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Downregulation of VEGF and KSP Gene Expression Inhibits Proliferation of A549 Cells

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Abstract: To investigate the proliferation and invasion of A549 cell by down-regulating KSP and VEGF gene. Designing and screening the siRNA of targeting VEGF and KSP promoter, transfected into A549 cells with transfetion reagent. According to the difference of transfection mixture the cells were divided into five groups. Western Blotting was used to detect the expression of VEGF and KSP. MTT assay was used to detect the cell proliferation, qPCR was used to measure the expression of VEGF and KSP, the MTT results indicated that the proliferation of the cells was significantly decreased; The results of qPCR showed that the siRNA obviously decreased the expression of VEGF and KSP gene;. The Western Blotting showed that the expression of VEGF and KSP were significantly reduced; The proliferation of the co-transfected cell were inhibited more than the single group transfected cell. These results indicate that co-regulation of VEGF and KSP gene expression more effectively inhibit A549 cell proliferation, better than only regulating VEGF or KSP gene expression.

Keywords: A549 cell, siRNA, Gene expression, *KSP*, *VEGF*

Intruduction

Non-small cell lung cancer is a common primary Lung cancer. Laboratory and clinical studies have demonstrated that cancer is a gradual process of long-term accumulation, involving multiple genetic and epigenetic variation, involving decrease of anti-oncogene gene expression and increase of oncogene expression[1]. VEGF is vascular endothelial growth factor gene, cancer cell growth and metastasis depend on angiogenesis, vascular endothelial growth factor is effective angiogenic growth factor[2]. VEGF and VEGFR has become targets for the treatment of cancer.Therefore, VEGF is expected to become a new molecular marker of cancer diagnosis and treatment[3]. KSP is a member of the kinesin family, and plays an important role in early mitosis, formation of the spindle, centrosome separation and chromosome division during mitosis[4]. In addition, KSP is also closely related to tumor development and progression, and highly expressed in many tumor cell lines[5]. It has become an important new target for cancer chemotherapy. Most research focused on siRNA or targeting a single gene, which is an effective way to control single-gene diseases[6]. There are various compensatory mechanisms and complex signal transduction pathway in cancer cell, therefore the effect becomes poor of siRNA controlling cancer[7]. We think that downregulate *KSP* and *VEGF* will have better effect in cancer therapy. In this study, we first designed and screened siRNA of cancer gene *VEGF and KSP*. Then, transfected siRNA into A549 cell respectively, detected its impact on proliferation, invasion, expression of the target gene. Finally,

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co-transfected to explore the application value cotroling proliferation and invasionon of A549 cell.

1 Materials and Methods

1.1 Cell cultures

A549 cells were acquired from the Biochemistry experiment center of Qingdao Medical College. The cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640), containing 10% fetal bovine serum, 100U/ml penicillin and 100 μg/ml strepto-mycin, and incubated at 37˚C in a humidified atmosphere with 5% CO_2. Cells in exponential growth phase were used throughout the experiments.

1.2 siRNA sequences design

The sequences of the RNAs oligonucleotides were designed as follows: *VEGF* siRNA, 5'-GGAGUACCCUGAUGAGAUCTT-3' (sense) and 5'-GAUCUCAUCAGGGUACUCCTT -3' (antisense); *KSP* siRNA, 5'-CUGAAGACCUGAAGACAAUTT-3' (sense) and 5'-AUUG UCUUCAGGUCUUCAGTT-3' (antisense); Negative control, 5'-UUCUCCGAACGUGUC ACGUTT-3' (sense) and 5'-ACGUGACACGUU CGGAGAATT-3' (antisense); The selected sequences did not share any homology with any of the known mRNA databases.

1.3 Transfection of RNAs oligonucleotides

The siRNAs were transfected into cultured cells at 65% confluence which had been seeded in 6-well plates 24h earlier. For transfection, Invitrogen™ Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.) was used as RNAs carrier. Five groups of cells were established as follows: CT (control group), NC (non-specific transfection group), VEGF groups, KSP groups, VEGF+KSP groups (co-transfection group). The cells in the CT group were treated with Opti-MEM (Thermo Fisher Scientific, Inc.), while the cells in the other groups were treated with Lipofectamine 2000-Opti-MEM. At 5-6 h post-transfect, the medium was replaced with 10% serum-supplemented RPMI-1640, and the cells were incubated for additional 24-96 h. Next, the cells were harvested by centrifugation, rinsed with phosphate buffered saline, and subjected to total RNA or protein extraction.

