested. The harvested cells were made into single cell suspension and adjusted to 5×10^5 cells/mL. After three times of washing by PBS, the cells were resuspended in 200 μL Binding Buffer. Then, 10 μL Annexin V-FITC and 10 μL propidium iodide solution were added and mixed gently. After 15 min of incubation at room temperature in dark, 300 μL Binding Buffer was added. One hour later, cell apoptosis rate and cell cycle distribution were determined by flow cytometry.

Cells received the same treatment were harvested for extracting the total protein by cell lysis kit. Then, a rabbit anti-human VEGF monoclonal antibody, rabbit anti-human AKT polyclonal antibody, rabbit anti-human ERK1/2 polyclonal antibody were used as the primary antibody, respectively, and a horseradish peroxidase-conjugated IgG antibody was used as the secondary antibody for Western-blot analysis. β-actin was used as a control. UVIDoc Imager was used for the gray value analysis of protein band.

2.3. Establishment of xenograft model of esophageal cancer in mice and grouping

Eca109 cells in logarithmic phase were made into cell suspension at 1×10^7 cells/mL by 0.25% trypsin. Then, 0.2 mL cell suspension was inoculated subcutaneously into right axilla of nude mouse. When the transplanted tumor grew to 150–250 mm^3 (about one week after inoculation), nudemice were divided into control group, radiotherapy group and combined group I, II, and III (n = 10), in accordance with the random number table.

From d 8 post-inoculation, the control group received normal saline 0.1 mL/d through intraperitoneal injection; the radiotherapy group received radiotherapy; the combined therapy group I, II, and III received radiotherapy plus daily injection of 2.5 mg/kg, 5 mg/kg, and 10 mg/kg RH-endostatin respectively. Radiation methods: After being anesthetized by 100 mg/kg ketamine and 10 mg/kg xylazine, nudemice were fixed and only exposed their legs growing with transplanted tumor; radiotherapy was conducted by medical linear accelerator, with 6 MV-X-ray, SSD 100 cm, a dose of 10 Gy for each time and a dose rate 300 cGy/min. After 21-day treatment, mice were sacrificed for collecting tumors.

2.4. Therapeutic evaluation

Before the first dose and since then, the length (L) and width (W) of transplanted tumor were measured every three days, the volume of transplanted tumor was calculated ($V = 1/2\ L \times W^2$), and the relative tumor volume (RTV) of all the groups were compared (RTV = volume after treatment/volume before treatment). Relative tumor proliferation rate (T/C, %) was used to reflect the effect of drug intervention. When $T/C(\%) \leq 40\%$ and $P < 0.05$ after statistical processing, the therapy was considered effective.

$$\text{Relative Tumor Proliferation Rate}\left(\frac{T}{C}\right) = \frac{\text{Average RVT of Therapy Group}}{\text{Average RVT of Control Group}} \times 100\%$$

2.5. Measurement of microvessel density (MVD) and the expression of VEGF in tumors

Tumors specimen were made into tissue slices, passing through fixing, routine decoloring, embedding and slicing. Immunohistochemical SABC staining was applied to CD34. The average number of positive blood vessels in 3 hotspots was recorded as MVD. The same method was conducted to detect the expression of VEGF protein. Image-Pro Plus 6.0 software was used to detect the absorbance value of the positive staining region, with the average integral optical density representing the content of VEGF.

2.6. Data statistics analysis

SPSSl8.0 software was used for statistical analysis and all the data were represented by mean ± SD. All the experiments *in vitro* were repeated three times. One-way ANOVA was adopted to compare means among groups. When $P < 0.05$, the difference was considered statistically significant.

3. Results

3.1. Evaluation of xenograft model and therapeutic effect

One week after inoculation, the average volume of the subcutaneous tumor nodules was 290 mm^3, and the tumor formation rate was 100%. There was no significant difference in body weight and tumor weight among groups before treatment ($P > 0.05$) (Table 1). After beginning the therapy, compared with control group, the tumor volume and the RTV in radiotherapy group and 3 combined therapy groups decreased significantly ($P < 0.05$); in the 3 combined therapy groups, only combined therapy group II was lower than radiotherapy group in tumor volume and RTV ($P < 0.05$) (Table 1).

At day 7 of treatment, the relative tumor proliferation rate (T/C, %) of combined therapy group II was lower than radiotherapy group ($P < 0.05$), and at day 21 of treatment, T/C of this group was less than 40% while the other groups was always more than 40% during treatment.

