Role of Vascular endothelial growth factor in radiotherapy resistance to Esophageal squamous cell carcinoma

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Research Article

Keywords: Esophageal carcinoma, Vascular endothelial growth factor, Radiotherapy, Curative effect, Anti-angiogenesis

Posted Date: May 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1653076/v1

Abstract

Vascular endothelial growth factor(VEGF) is related to the radiation resistance of tumors, resulting in the failure of tumor radiotherapy. The purpose of this study was to discuss the role of VEGF in radiotherapy resistance of esophageal squamous cell carcinoma(ESCC). We used the VEGF kit by ELISA to detect the serum VEGF level of ESCC patients who only received radiotherapy. The expression of VEGF in ESCC cells after siRNA treatment was verified by Western blot. The sensitivity of ESCC cells to radiation after knocking down VEGF was analyzed by Clonogenic assay and Cell counting kit(CCK-8). The results showed that the level of serum VEGF in patients with ESCC before and after radiotherapy was related to the clinical response, and it was confirmed that knocking down the expression of VEGF in ESCC cells improved the sensitivity to radiation.

1 Introduction

Esophageal cancer(EC) is a common tumor of the digestive system, and its incidence and mortality are increasing year by year. It has become one of the main reasons for the global cancer burden (Sung et al. 2021). Nearly half of the new cases of esophageal cancer in the world are in China, and ESCC is the main histological type (Li et al. 2021). Radiotherapy plays an important role in the multidisciplinary treatment of esophageal cancer (Cummings et al. 2021). Despite the continuous advancement of radiotherapy technology, the 5-year survival rate of esophageal cancer patients receiving radiotherapy is still only 19.9% (Miller et al. 2019). As radiotherapy resistance is one of the main reasons for the recurrence and progression of esophageal cancer after treatment (Buckley et al. 2020), enhancing the sensitivity of esophageal cancer to radiotherapy and improving the therapeutic effect of patients with ESCC are problems that need to be resolved urgently in clinical practice.

VEGF was first identified in bovine pituitary secretions and was not isolated and purified by researchers until 1989. The family of VEGF proteins identified so far includes, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and and placenta growth factor(PlGF), which exert their biological functions by targeting the receptors VEGFR-1, VEGFR-2 and VEGFR-3, mainly inducing the proliferation of vascular endothelial cells, promoting angiogenesis and increasing vascular permeability (Qin et al. 2019). Tumor cells and their surrounding stromal cells can secrete VEGF to promote neoangiogenesis and form a unique vascular system within the tumor tissue. Studies have shown that high VEGF expression is often associated with highly aggressive tumors and poor prognosis (Poon, Fan, and Wong 2003). Furthermore, VEGF is found to have relaitionship with radioresistance in solid tumors recent years (Buckley et al. 2020). In the study of Wang et al. (Wang et al. 2020), VEGF-C stable and silent nasopharyngeal carcinoma cells were established, and it was found that combined radiotherapy significantly inhibited the proliferation and growth ability, and induced nasopharyngeal carcinoma. It makes cell apoptosis and DNA damage. There are similar research results in cervical cancer (Dong et al. 2021) and soft-tissue sarcoma (Lee et al. 2015). However, the role of VEGF in esophageal squamous cell carcinoma resistance to radiotherapy is rarely studied. In our research, we confirmed the important role of VEGF in radiotherapy of ESCC through

clinical data and conclusion in vitro experiments, so as to provide a basis for radiotherapy combined with antiangiogenic therapy mode.

2 Materials And Methods

2.1 Patient collection.

From September 2019 to June 2021, a total of 167 patients with ESCC were admitted to the institute. Screening is performed according to the following criteria: 1.Clear pathological basis for the diagnosis of esophageal squamous cancer and no history of other malignant tumors before treatment; 2.Patients only received simple radiotherapy and completed all radiotherapy plans; 3.KPS score ≥ 70 points; 4.Complete CT and barium meal review data after radiotherapy; 5.No other diseases related to VEGF elevation in the past. A total of 68 cases were enrolled, and the peripheral blood of the patients before the start of radiotherapy and the 4th week after radiotherapy were collected for serum VEGF determination. This study was approved by the patients' consent and the ethics committee of Huai'an Hospital Affiliated to Xuzhou Medical University.