1.4 Cell proliferation assay

Cell viability was examined by MTT assay (Roche Diagnostics). For the experiment, A549 cells were seeded in a 96-well plate (Corning Life Sciences, New York, NY, USA) at a density of $5x10^3$ cells/well, and transfected with siRNA and then we need to change the medium six hours after transfection. At 24, 48 and 72 h post-transfection, MTT (5 mg/ml) was added to the cells, which were incubated at 37˚C for additional 4 h. Next, the supernatant was removed, and 150 μl dimethyl sulfoxide (Sigma-Aldrich) was added to each well, followed by 10-min agitation. The absorbance of each well at 490 nm was measured using an ELISA plate reader (Thermo Multiskan MK3; Thermo Fisher Scientific, Shanghai, China). Quintuplicate wells were used for each experimental condition, and all the experiments were repeated at least three times.

1.5 Total RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from the cells using Invitrogen™ TRIzol reagent (Thermo Fisher Scientific, Inc.), and cDNA was prepared with First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Next, the cDNA amplified by qPCR, using the following primers (Shanghai Sangon Biotech Co., Ltd., Shanghai, China): *VEGF*, forward 5´-TGCCCACTGAGGAGTCCAAC-3´and reverse 5´-TGGTTCCCGAAACGCTGAG-3´; KSP, forward 5´-CCCCGTAACAAGAGAGGAGTG -3´and reverse 5´-TCCTTTTTGCTGCCCCCTTT-3´; The reaction conditions were as follows: 94˚C for 30 sec, followed by 40 cycles of annealing at 60 or 72˚C (*KSP* or *VEGF* respectively) for 30 sec, and extension at 72˚C for 30 sec. qPCR amplification was performed on a Corbett Rotor-Gene 3000 (Qiagen, Inc., Valencia, CA, USA),using FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics).

Each RNA sample was analyzed in triplicate.

1.6 Western blot analysis

The western blot was used to detected the expression of VEGF, KSP and bcl2. Total protein content was extracted from the cells with RIPA lysis buffer (Beyotime Institute of Biotechnology, Nantong, China). The protein concentration in the cell lysates was determined with A280 (nm) ultraviolet absorption method. The samples were separated by SDS-PAGE using 10% polyacrylamide gels (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and then transferred to PVDF membranes. Membranes were blocked for 1 h with blocking buffer (5% BSA or 5% non-fat milk) at room temperature. Subsequently, primary antibodies were added to the membrane and incubated overnight at 4˚C. Antibodies were diluted 1:1,000. while the mouse monoclonal antibodies against GAPDH was diluted 1:2,000. Following 3 washes of 5 min each with TBST, the membrane were incubated with secondary antibody for 1 h at room temperature, following 3 washes of 5 min each with TBST, and then proteins were visualized using the enhanced chemiluminescence method, and protein expression was quantified with a Gel EDAS 293 analysis system.

1.7 Statistical analysis

All of the data were used statistical software SPSS17.0 do statistical analysis , and the results expressed in $\bar{x}\pm s$, using the single factor ANOVA method do multiple comparison and the LSD methodhe do comparison between two groups.

2 Results

2.1 Cell morphology was observed in all groups after transfection 48h

cells in NC and CT groups has a good shape, fusiform. The difference was not significant. KSP and VEGF groups cells increased apoptosis, cell rounding, and the two groups has no significant difference of apoptosis number. the number of apoptotic cells and round cells of KSP+VEGF groups was significant increased. Comparing VEGF+KSP group with KSP or VEGF group, the difference of was also significant .Figure 1.

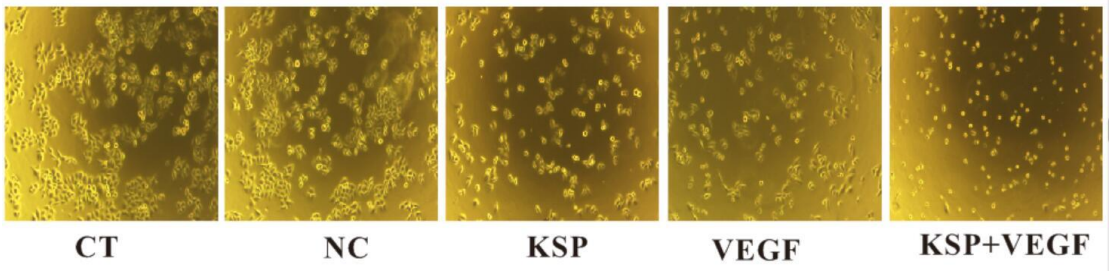

Figure 1. Morphology of each groups after transfection 48 h

2.2 siRNA effectively inhibited the proliferation of A549 cells.

