

# VEGF<sub>165</sub> antisense RNA suppresses oncogenic properties of human esophageal squamous cell carcinoma

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## Abstract

**AIM:** To investigate the effect of antisense RNA to vascular endothelial growth factor 165 (VEGF<sub>165</sub>) on human esophageal squamous cell carcinoma cell line EC109 and the feasibility of gene therapy for esophageal carcinoma.

**METHODS:** By using subclone technique, the full length of VEGF<sub>165</sub> amino acid cDNA, which was cut from pGEM-3Zf (+), was cloned inversely into the eukaryotic expression vector pCEP4. The recombinant plasmid pCEP-AVEGF<sub>165</sub> was transfected into EC109 cell with lipofectamine. After a stable transfection, dot blot, enzyme-linked immunosorbent assay (ELISA), laser confocal imaging system analysis, transmission electron microscopy and flow cytometry were performed to determine the biological characteristics of EC109 cell line before and after transfection *in vitro* and whether there was a reversion in the tumorigenic properties of the EC109 cell *in vivo*.

**RESULTS:** The eukaryotic expression vector pCEP-AVEGF<sub>165</sub> was successfully constructed and transfected into EC109 cells. The expression of VEGF<sub>165</sub> was significantly decreased in the transfected cells while the biological characteristics of the cells were not influenced by the expression of antisense gene. The tumorigenic and angiogenic capabilities were greatly reduced in nude mice, as demonstrated by reduced tumor end volume (820±112.5)mm<sup>3</sup> vs (7930±1035)mm<sup>3</sup> and (7850±950)mm<sup>3</sup>,  $P=1/4.01 \times 10^{-2}$  and microvessel density (8.5±1.2)mm<sup>-2</sup> vs (44.3±9.4)mm<sup>-2</sup> and (46.4±12.6)mm<sup>-2</sup>,  $P<0.01$  in comparison between experimental groups empty vector transfected group and control group.

**CONCLUSION:** The angiogenesis and tumorigenicity of human esophageal squamous cell carcinoma were effectively inhibited by VEGF<sub>165</sub> antisense RNA. Antisense RNA to VEGF<sub>165</sub> can potentially be used as an adjuvant therapy for solid tumors.

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## INTRODUCTION

Angiogenesis, which is defined as the formation of new blood vessel from the pre-existing vascular bed, is essential for solid tumor

growth, for the entrance of tumor cell into the circulation, and for the subsequent establishment and growth of metastasis. Many studies demonstrated that tumor angiogenesis is associated with patient outcome and is an independent prognostic marker in almost all solid tumors, including esophageal carcinoma<sup>[1-10]</sup>. Tumor angiogenesis is a complex process, involving growth factors and extracellular matrix enzymes. Among the many known triggers of tumor angiogenesis, vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is an endothelial cell-specific mitogen and an angiogenesis inducer released by a variety of tumor cells and expressed in human tumors *in situ*. VEGF<sub>165</sub> is the most effective angiogenic factor in the VEGF family. Tumor cells engineered to express VEGF constitutively exhibit enhanced tumor growth and angiogenic phenotypes<sup>[11-13]</sup>. Conversely, inhibition of the expression of VEGF<sub>165</sub> was considered as a therapeutic strategy for the treatment of solid tumors<sup>[14-24]</sup>.

In this report, we constructed antisense RNA to VEGF<sub>165</sub> eukaryotic expression vector and applied gene transfer technology to modulate the expression in stably transfected human esophageal squamous cell carcinoma cells. We assessed the effects of down-regulation of VEGF expression on the biological characteristics *in vitro*, microvessel density and tumorigenic capability in nude mice.

## MATERIALS AND METHODS

### Cell line and vector

The EC109 human esophageal squamous cell carcinoma cell line was generously provided by Dr. Sun (Department of Thoracic Surgery, Tangdu Hospital, Fourth Military Medical University). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM), high glucose media (Life Technologies) and supplemented with 100mL·L<sup>-1</sup> fetal calf serum (HyClone Laboratories), penicillin, streptomycin, and nonessential amino acids (Life Technologies). The vector pGEM-3Zf (+) (carrying the full length aminoacids cDNA of VEGF<sub>165</sub>) was kindly provided by Dr. Abraham (Columbia University, USA) and vector pCEP4 was a gift from Dr. Li (Department of Infectious Disease, Tangdu Hospital, Fourth Military Medical University, China).

