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Effect and mechanism analysis of siRNA in inhibiting VEGF and its anti-angiogenesis effects in human osteosarcoma bearing rats

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Abstract. – OBJECTIVE: To explore the effect of siRNA in inhibiting vascular endothelial growth factor (VEGF) expression in tumor cells from human osteosarcoma bearing rats and its anti-angiogenesis effect, to further study the reliability, effectiveness and safety of VEGF as a therapeutic target in treating osteosarcoma.

MATERIALS AND METHODS: After treatment, the long diameter and short diameter of tumor lesion were detected by Vernier caliper, and the tumor volume and tumor inhibition rate were calculated. The whole-body fluorescence imaging was used to detect the general morphology and volume change of tumor lesion before and after treatment. The rats were killed after treatment, RT-PCR and Western blot were used to detect VEGF expression. MTT was used to detect the proliferative ability of tumor cells *in vitro*.

RESULTS: Three chemotherapies could inhibit the growth of tumor lesion, the decrease of tumor volume was significant ($p < 0.05$), the therapeutic effect in Ad-VEGF-siRNA + neoadjuvant chemotherapy group was better than the other two groups, the differences were statistically significant ($p < 0.05$). Furthermore, the three chemotherapies could inhibit the invasiveness of tumor cells, which was most significant in Ad-VEGF-siRNA + neoadjuvant chemotherapy group ($p < 0.05$).

CONCLUSIONS: The growth of tumor tissue in osteosarcoma bearing rats is inhibited in Ad-VEGF-siRNA group, Ad-VEGF-siRNA + neoadjuvant chemotherapy group and Ad-VEGF-siRNA + anti-angiogenesis chemotherapy group. The effect in Ad-VEGF-siRNA + neoadjuvant chemotherapy is more significant than simple biological therapy or Ad-VEGF-siRNA + anti-angiogenesis chemotherapy.

Key Words:
Small interference RNA (siRNA), Vascular endothelial growth factor (VEGF), Osteosarcoma bearing rat.

Introduction

The growth, metastasis, treatment and prognosis of osteosarcoma are affected by many factors, one important factor of which is the formation of new vessels in tumor[1-3]. The formation of new vessels is the important basis of tumor growth or metastasis[3-5]. The vessels not only supply nutrition for tumor tissue but also export tumor cells to the other locations in body; thus, the growth and malignant metastasis of tumor are promoted[6-8].

The inducers of neovascularization in tumor tissue are various vascular growth factors[9-11]. These vascular growth factors are secreted by tumor cells and among them the most important factor is vascular endothelial growth factor (VEGF)[11,12]. Human VEGF gene locates at 6p21.3 on chromosomal, and the total length is 28kb. The gene length to encode VEGF gene is around 14kb, which is consisted of 8 exons and 7 introns alternatively[9,13]. VEGF gene products include 5 isomers of VEGF-A, B, C, D and E according to different splicing mode of mRNA, which react with VEGFR-1, 2, 3 respectively[14,15].

RNA interference (RNAi) is a sequence-specific post-transcriptional silencing, the mechanism is that the double-stranded DNA silences the expression of the corresponding gene[16]. It is reported that during the occurrence and development of tumor tissue, VEGF can promote the formation of new vessels in tumor tissue and formation of ascites to promote tumor growth. VEGF is an important indicator to evaluate tumor prognosis, which can be secreted by almost all the solid tumor cells[17,18]. Thus, VEGF has gradually become a novel target spot for tumor treatment, and the growth of tumor can be effectively inhibited or blocked by blocking VEGF or its receptor. If the siRNA with homologous sequences to VEGF and/or VEGFR mRNA is transfected into tumor cells, the expression of VEGF and/or VEGFR can be blocked to inhibit tumor angiogenesis to further conduct the anti-tumor growth, anti-infiltration and anti-metastasis effect[19,20].