2.2 Determination of serum VEGF.

The VEGF Kit was purchased from Beijing Jianping Jiuxing Biology Medicine Technology Co. Enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturer's instructions to detect the serum VEGF level of patients, the reference range of normal human serum VEGF is 0-142.2 pg/ml.

2.3 Evaluation of clinical efficacy.

All patients underwent a positioning scan on a Computer Tomography (CT) machine (Siemens, Germany), and two doctors performed a target area delineation based on the CT scan results, and finally completed radiotherapy on 600C/D X-ray linear accelerator (Varian, USA). Repeat CT and barium meal 4 weeks after radiotherapy, and evaluate the clinical efficacy according to the guidelines for the evaluation of the efficacy of solid tumors (RECIST 1.1) (Eisenhauer et al. 2009), which are divided into 4 levels:1.Complete Response (CR):Disappearance of all target lesions.Any pathological lymph nodes (whether targetor non-target) must have reduction in short axis to < 10 mm;2.Partial Response (PR): At least a 30% decrease in the sum of thediameters of target lesions, taking as reference the baseline sum diameters；3.Progressive Disease (PD): At least a 20% increase in the sum of the diameters of target lesions, taking as reference the smallest sum on study(this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%,the sum must also demonstrate an absolute increase of at least 5 mm. (Note: the appearance of one or more new lesions is also consideredprogressions)；4.Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficientincrease to qualify for PD, taking as reference the smallest sum diameterswhile on study.

2.4 Cell culture and processing.

The human esophageal cancer cell line KYSE-450 (K450) and KYSE-510 (K510) came from the Central Laboratory of Huai'an Hospital Affiliated to Xuzhou Medical University. The cancer cells were cultured in RPMI 1640 medium (Shanghai Basalmedia) supplemented with 10% fetal bovine serum (America Zeta-Life) and 1% penicillin/streptomycin (Shanghai Basalmedia), placed in a 5% CO_2 37°C incubator. The medium of the cells was renewed after they were washed with PBS solution (Shanghai Basalmedia) two times every day. The cell passage was done every 2–3 days. All cells are pretreated before radiation.

2.5 Cell transfection.

Three VEGF siRNA was purchased from Huzhou Hema Biology Co., Ltd. Lipofectamine 3000 Transfection Reagent (Thermofisher, USA) was used to transfect VEGF siRNA into esophageal cancer cells and the transfection efficiency was verified by western blotting.

The VEGF siRNAs double-stranded sequences is as follows:

si-VEGF-1: 5'-CCUCCGAAACCAUGAACUUTT-3'

si-VEGF-2: 5'-GCAGAUUAUGCGGAUCAAATT-3'

si-VEGF-3: 5'-CCAAAGAAAGAUAGAGCAATT-3'

2.6 Western Blot Analysis.

Esophageal cancer cells were transfected with siRNA for 48h, then the medium was aspirated, and the cells were rinsed with ice-cold PBS solution 3 times, appropriate amount of ice-cold RIPA buffer was added in and cells were suspended evenly by shaking and placed on ice for 20min. The cells were collected and then centrifuged in an centrifuge tube (Eppendorf, German) at 12000 rpm for 10 min at 4°C, the supernatant was collected. The protein concentration was determined by the BCA assay. The total protein was separated by 10% SDS-PAGE and transferred to a PVDE membrane at 25V overnight. The membrane was blocked with 5% skim milk and TBST for 1 hour, and incubated with the primary antibody at 4°C overnight. The membrane was washed with TBST for 3 times at room temperature, incubated with secondary antibody for 30 min at room temperature, protein bands were detected by ECL method, images were collected by chemiluminescence imaging system, and the ChemiScope software on the instrument was used for grayscale analysis.