### Plasmid construction

The expression vector for VEGF<sub>165</sub> antisense RNA was constructed by subcloning cDNA fragment that code for VEGF<sub>165</sub> into the eukaryotic expression vector pCEP4. pGEM-3Zf (+) was digested by *Kpn*I and *Hind* III. The fragment was purified by gel. The VEGF<sub>165</sub> amino acids cDNA was cloned inversely in the *Hind* III/*Kpn*I site of pCEP4 to generate plasmid pCEP-AVEGF<sub>165</sub> (Figure 1).

### Transfection and selection

The transfection and selection of the EC109 cells were carried out in a 6-well plate. When the cells reached 70% confluence, the transfection process began. Briefly, solution A was prepared by diluting 10μg of pCEP-AVEGF<sub>165</sub> into 200μL serum-free medium, and solution B was prepared by diluting 20μL Lipofectimine 2000 (Life

Technologies) into 200 $\mu$ L serum-free medium. The two solutions were combined for 20 min at room temperature, and then 0.6mL serum-free medium was added to the tube containing the complex, and subsequently added to the rinsed cells. The medium was replaced with fresh and complete medium 18 h after the start of transfection. Seventy-two hours after transfection, it was replaced again with the selective medium containing 200g $\cdot$ L<sup>-1</sup> hygromycin B (Boehringer Mannheim). Once stable transfections were obtained, the cells were maintained in 100g $\cdot$ L<sup>-1</sup> of hygromycin B. The EC109 cells were transfected with either the empty pCEP4 vector or pCEP-AVEGF<sub>165</sub>.

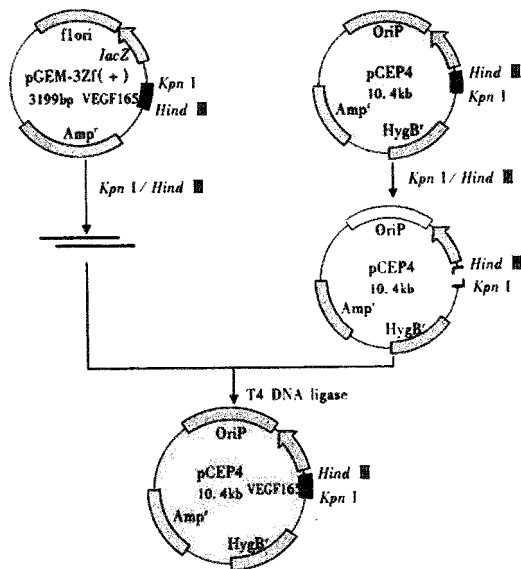


Figure 1 Diagram of the construction of the vector pCEP-AVEGF<sub>165</sub>.

### Dot blot analysis

Total cellular RNA was extracted from the cultured cells using the Trizol isolation kit (Life Technologies) according to the manufacturer's instruction. The recovered total RNA was redissolved in diethyl pyrocarbonate-treated water and 20 $\mu$ g was immobilized onto a gene screen plus membrane (DuPont) by gentle suction with a blotting manifold (Bethesda Research Laboratories). The membrane was then probed with a 5'-end-radiolabeled synthetic oligodeoxyribonucleotide complementary.

### Flow cytometry analysis

Approximately 5 $\times$ 10<sup>6</sup> centrifugal sedimentation cells were immediately fixed in 700mL $\cdot$ L<sup>-1</sup> ethanol and stored at 4 $^{\circ}$ C in PBS in preparation for fluorescent-activated cell sorting. Flow cytometry analysis was performed on a FACStar flow cytometer (Becton Dickinson). Histograms of cell number logarithmic fluorescence intensity were recorded for 10 000 cells per sample.