Thus, we specifically inhibited VEGF expression by artificially synthesized siRNA targeting

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on VEGF gene in UMR106 cells. Through expression of target gene, change of tumor cell growth and angiogenesis in tumor tissue, we explored the effect of siRNA in inhibiting VEGF expression and its anti-angiogenesis effect in tumor cells from human osteosarcoma bearing rats, and further studied the reliability, effectiveness and safety of VEGF as a therapeutic target in treating osteosarcoma.

Materials and Methods

Cell Culture and Establishment of Osteosarcoma Model

The cell line was UMR106 human osteosarcoma cell line from Lanzhou University. The cells were cultured in RPMI Media1640 culture medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 10% fetal calf serum (Thermo Fisher Scientific Inc., Waltham, MA, USA), and had suspension culture at 5% CO_2 and 37°C. Male Sprague-Dawley (SD) rats and BALB/c male nude mice were purchased from the Experimental Animal Center of Lanzhou University. After several generations, the UMR106 cells were implanted into BALB/c nude mice for passage to enhance the tumorigenicity. Then 5×10^6 UMR106 cells were injected into the armpit of foreleg in 3 BALB/c nude mice.

Grouping of Rats and Chemotherapy

130 SD rats were randomly divided into 5 groups, including 5 rats in the blanket control group (group A), 30 rats in the mock-vehicle control group (group B), 30 rats in the Ad-VEGF-siRNA group (group C), 30 rats in the Ad-VEGF-siRNA + neoadjuvant chemotherapy group (group D) and 30 rats in the Ad-VEGF-siRNA + anti-angiogenesis group (group E). After the rats were weighted, the drug dose was calculated: in Ad-VEGF-siRNA group (0.2 ml Ad-VEGF-siRNA virus purification solution was injected every other day, the dose was around l×109 pfu/m1, constructed in our lab), intratumoral injection was used since the 8th day after inoculation, there were 5 times in total. Administration of neoadjuvant chemotherapy: 45 mg/kg 5-fluorouracil, 6.2 mg/kg epirubicin and 61.8 mg/kg cyclophosphamide were dissolved in normal saline, which was then injected through caudal vein. Administration of anti-angiogenesis chemotherapy: after the rats were weighted, the drug dose was calculated. 0.256 mg/kg (7.5 mg/m^2) ENDOSTAR (Recombinant Human Endostatin Injection) was dissolved in normal saline and injected through caudal vein; the control group: the same amount of normal saline solution was injected through caudal vein. The rats were treated for 4 weeks. After completing the treatment, Vernier caliper was used to measure the long diameter and short diameter of tumor lesion, and the tumor volume and tumor inhibition rate were calculated, formula: Tumor volume = 0.52 × long diameter × short diameter.

The Whole-Body Fluorescence Imaging of Osteosarcoma Bearing Rats Before and After Chemotherapy

The process of whole-body fluorescence imaging was as follows: the rats in different groups received whole-body fluorescence imaging before and after chemotherapy to compare the size change of tumor lesion before and after chemotherapy. The fluorescence imaging system is consisted of light source, emission filter, laser filter and viewfinder. Before imaging, SD rats were slightly anesthetized, and received whole-body fluorescence imaging after every time interval, the rats with osteosarcoma metastasis received whole-body fluorescence imaging from flap window.

VEGF Expression Detected by RT-PCR

The DNAs in UMR106 cells were extracted according to the protocol in the kit. There were 2 primers in PCR, including 5′ primer and 3′ primer. One DNA strand was used as template while designing primer (usually the information strain was used as template), the sequence of 5′ primer is the same as the sequence of DNA fragment at upstream of 5′ terminal; the sequence of 3′ primer is complementary to the sequence of DNA fragment at upstream of 3′ terminal. The sequence of upstream primer was 5'-GGTGAGAGGTCTAGTTCCCGA-3', the sequence of downstream primer was 5'-CCATGAACTTTCTGCTCTTC-3', and the annealing temperature was 58.3°C. The following reaction conditions were pre-denaturation at 96°C for 10 minutes and denaturation at 94°C for 0.5 minutes. The hydrogen bond in the double-stranded DNA template was broken by heating to form single-stranded DNA. Then the conditions were annealing at 63°C for 0.5 minutes and at 72°C for 0.5 minutes, including 32 cycles in total.