3.2. Analysis of MVD and expression of VEGF protein

Table 2 showed that after treatment, the average MVD and VEGF expression of all therapy groups were lower than control group; the average MVD and VEGF expression of combined therapy group II were lower than radiotherapy group ($P < 0.05$).

3.3. Cell cycle distribution and apoptosis rate

Results showed that the percentage of cells in S phase and G_2/M phase of combined therapy group significantly decreased and that in G_0/G_1 phase was obviously increased ($P < 0.05$), compared with control group and radiotherapy group. The apoptosis rate of two therapy groups were both higher than control group and the combined group was higher than radiotherapy group ($P < 0.05$) (Table 3).

In order to explore the mechanism of RH-endostatin inhibiting the neovascularization in which VEGF participated, we detected the expression of VEGF and VEGF signal paths related proteins (AKT, p-AKT, ERK1/2, and p-ERK1/2) in cell. Table 4 showed that the protein expressions of two therapy groups decreased obviously compared with control group, and the combined therapy group was lower than radiotherapy group ($P < 0.05$).

Table 2

Levels of MVD and VEGF protein in tumor tissues.

Groups	MVD (mm^2)	VEGF protein
Control group	108.22 ± 16.22	0.43 ± 0.06
Radiotherapy group	73.95 ± 13.72[a]	0.38 ± 0.03
Combined therapy group I	60.14 ± 11.88[a]	0.34 ± 0.04[a]
Combined therapy group II	40.52 ± 9.06[ab]	0.23 ± 0.03[ab]
Combined therapy group III	54.98 ± 10.25[a]	0.30 ± 0.03[a]

Note: [a] *vs.* control group, $p < 0.05$; [b] *vs.* radiotherapy group, $p < 0.05$.

Table 1

Tumor volume, RTV and relative tumor proliferation rate (T/C, %) of each group at different time points before and after beginning the treatment.

	Before treatment	Day 7	Day 14	Day 21
Tumor volume (mm^3)				
Control group	263.54 ± 10.94	521.93 ± 14.52	797.81 ± 19.13	1049.24 ± 70.17
Radiotherapy group	262.31 ± 12.16	442.23 ± 16.64	530.02 ± 17.68[a]	621.78 ± 21.11[a]
Combined treatment group I	258.57 ± 12.32	397.28 ± 14.97[a]	476.77 ± 15.46[a]	532.18 ± 17.45[a]
Combined treatment group II	261.92 ± 10.74	360.65 ± 13.05[ab]	402.03 ± 13.85[ab]	429.82 ± 14.29[ab]
Combined treatment group III	260.63 ± 12.16	368.14 ± 13.79[a]	443.50 ± 14.23[a]	491.31 ± 15.94[a]
Relative tumor volume (RTV)				
Control group	–	1.99 ± 0.29	3.03 ± 0.32	3.99 ± 0.37
Radiotherapy group	–	1.69 ± 0.23	2.01 ± 0.24[a]	2.38 ± 0.27[a]
Combined treatment group I	–	1.53 ± 0.20[a]	1.85 ± 0.21[a]	2.05 ± 0.23[a]
Combined treatment group II	–	1.37 ± 0.18[ab]	1.52 ± 0.19[ab]	1.63 ± 0.21[ab]
Combined treatment group III	–	1.41 ± 0.22[a]	1.70 ± 0.25[a]	1.88 ± 0.26[a]
Relative tumor proliferation rate (T/C, %)				
Radiotherapy group	–	0.86 ± 0.07	0.67 ± 0.06	0.60 ± 0.05
Combined treatment group I	–	0.76 ± 0.07	0.60 ± 0.05	0.51 ± 0.04
Combined treatment group II	–	0.67 ± 0.05[b]	0.49 ± 0.03[b]	0.40 ± 0.03[b]
Combined treatment group III	–	0.71 ± 0.06	0.56 ± 0.04	0.46 ± 0.04

Note: [a] *vs.* control group, $p < 0.05$; [b] *vs.* radiotherapy group, $p < 0.05$.

Table 3
Cell cycle distributions and apoptosis rate in each groups (%).

Groups	G_0/G_1 phase	S phase	G_2/M phase	Apoptosis rate
Control cells	40.02 ± 1.25	36.31 ± 0.94	23.67 ± 0.78	6.32 ± 0.81
Radiotherapy cells	46.22 ± 1.36	35.97 ± 1.03	17.81 ± 1.16[a]	10.41 ± 0.96[a]
Combined therapy cells	71.45 ± 2.39[ab]	22.38 ± 0.89[ab]	6.17 ± 0.54[ab]	18.92 ± 1.44[ab]

Note: [a] *vs.* control group, $p < 0.05$; [b] *vs.* radiotherapy group, $p < 0.05$.