2.7 Clonogenic assay.

Cell suspension containing 200 cells was added to a six-well plate. After the cells are fully adhered overnight, 600C/D X-ray was used to irradiate different dose of 0, 2, 4, 6, 8 Gy to cancer cells, and then they were cultured in an incubator for 12 days. Then the cell culture was stained with crystal violet, more than 50 cells were considered as valid colonies, and the colony formation rate (PE) and survival score (SF) were counted. PE = number of effective colonies/number of cells seeded, SF = PE of irradiated cells/PE of unirradiated cells.

2.8 Cell counting kit 8.

$2x10^3$ cells suspension was added to 96-well plate, when the cells were fully attached overnight, the cancer cells were irradiated with 600C/D X-ray at the dose of 6Gy, and then they were cultured in an incubator. After 24h, 48h and 72h, 100μL medium and 10μL CCK-8 reagent (Dojindo, Japan) were added to each well. After incubating for 2h, a microplate reader (Beijing Pulang, China) was used to measure the absorbance of each well at 450nm(OD).

2.9 Statistical analysis.

Statistical analysis including ANOVA and Student's t-test wasconducted using Prism 9.0 (San Diego, USA) and SPSS 23.0 (Chicago, USA) software.Each experiment was repeated three times. All methods were performed in accordance with the relevant guidelines and regulations.

3 Results

3.1 Serum levels of VEGF before and after radiotherapy.

Patients with ESCC who received radiotherapy were evaluated based on their CT and barium meal 4 weeks after radiotherapy, according to the Resicist 1.1 We evaluated the efficacy as complete remission (CR) and partial remission (PR) according to the standard. It is classified as the radiotherapy effective group, and patients with stable disease (SD) and disease progression (PD) are classified as the radiotherapy ineffective group. Among them, 50 patients obtained effective clinical response, and 18 patients obtained low-efficiency clinical response. The serum VEGF levels of all patients before and after radiotherapy are as follows (Table.1). The serum VEGF level of patients in the effective group before radiotherapy was 147.8 ± 53.5ng/ml, and the serum VEGF level after radiotherapy was 119 ± 86.6ng/ml; the serum VEGF level of patients in the low-efficiency group before radiotherapy was 190.1 ± 44.2ng/ml, and the serum VEGF level after radiotherapy was 196.8 ± 74.0ng/ml.

3.2 Changes of serum VEGF levels with radiotherapy.

Further we found that the level of serum VEGF before radiotherapy in the effective radiotherapy group was lower significantly than that in the ineffective radiotherapy group, and the gap was statistically significant ($p = 0.0062$) (Figure.1A). The changes in serum VEGF levels of the effective group and the inefficiency group before and after radiotherapy were statistically significant ($p = 0.0092$) (Figure.1D), and the serum VEGF levels of the effective group after radiotherapy were generally lower than before radiotherapy ($p = 0.0029$) (Figure.1B), but there was no significant difference in serum VEGF level after radiotherapy in the ineffective group compared with before radiotherapy ($p > 0.05$) (Figure.1C).

3.3 VEGF was knocked down by siRNAs.

In order to explore the role of VEGF in radiotherapy, we purchased siRNA. The results of Western Blot showed that transfection of siRNA-VEGF-2 and siRNA-VEGF-3 can down-regulate the expression of VEGF in ESCC cells, and transfection for the down-regulation of siRNA-VEGF-3 is more pronounced (Figure.2A),

so in subsequent experiments, we chose to transfect SiRNA-VEGF-3 (hereinafter referred to as si-VEGF) into ESCC cells to knock down the expression of VEGF.

3.4 Knockdown of VEGF inhibited the growth and proliferation of irradiated ESCC cells.

We transfected si-VEGF into K450 and K510 cells, and evaluated the growth and proliferation ability of the cells in the transfection group and the control group after irradiation with the clone formation experiment and CCK-8 reagent. The clone formation results showed that after different doses of radiation, VEGF knockdown reduced the number of K450 and K510 cell colonies (Figure.2C and D) significantly. CCK-8 results showed that the 450mm OD of the transfected cells decreased significantly after 6Gy X-ray irradiation (Figure.2B) compared with the control group. The above results indicate that knocking down the expression of VEGF causes ESCC cells to be more sensitive to the radiation.