When the system temperature decreases, the primer combines with DNA template to form local double strands. After that the condition was extension at 72°C for 5 minutes. The DNA strand complementary to the template was extended from 5′ terminal to 3′ terminal by the action of Taq enzyme (the activity was optimal at around 72°C) with dNTP as material.

Expression of VEGF Detected by Western Blot

All the proteins were extracted by the conventional method. After being cooled to room temperature, the protein samples were loaded in the wells of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and received electrophoresis. The proteins were then transmembraned onto polyvinylidene fluoride (PVDF) membrane (FFP30/FFP33) by Bio-Rad wet-type transmembrane apparatus (Hercules, CA, USA).

After transmembrane, the membrane was immersed in prepared Western washing solution (P0023C) for 1-2 minutes to remove the transmembrane solution. Then the primary and secondary antibodies were added respectively. Electrochemiluminescence (ECL) reagent was used to detect the proteins according to the protocol. The X-Ray film of the protein bends was developed.

The Proliferative Ability in vitro Detected by MTT Assay

1. The tumor cells were collected and made into cell suspension, 100 μl suspension was added in every well and the cell concentration was adjusted to 1000-10000/well (the margin wells were filled with phosphate buffered saline – PBS).
2. The cells were incubated at 5% CO_2 and 37°C, and the drugs were added when the bottom was paved with single cell layer (96-well plate). Then the cells were incubated at 5% CO_2 and 37°C for 16-48 hours, and observed under inverted microscope.
3. 20 μl MTT solution was added (5 mg/ml of 0.5% MTT) and incubated again for 4 hours. The culture was terminated, and the culture medium was discarded.
4. 150 μl dimethyl sulfoxide (DMSO) was added in every well and shaked by a low speed for 10 minutes to dissolve the crystal substance. The optical density of every well was detected by microplate reader at 490 nm wave length. E. zero setting wells (culture medium, MTT and DMSO) and control wells (cells, the drug dissolution medium of the same concentration, culture medium, MTT and DMSO) were set.

Statistical Analysis

Data were expressed as mean±SD for each group and analyzed in SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed by using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test. All tests were two-tailed, and the level of significance employed was $p < 0.05$.

Results

The Whole-Body Fluorescence Imaging of Osteosarcoma Bearing Rats Before and After Chemotherapy

UMR106 cells were implanted into armpit of foreleg, after 60 days the metastasized tumor was detected by whole-body fluorescence imaging system combined with skin flap technology. The fluorescence imaging showed that there were many subcutaneous tumor lesions in armpit of foreleg with diameter of 0.4 mm-1.9 mm. In Figure 1, the fluorescence imaging showed that the tumor sizes in group A and group B were not statistically different ($p > 0.05$). However, tumor sizes in group C, D and E were significantly smaller than group A and B ($p < 0.05$); the tumor sizes in group C and E were not statistically different ($p > 0.05$). However, the tumor size in group D was significantly smaller than group C and E ($p < 0.05$). There was almost no tumor tissue in group D.

The Change of Tumor Volume Before and After Chemotherapy

The change of tumor volume before and after treatment in Ad-VEGF-siRNA + neoadjuvant chemotherapy group is shown in Figure 2. The tumor volume was decreased, which could not be observed by naked eye in the 4th week. The tumor volumes in group A and B were not changed before and after treatment. The changes of tumor volume in group C and E were evident. However, it was not as significant as group D, thus the images of group C and E are not shown. The comparison of tumor volume change is shown in Table I. The three chemotherapies could inhibit the tumor growth, the tumor volumes in group C,