### Transmission electron microscope examination

The centrifugalized cells were placed in 40g $\cdot$ L<sup>-1</sup> glutaraldehyde and then post-fixed in osmium tetroxide and embedded in Epon. Routine thin sections were stained with uranyl acetate and lead citrate. Thin sections were mounted on grids and examined under a transmission electron microscope (JEM-2000EX) at 60kV.

### Laser confocal microscope analysis

Indirect immunofluorescence techniques were applied in the transfected EC109 cells and the parental cells. VEGF<sub>165</sub> protein was detected with mouse anti-human VEGF<sub>165</sub> antibody and sheep anti-mouse IgG-FITC (Dako A/S Denmark). FITC was activated by light with a wavelength of 488nm. The data of laser scanning were 3%. The

expression of VEGF<sub>165</sub> was analyzed by confocal microscope system controlled by software obtained by Bio-Rad.

### Tumorigenicity assay

Athymic Balb/c nude mice were obtained from the Animal Center of Fourth Military Medical University. The mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions and used at 8-12 weeks of age. Cells used for injection were grown to subconfluence, trypsinized, washed once, and resuspended in serum-free DMEM. The cell suspensions were examined microscopically to ensure that they were composed of single-cell suspensions. Mice were injected s.c. on the hind leg with 5 $\times$ 10<sup>6</sup> single cells in 0.1mL. The mice were then separated into three groups, depending on whether they were injected with pCEP-AVEGF<sub>165</sub> transfected cells, pCEP4 empty vector transfected cells, or control cells. Each group contained five mice. Calipers was used for the calculation of tumor size. Microvessel density was determined under light microscopy after immunostaining of sections with anti-CD34 monoclonal antibody according to the strepto ABC kit (Dako A/S Denmark) instruction.

### Statistical analysis

The data were analyzed for significance by ANOVA.

## RESULTS

### VEGF<sub>165</sub> antisense vector construction

After ligation, transformation and selection, three clones were found likely to contain the desired recombinant. These clones were digested by restriction enzymes KpnI/Hind III or KpnI/SfiI. The 640bp or the 660bp fragment was found by using polyacrylamide gel electrophoresis. These recombinant plasmids were the eukaryotic expression vectors of antisense RNA to VEGF<sub>165</sub> (Figure 2).

### Expression of VEGF<sub>165</sub> antisense RNA

Two weeks after being transfected and selected by hygromycin B, the EC109 cells transfected by pCEP-AVEGF<sub>165</sub> expressed antisense RNA to VEGF<sub>165</sub> which was confirmed by dot blot analysis, whereas the cells transfected by pCEP4 empty vector and control group cells were negative (Figure 3).

### Expression of VEGF<sub>165</sub> in vitro

ELISA showed that a great number of VEGF<sub>165</sub> accumulated in the pCEP4 empty vector transfected group and control group cells, whereas in the pCEP-AVEGF<sub>165</sub> transfected group cells, the level of VEGF<sub>165</sub> was very low. The level of VEGF<sub>165</sub> expression was significantly lower in EC109 cells transfected by pCEP-AVEGF<sub>165</sub> than that in the pCEP4 empty vector transfected group and control group cells ( $P<0.01$ ) determined under confocal microscope, as indicated in Figure 4.

### The change of ultrastructure and cell cycle

There was no substantial change neither in the ultrastructure examined under transmission electron microscope nor in the cell cycle determined by flow cytometer.

### The change of tumorigenic capacity in vivo

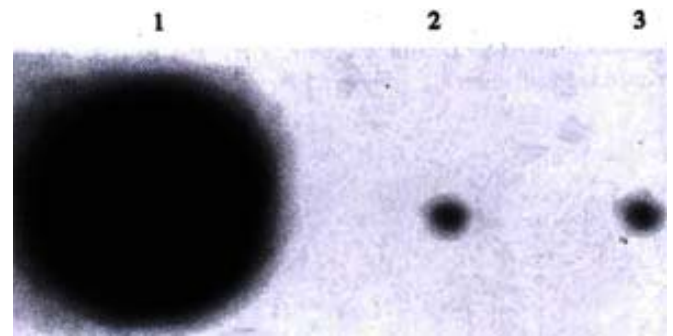
The nude mice were sacrificed at week 5. Tumor volume was measured and morphological characteristics were assessed in HE stained sections. pCEP-AVEGF<sub>165</sub> transfected xenografts grew very slowly, pCEP4 empty vector transfected group and nontransfected control xenografts were significantly larger than pCEP-AVEGF<sub>165</sub> transfected xenografts ( $P<0.01$ ), and the mean tumor volumes were (820 $\pm$ 112.5)mm<sup>3</sup>, (7930 $\pm$ 1035)mm<sup>3</sup> and (7850 $\pm$ 950)mm<sup>3</sup>,