4 Discussion

Our research showed that the serum VEGF level of patients with ESCC before and after radiotherapy is related to the curative effect, and knocking down the expression of VEGF in ESCC cells can improve the sensitivity to radiation in vitro, which suggested that VEGF plays an important role in radiation resistance, provided ideas for future clinical radiotherapy sensitization.

In China, esophageal cancer is one of the digestive tract tumors that seriously endanger people's health, of which more than 90% are squamous cell carcinoma, while adenocarcinoma is the main esophageal cancer in occident countries (Huang et al. 2021). At present, the treatment of ESCC is mainly based on a combination of various treatment strategies, including surgery, chemotherapy, radiotherapy and targeted therapy. Radiotherapy combined with anti-angiogenesis therapy has been proved to be a good way to improve the anti-tumor effect in many types of cancer. Liu et al. (Liu et al. 2020) evaluated the therapeutic effect of radiotherapy combined with apatinib (anti-angiogenesis drug) on mouse nasopharyngeal carcinoma xenograft model, and found that the tumor inhibition effect of combined therapy was stronger than that of apatinib alone or radiotherapy alone. This is similar to the research results of Koo et al. (Koo et al. 2016) and Gao et al. (Gao et al. 2015), which all indicate the synergistic effect of anti-angiogenesis therapy on radiotherapy. Moreover, compared with radiotherapy and chemotherapy, the tumor volume of patients with non-small cell lung cancer with multiple brain metastases after whole brain irradiation combined with apatinib is significantly reduced, and patients can get better objective remission rate and longer median progression-free survival time, but there is no significant difference in median overall survival time (Ren et al. 2021). Another study showed that the curative effect of radiotherapy combined with antiangiogenesis for nasopharyngeal carcinoma patients was similar to that after radiotherapy and chemotherapy, but the acute adverse reactions were significantly reduced (Kang et al. 2018). At present, there are few studies on the combination of antiangiogenesis and radiotherapy in esophageal cancer. Hu et al. (Hu et al. 2020) found that the combined use of apatinib in patients with ESCC undergoing radiotherapy and chemotherapy can significantly improve the median survival time of patients, but not

the total survival rate. Shi et al. (Shi et al. 2020) also have similar results. Radiotherapy for esophageal cancer combined with anlotinib can achieve better anti-tumor effect than single drug and radiotherapy alone. Our previous research (Ma et al. 2021) found that VEGF was related to the prognosis of ESCC during concurrent radiotherapy and chemotherapy. In this study, it was further found that the change of VEGF was still related to the prognosis and curative effect of patients who only received radiotherapy for ESCC, suggesting that VEGF (degree of change before and after radiotherapy) played a role in the radiotherapy resistance of ESCC, and it was a potential target for radiotherapy sensitization of ESCC in the future.