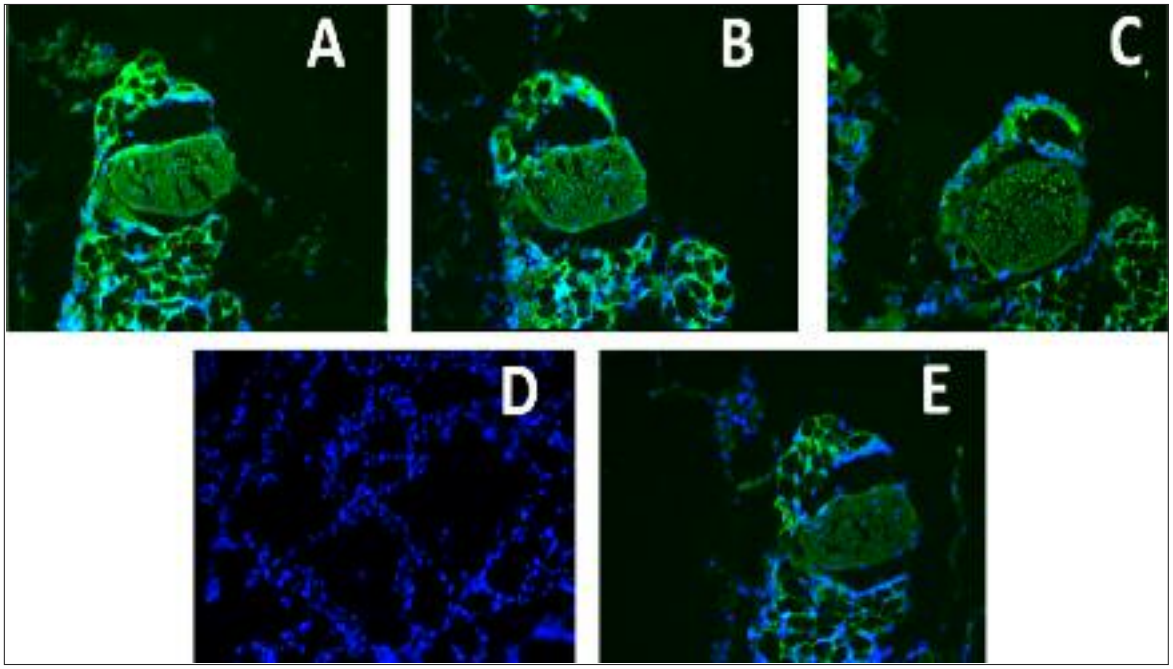

Figure 1. The whole-body fluorescence imaging of osteosarcoma bearing rats before and after chemotherapy. ***A,*** The control group; ***B,*** The mock-vehicle control group; ***C,*** Ad-VEGF-siRNA group; ***D,*** Ad-VEGF-siRNA + neoadjuvant chemotherapy group; ***E,*** Ad-VEGF-siRNA + anti-angiogenesis group.

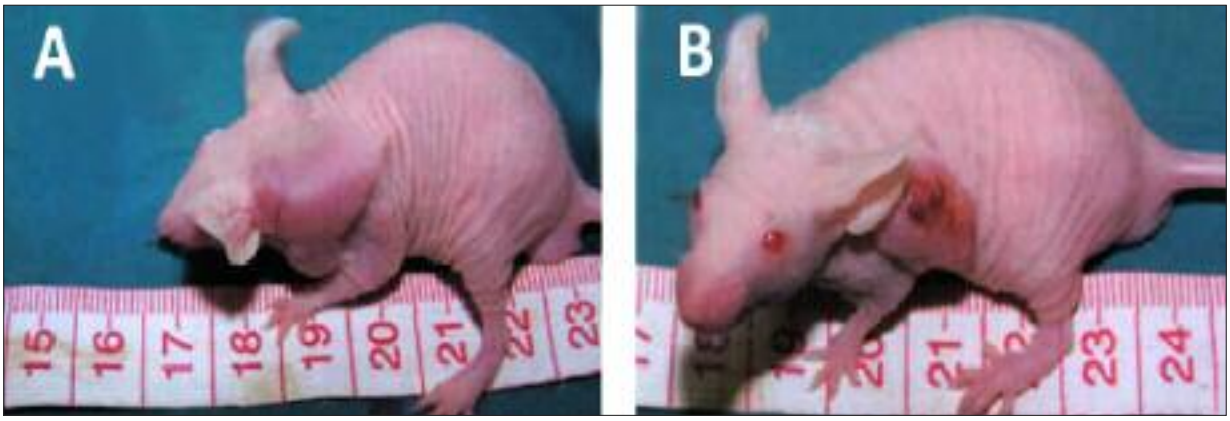

Figure 2. The change of tumor tissue in Ad-VEGF-siRNA + neoadjuvant chemotherapy group before and after treatment. ***A,*** The tumor in armpit of foreleg was very evident in the 1st week. ***B,*** The tumor in armpit of foreleg was almost disappeared in the 4th week.