respectively. pCEP-AVEGF<sub>165</sub> transfected xenografts had a relatively large area of central necrosis. Immunohistochemical staining for CD34 was performed to evaluate tumor microvessel density. The microvessel density was expressed as the average number of the five highest areas

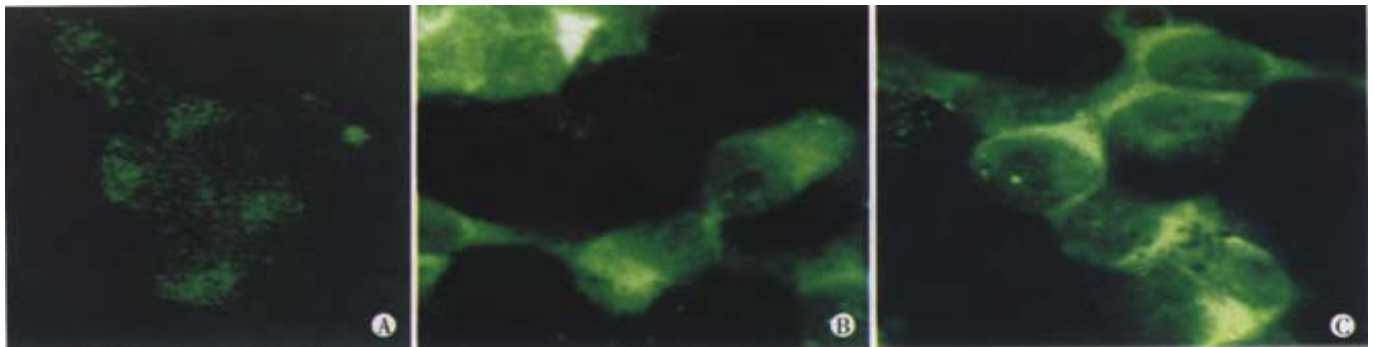


**Figure 2** Identification of recombinant clone by restriction enzyme.  
1: Fragment of 640bp digested with *KpnI/Hind III*  
2: DL2000 markers (2000,1000,750,500,250,100bp)  
3: Fragment of 660bp digested with *KpnI/SfiI*

identified within a single  $\times 200$  field, for the pCEP-AVEGF<sub>165</sub> transfected mice, pCEP4 empty vector group and nontransfected controls were  $(8.5 \pm 1.2) \text{ mm}^2$ ,  $(44.3 \pm 9.4) \text{ mm}^2$  and  $(46.4 \pm 12.6) \text{ mm}^2$ , respectively ( $P < 0.01$ ).



**Figure 3** Expression of antisense RNA to VEGF<sub>165</sub> in EC109 cell  
1: Transfected by pCEP-AVEGF<sub>165</sub>  
2: Transfected by empty vector  
3: Control group



**Figure 4** Expression of VEGF<sub>165</sub> in EC109 cell ( $\times 40$ ): transfected by pCEP-AVEGF<sub>165</sub> (A), transfected by empty vector (B), and control group (C).