VEGF has been proved to be involved in the occurrence and development of tumors, and is closely related to the prognosis of patients (Siemann, Chaplin, and Horsman 2017). In recent years, many studies have found that inhibiting VEGF expression can improve the sensitivity of many types of cancer to radiotherapy (Chen et al. 2020, Hu et al. 2012, Yang et al. 2012, Wang et al. 2020). In this study, we also found that siRNA knocks down VEGF expression in ESCC cell line to significantly inhibit the growth and proliferation of cells after radiotherapy, which is similar to Chen's research results (Chen et al. 2015). VEGF has the function of regulating tumor angiogenesis. The distorted neovascularization of tumor tissue forms the microenvironment of local hypoxia, which leads to radiation resistance. Inhibition of VEGF can normalize tumor blood vessels, improve local hypoxia and thus improve the sensitivity of tumor to radiation (El Alaoui-Lasmaili and Faivre 2018). On the other hand, tumor cells may undergo gene mutation after being irradiated, which may repair their damaged functions, restore or even enhance their growth and proliferation ability, and lead to radiation resistance. Studies have proved that receiving radiation after knocking down VEGF expression can activate NF-kB pathway or PI3K/mTOR and other signal pathways (Wang et al. 2020, Chen et al. 2020), induce DNA damage of tumor cells, help to activate the apoptosis or death process of tumor cells, and further improve the lethality of radiation to tumors. In addition, radiotherapy can promote immune response and also lead to immunosuppression(McLaughlin et al. 2020). Immunosuppressive effects of radiotherapy include recruiting specific immune subsets and differentiating immune subsets into tumor-promoting phenotypes, such as regulatory T cells (Tregs), Myeloid derived suppressor cells (MDSCs), Th2 cells, Th2 CD4 + T cells and M2-tumor-associated macrophages (Lee et al. 2020). Studies have proved that inhibition of VEGF can effectively inhibit the maturation of dendritic cells, reduce the recruitment of Tregs, reduce the number and effectiveness of MDSCs (Hu and Jiang 2017), and thus improve the immunosuppression caused by radiotherapy, showing a better anti-cancer effect.

VEGF-targeted anti-angiogenesis therapy combined with radiotherapy to inhibit tumor has been the research focus and direction at present, but the mode and time of synergistic therapy still need to be further explored. There is a window period for VEGF to "normalize" tumor blood vessels, which is different for different drugs and different cancer types. The "normalization" of tissues and blood vessels in esophageal cancer occurred on the 5th day after the use of recombinant human endostatin (Zhu et al. 2015), and the "normalization" of tissues and blood vessels in colon cancer occurred on the 12th day after the use of Bevacizumab (Willett et al. 2004, Willett et al. 2005). During this time period, the combined radiotherapy can obviously inhibit the tumor. In the future, we will judge the time of vascular

normalization of esophageal cancer from the changes of serum VEGF in patients receiving anti-angiogenesis therapy, and explore a more appropriate cut-in time of radiotherapy, so as to obtain better clinical effect.

Our research is limited by the small sample size and single-center research, so we need to further expand the sample size and in vitro experiments to verify the conclusion. It is still necessary to further clarify the mechanism, so as to provide the basis for becoming a radiosensitizer. Although we found that VEGF is related to radiotherapy resistance, the timing of the combined application of targeted VEGF therapy and radiotherapy still needs further exploration.

In a word, we found that the serum VEGF level is related to the radiotherapy effect in patients with ESCC, and inhibiting VEGF can enhance the sensitivity of ESCC to radiation, which indicates that VEGF is a potential target of radiotherapy for ESCC, and provides ideas for clinical targeted VEGF anti-angiogenesis therapy combined with radiotherapy to improve the curative effect of patients.

Declarations

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

XYS conceived and designed the expriment. CY and YYM and HL collected the patient's peripheral blood. JHX and HLL and QJ outline the patient's radiotherapy field and verify the treatment plan. XL perfomed the experiments and wrote the manuscript draft. XYS and ZYZ and LQZ edited the paper. All the authors have read and approved the final manuscript and agree to be accountable for all aspects of the study.

Ethics approval and consent to participate

Peripheral blood was collected with the permission of the ethics society of Huai'an Hospital Affiliated to Xuzhou Medical University(HEYLL No.201932) and the written consent of the patient.

Funding

This research was supported by Huai'an Science Project (No.HAB202029); National Natural Science Foundation of China (No.81872484; No.82073365); Jiangsu Science and technology planning project (No.BE2019605).

Acknowledgments

Thanks to all participants in this study.

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Tables

Table.1.Comparison of clinic-pathological characteristics between the effective group and the ineffective group.