Table I. Comparison of tumor volume in rats of different groups (cm, ± s).

Group	n	1st week	2nd week	3rd week	4th week
Group A	10	2.41 ± 0.06	2.45 ± 0.05	2.51 ± 0.04	2.55 ± 0.09
Group B	40	2.35 ± 0.04	2.35 ± 0.09	2.41 ± 0.08	2.48 ± 0.14
Group C	40	2.34 ± 0.11	1.51 ± 0.02*	0.74 ± 0.09*	0.47 ± 0.14*
Group D	40	2.27 ± 0.09	1.10 ± 0.03*,#	0.38 ± 0.08*,#	0.29 ± 0.09*,#
Group E	40	2.42 ± 0.04	1.64 ± 0.11**	0.54 ± 0.14*	0.37 ± 0.11*

Note: *Means $p < 0.05$ compared with group A and B; #Means $p < 0.05$ compared with group C and E.

D and E were decreased since the 1st week, which were significantly different from group A and B ($p < 0.05$). However, the tumor volumes in group A and B were not statistically different. Since the 2nd week, the tumor volume in group D was significant smaller than group C and E ($p < 0.05$). Ad-VEGF-siRNA could significantly inhibit the tumor volume ($p < 0.05$). Ad-VEGF-siRNA + neoadjuvant chemotherapy could most significantly inhibit the tumor volume ($p < 0.05$). However, the tumor volumes were not statistically different between Ad-VEGF-siRNA + anti-angiogenesis chemotherapy group and Ad-VEGF-siRNA group ($p > 0.05$).

VEGF Expression Detected by RT-PCR and Western Blot

As shown in Figure 3, the expressions of VEGF in tumor tissue in different groups were detected by RT-PCR. The expressions of VEGF in group A and group B were not changed since the 1st week, and there was no statistical difference between two groups ($p > 0.05$). However, the expressions of VEGF in the other three groups were significantly inhibited compared with group A and B ($p < 0.05$). The inhibition was obvious in group D, which was less significant than group C and E ($p < 0.05$). The expressions of VEGF at different time points in group C and E were not statistically different ($p > 0.05$). Ad-VEGF-siRNA could significantly inhibit the expression of VEGF in tumor tissue ($p < 0.05$). Ad-VEGF-siRNA + neoadjuvant chemotherapy could most significantly inhibit VEGF expression ($p < 0.05$). However, there was no difference between Ad-VEGF-siRNA + anti-angiogenesis chemotherapy group and Ad-VEGF-siRNA group ($p > 0.05$).

Furthermore, we used Western blot to detect the expressions of VEGF gene in tumor tissue in different groups. As shown in Figure 4, the change trend of VEGF protein was in accordance with mRNA.

Apoptotic Rates of Cells After Treatment Detected by MTT Assay

The apoptotic rates of cells after treatment detected by MTT in different groups were shown in Figures 2 to 4. The results showed that three chemotherapies could effectively inhibit tumor growth. The inhibition rates in group A, B and C were significantly higher than group D, the differences were statistically significant ($p < 0.05$); MTT result showed that the inhibition rate in group C was highest, the rate in group A was secondary to it and the rate was lower in group B, the inhibition rate in group C was significantly different from group A and B ($p < 0.05$).