Table 4
Ratio of gray value of target protein/β-actin.

Groups	VEGF	AKT	p-AKT	ERK1/2	p-ERK1/2
Control cells	1.05 ± 0.23	0.65 ± 0.13	0.48 ± 0.08	0.88 ± 0.09	0.67 ± 0.05
Radiotherapy cells	0.82 ± 0.15[a]	0.50 ± 0.09[a]	0.33 ± 0.05	0.69 ± 0.12[a]	0.50 ± 0.06[a]
Combined therapy cells	0.45 ± 0.07 [ab]	0.29 ± 0.04 [ab]	0.20 ± 0.03 [ab]	0.33 ± 0.06 [ab]	0.28 ± 0.03 [ab]

Note: [a] *vs.* control group, $P < 0.05$; [b] *vs.* radiotherapy group, $P < 0.05$.

4. Discussion

Surgical excision is the main method to treat esophageal cancer, which can cure patients at the early stage. Al-Herz found that the 1-year survival rate and 5-year survival rate of esophageal cancer were respectively 78.3% and 30.3% [12]. Radiotherapy is considered as an effective local therapy not requiring cutting off organs, which makes it an option for patients unable or unwilling to be operated on. In contrast with surgery, radiotherapy brings less pain, no hospitalized mortality and severe complications.

At present, in China most confirmed esophageal cancer patients are in mid and late stage, and radiotherapy is the main option of therapy. It is difficult to diagnose esophageal cancer in early stage, only 20% of patients in mid or late stage are suitable for surgery and about 80% of patients receiving operation also need radiotherapy after resection.

In the study, radiotherapy plus RH-endostatin was used to treat xenograft of esophageal cancer in nudemice, and the results showed that radiotherapy plus 5 mg/kg/d RH-endostatin could significantly inhibit tumor proliferation. However, radiotherapy plus 2.5 mg/kg or 10 mg/kg RH-endostatin did not display the same anti-tumor effect. Celik *et al.* [13] used RH-endostatin to intervene in transplanted pancreatic cancer, and found the optimal dosage of intervention was 250 mg/kg/d, while less or more than the dosage showed the less inhibition of tumor, which is similar to our study. The treatment of anti-tumor angiogenesis drug bevacizumab in colorectal cancer and the intervention of ATN-161 in Lewis lung cancer also showed the same characteristic [14,15]. Peyman *et al.* evaluated the effects of ascorbic acid on experimentally induced corneal neovascularization in the rat model, and in the 7 working concentrations from 250 μg/mL to 100 mg/mL, they found the optimal dose–effect relation was in concentrations between 1 mg/mL and 500 μg/mL [16]. In the study, when the dose of RH-endostatin was 5 mg/kg/d, there was the best inhibition of tumor angiogenesis, which indicates that this dosage was the most optimal working concentration.

In accordance with the above results, when radiotherapy plus 5 mg/kg/d RH-endostatin was given, MVD and VEGF expression in tumors both reached the lowest level. Meanwhile, *in vitro*, the expression level of proteins related to VEGF signal pathways (AKT, p-AKT, ERK1/2, and p-ERK1/2) was the lowest when cells received combined therapy. As the PI3K/AKT path and PLC-γ/PKC/ERK1/2 path are both related to angiogenesis [17,18], angiogenesis would be weakened when the expression of VEGF is inhibited. But now the mechanism of RH-endostatin is not yet completely understood, it is unclear whether RH-endostatin will influence tumor proliferation and angiogenesis through other ways. For example, Xiao L *et al.* found treatment of RH-endostatin could attenuate b-FGF-activated phosphorylation of p38 and ERK1/2 in human umbilical vein endothelial cells which indicate that endostatin might exert its anti-tumor effect via suppressing b-FGF-induced angiogenesis and b-FGF-activated MAPK signaling pathway [19]. Wan [20] even found that endostatin had an epithelium-protective effect and inhibited the inflammation in the pathogenesis of pulmonary fibrosis.

In conclusion, radiotherapy plus RH-endostatin could significantly improve the efficacy of radiotherapy for esophageal cancer, which may be partly realized by inhibiting the VEGF signal paths, and then inhibits tumor angiogenesis.

Conflict of interest statement

We declare that there is no conflict of interests in this study.

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