Patient characteristics		The ineffective group(n=18)	The effective group(n=50)	*c2*	*P*
Sex				0.622	0.430
	Male	12(67%)	28(56%)		
	Female	6(33%)	22(44%)		
Age(years)				0.302	0.582
	≤75	8(44%)	26(52%)		
	75	10(56%)	24(48%)		
Tumor Location				2.361	0.519
	Cervical	2(11%)	3(6%)		
	Upper thoracic	3(17%)	10(20%)		
	Middle thoracic	4(22%)	19(38%)		
	Lower thoracic	9(50%)	18(36%)		
Tumor Size(cm)				1.209	0.272
	≤ 4.8cm	7(39%)	27(54%)		
	4.8cm	11(61%)	23(46%)		
Grade				2.090	0.148
	1-2	10(56%)	18(36%)		
	3-4	8(44%)	32(64%)		
TNM Stage				3.895	0.158
	I	2(11%)	10(20%)		
	II	7(39%)	28(56%)		
	III	9(50%)	12(24%)		

Table.2 Serum VEGF levels in patients with ESCC before and after radiotherapy

Clinical response	N	VEGF(pg/ml)	
		Before IMRT	After IMRT
The effective group(CR+PR)	50	147.8±53.5	119.2±86.6
The ineffective group(PD+SD)	18	190.1±44.2	196.8±74.0