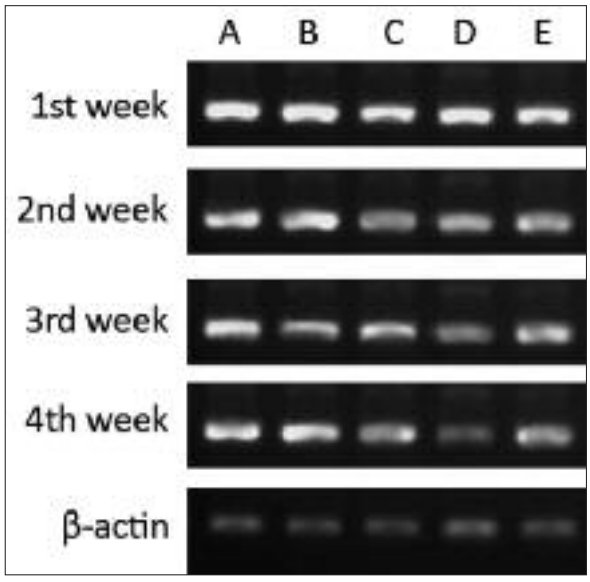

Figure 3. The change of VEGF expression in cells detected by PCR. ***A,*** The control group; ***B,*** The mock-vehicle control group; ***C,*** Ad-VEGF-siRNA group; ***D,*** Ad-VEGF-siRNA + neoadjuvant chemotherapy group; ***E,*** Ad-VEGF-siRNA + anti-angiogenesis group.

Discussion

Angiogenesis in tumor and its significant effect in occurrence and development of tumor have become the major breakthroughs in cancer research in the recent 30 years[21,22]. It has been proven that the rapid growth, infiltration and metastasis of tumor are the main features of tumor, which are also the main reasons that cause death. There are new vessels formed in tumor lesion of more than 3[23,24] mm^3. Effectively inhibit-

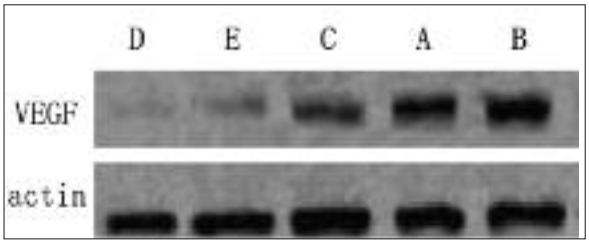

Figure 4. The expressions of VEGF in different groups in the 4th week.