## DISCUSSION

Mammalian cells require oxygen and nutrients for their survival and are therefore located within  $100 \mu\text{m}$ - $200 \mu\text{m}$  blood vessels—the diffusion limit for oxygen. For multicellular organisms which grow beyond this size, they must recruit new blood vessels by angiogenesis and vasculogenesis. This process is regulated by a balance between pro- and anti-angiogenic molecules, and is derailed in various diseases, especially cancer. Without blood vessels, tumor can not grow beyond a critical size or metastasize to another organ<sup>[25-29]</sup>. In 1971, Folkman<sup>[30]</sup> proposed that solid tumor growth and metastasis are critically dependent on angiogenesis, the formation of new blood vessels from pre-existing vasculature, and hence, blocking angiogenesis could be a strategy to arrest tumor growth. The induction of angiogenesis is mediated by several factors released by both tumor and host cells. One of the key mediators of angiogenesis is VEGF, a multifunctional growth factor that is overexpressed and secreted by a majority of human and animal tumors. VEGF was purified by Ferrara *et al*<sup>[31]</sup> from the conditioned medium of bovine pituitary folliculo stellate cells. VEGF is a homodimeric 46ku heparin-binding glycoprotein with potent angiogenic, mitogenic, and vascular permeability-enhancing activities specific for endothelial cells. By alternative splicing of messenger RNA, VEGF may exist in at least four different homodimeric molecular species each monomer having 121, 165, 189 or 206 amino acids, respectively (VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, VEGF<sub>206</sub>). Among this family, VEGF<sub>165</sub> is the most important effector. Antiangiogenic therapy targeting VEGF has been

proposed as a means of inhibiting VEGF-dependent tumor growth and metastasis<sup>[32-40]</sup>.

It has been suggested that antisense RNA could block the translation progress of aim protein effectively and inhibit expression<sup>[41-45]</sup>. DeFatta *et al*<sup>[46]</sup> found that reducing eIF4E express on *via* antisense RNA suppressed both the tumorigenic and angiogenic properties of the head and neck squamous cell cancers, cell line FaDu, as demonstrated by lowered capacity to grow in soft agar, reduced expression of angiogenic factors, and loss of tumorigenicity in nude mice. Oku and associates<sup>[47]</sup> transfected human SK-MEL-2 melanoma cells with antisense VEGF which resulted in substantial inhibition of intracerebral tumor growth in nude mice, and a decrease in tumor vascularity, blood flow, and permeability.

The prognosis of human esophageal squamous cell carcinoma after curative resection is dismal. Radiotherapy and several conventional chemotherapeutic agents have been tried to improve the prognosis, but the results are generally disappointing. In this regard, antiangiogenic therapy could be a promising and hopeful strategy for esophageal cancer<sup>[48-51]</sup>. In this study, an antisense RNA to VEGF<sub>165</sub> eukaryotic expression vector pCEP-AVEGF<sub>165</sub> was constructed successfully. We transfected it into human esophageal squamous cell carcinoma cell line EC109. Under immunohistochemistry and confocal microscopy, it was found that the expression of VEGF<sub>165</sub> decreased significantly in the cells transfected with VEGF<sub>165</sub> antisense RNA compared with the empty vector transfected and control group. Under transmission electron microscopy and flow cytometry, we observed that the ultrastructure

and cell cycle had no change among transfected and control groups. In the nude mice tumor model, the tumorigenicity, the rate of tumor growth, and microvessel density were significantly decreased for the tumors derived from antisense RNA transfected cells as compared with the empty vector transfected and parental cells. pCEP-AVEGF<sub>165</sub> transfected tumors had a very low initial growth rate with central necrosis. These results suggested that inhibition of tumor growth might be achieved by VEGF<sub>165</sub> antisense RNA's down-regulation of endogenous VEGF expression in tumor tissues. In the meantime, we found that the VEGF<sub>165</sub> antisense RNA therapy could slow the rate of tumor growth and not inhibit completely the tumorigenicity. This demonstrated that the process of angiogenesis and tumorigenicity is complex and involves multifactors. To the best of our knowledge, this is the first experimental report which shows that VEGF<sub>165</sub> antisense RNA suppresses the growth of human esophageal squamous cell carcinoma *in vivo* in association with decreased vessel number in the treated tumors.

Esophageal carcinoma is still common in China<sup>[52-58]</sup>, and the treatment remains a big problem up to date<sup>[59-65]</sup>. Our present study suggests that antisense RNA to VEGF<sub>165</sub> can potentially be used as an adjuvant therapy for human esophageal squamous cell carcinoma. Further studies are needed to understand the details of the mechanisms for appropriate clinical application.

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