Figures

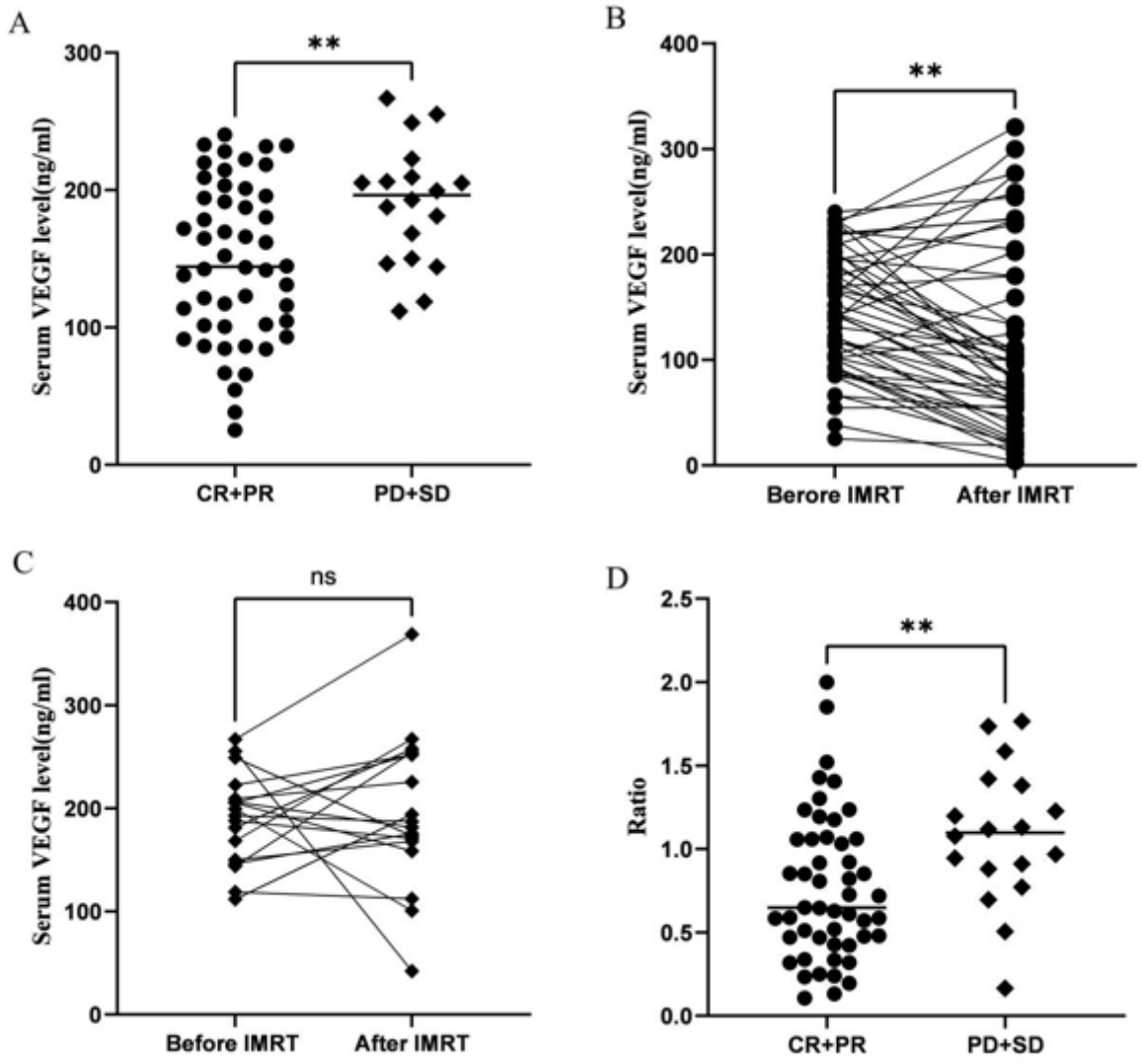

Figure 1

(A)The serum VEGF level before radiotherapy in the effective group was significantly lower than that in the ineffective group. (B)The serum VEGF of patients in the effective group decreased significantly after radiotherapy compared with before radiotherapy. (C)There was no significant difference of serum VEGF in the ineffective group before and after radiotherapy. (D)There was significant difference in the level of serum VEGF between the two groups before and after radiotherapy, and the level of serum VEGF in the

effective group decreased after radiotherapy. (ratio=serum VEGF after radiotherapy/serum VEGF before radiotherapy)

(*p<0.05;**p<0.01;***p<0.001)

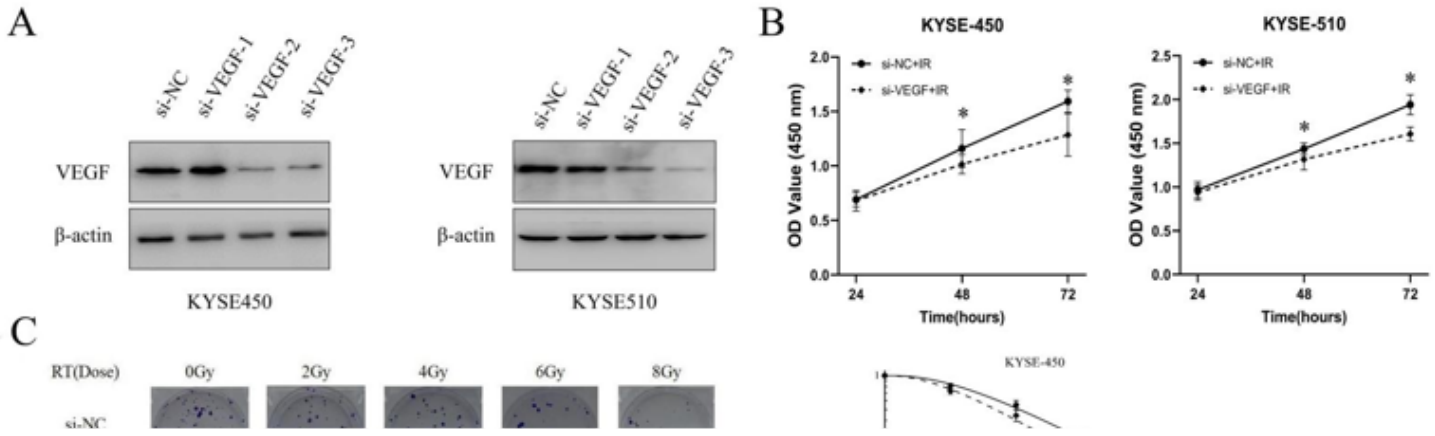

Figure 2

(A) The expression of VEGF in ESCC cells after transfection was detected by Western blot. (B)450nm OD were detected by CCK-8 assay after 6Gy x-ray irradiation of ESCC cells treated with siRNAs. (C, D)The number and size of colonies were analyzed by clonogenic assay after irradiation of ESCC cells transfected with siRNA. (*p<0.05;**p<0.01;***p<0.001)