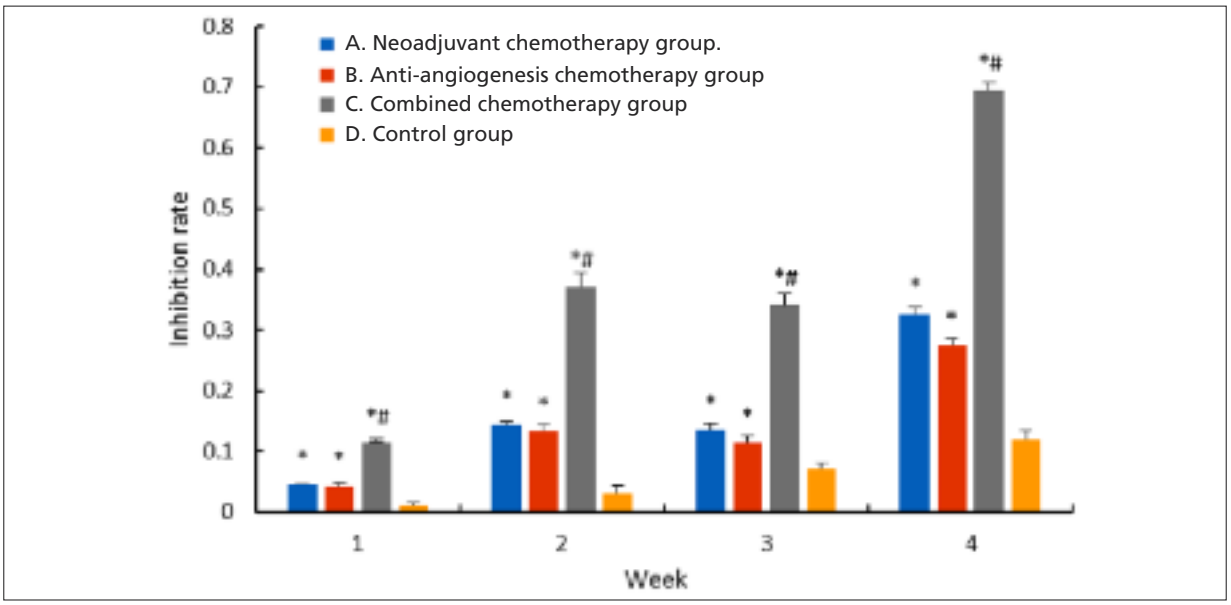

Figure 5. The cell apoptotic rate after treatment analyzed by MTT. *Means $p < 0.05$ compared with group D; #Means means $p < 0.05$ compared with group A and B.

ing angiogenesis and cutting off blood supply in tumor are expected to inhibit tumor growth, invasion and metastasis[25,26].

The new vessels in tumor can persistently supply nutrient and oxygen for tumor cells, and remove metabolites of tumor. Besides, the growth of tumor is dependent on the paracrine of capillary endothelial cells, and tumor cells can directly activate cell growth factor by endothelial cells. On the other hand, the VEGF secreted by tumor cells can activate the growth of endothelium[27,28]. Tumor vessels not only supply nutrient to tumor, but also export a large number of tumor cells to the body to cause the malignant growth and malignant metastasis of tumor[29].

In the recent years, it is found that among all tumor vascular growth factors, only VEGF can directly stimulate the division and proliferation of endothelial cells, which is a highly specific mitogen for vascular endothelial cells. It can directly induce the tumor angiogenesis. Other vascular growth factors are considered to act through VEGF[30-33]. VEGF is known as the most effective angiogenic protein, which is a diffusible specific mitogen and vascular growth factor for endothelial cells. At present, there are 6 members in VEGF family: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PLGF). The members have similar structure, function and distribution. And the confirmed VEGF receptors include VEGFR-l (flt-1), VEGFR-2 (KDR/flk-l), VEGFR-3 (flk-4), neuropilin-l (np-l) and neuropilin-2 (np-2).VEGFR-l and VEGFR-2 are tyrosine kinase receptors on cell surface which mainly locate at cell surface of tumor cells. Flt-1 and KDR have differential functions to induce the physiological function of VEGF[34,35].

In this study, we detected the tumor tissue of osteosarcoma bearing rats, the results showed that in the detection of tumor volume, the growth speed of tumor lesion in Ad-VEGF-siRNA group was significantly lower than the control group, the difference was significant. That is because the growth of tumor is closely related to the blood supply in tumor tissue, and Ad-VEGF-siRNA inhibits the growth of tumor by inhibiting neovascularization. MTT result showed that transfection of a certain amount of Ad-VEGF-siRNA could significantly inhibit the proliferation of UMR106 cells *in vitro*, and the inhibitory effect was gradually increased as the transfection dose increased and the reaction time prolonged.

VEGF can directly or indirectly act on the every link to promote angiogenesis. Being different from physiological or non-tumor angiogenesis effect of VEGF, in tumor tissue the secretion of VEGF, increase of vasopermeability, exosmose of plasma proteins and deposition of

fibrin gel are persistent. Thus, there is abundant angiogenesis in tumor tissue[36,37]. On the one hand, the abundant new vessels to transport oxygen, nutrient and metabolites can satisfy the need of indeterminate growth of tumor tissue; on the other hand, the increase of vasopermeability and incomplete of basement membrane of blood vessels also provide the condition for the invasion and metastasis of tumor[38]. Thus, VEGF can stimulate the invasion of tumor cells, maintain vessel dilution in tumor tissue and increase vasopermeability to induce the nutrient substances and oxygen to effuse into tumor cells, providing the sufficient material basis for the division, proliferation and infiltration of tumor cells.

Conclusions

The chemotherapeutical effect of Ad-VEGF-siRNA + neoadjuvant chemotherapy is more significant than simple biological therapy or Ad-VEGF-siRNA + anti-angiogenesis chemotherapy. This scheme may bring a novel thought for the future treatment and further understanding of human osteosarcoma.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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