The results showed in Table 2. When siRNA independent and co-transfected A549 cells for 24h, 48h, 72h, the optical density of KSP group or VEGF group was significantly lower than that of CT group ($p<0.05$), while the optical density of KSP+VEGF group was much lower. Comparing VEGF or KSP group with KSP+VEGF group, the difference of ODs was also significant($p<0.05$). However, there was no difference between NC group and CT group, on the other words, the difference was not significant ($p>0.05$).

Table 2 . cell proliferation rate (n = 5 , x±s)

GROUP	24 h	48 h	72 h
CT	1.131±0.044	1.343±0.031	1.448±0.023
NC	1.124±0.034	1.333±0.013	1.427±0.028
VEGF	0.834±0.008**	0.654±0.017**	0.635±0.012**
KSP	0.732±0.035**	0.623±0.012**	0.521±0.015**
VEGF+KSP	0.623±0.066*	0.565±0.032*	0.433±0.069*

2.3 the protein and miRNA expression levels after transfection

The results showed that the expressions of KSP, VEGF, bcl2 protein and mRNA in KSP, VEGF and KSP+VEGF groups were significantly lower than the CT and NC groups ($p<0.05$); The expressions of mRNA and protein in VEGF+KSP groups were significantly lower than KSP or VEGF groups ($p<0.05$). Table 3.

Table 3. The protein and miRNA expression levels of each groups

GROUP	VEGF		KSP		BCL2
	protein	mRNA	protein	mRNA	mRNA
CT	1.25±0.01	1.10±0.01	1.50±0.06	1.12±0.02	0.94±0.05
NC	1.32±0.01	0.97±0.03	1.46±0.05	0.85±0.10	0.91±0.05
VEGF	0.89±0.01	0.10±0.01	—	—	0.72±0.05
KSP	—	—	1.08±0.05	0.13±0.03	0.69±0.02
VEGF+KSP	0.51±0.01	0.06±0.01	0.83±0.04	0.07±0.01	0.50±0.01

3 Discussion

Nearly half a century, with the development of industrialization, lung cancer incidence and mortality has been on a clear upward trend[8], but there is no fully effective drug to treat lung cancer. More and more studies show that siRNA technology can effectively inhibit the proliferation of cancer cells[9]. Therefore, finding an effective siRNAs target site may be one of the most popular cancer treatment.

In recent years studies have shown that lung cancer development, metastasis and prognosis had closely related to angiogenesis[10]. Some studies suggest that immortalized tumor cell formation and angiogenesis are closely related. In recent years, we found that many of angiogenesis factors, including *VEGF* is a new discovery of the role of the current strong and specific vascular regulatory factors[11]. *VEGF*, also known as vascular permeability factor, vascular endothelial cell proliferation, vascular basement membrane hydrolysis and build stronger role, and high specificity, tumor angiogenesis is induced strong and specific role of regulator[12]. Transfection of targeting *VEGF* siRNAs, which can effectively lower mRNA and Protein levels. With the reduced *VEGF* expression, Bcl2 expression was significantly reduced, the degree of apoptosis was significantly increased.

KSP has conserved ATP enzyme domain and dynamic domains, it play an important role in formation of a bipolar spindle and separation processes in mitotic metaphase[13]. Studies have shown, the *KSP* siRNA can lead to the generation of a single star spindle promote mitotic arrest in M phase and induce apoptosis of tumor cells[14]. Studies

have shown that, KSP is highly expressed in human malignant proliferation of tissue cells, the expression level of cell proliferation and mitosis and tumor proportion of students Growth rate correlated. *KSP* protein involved in cell mitosis[15], the body's normal proliferation of cells, there are also the expression of *KSP*, but its expression level was significantly lower than the malignant tissue; the body's normal differentiation and maturation of cells and tissues, such as nerve cells were not detected in the *KSP* expression[16]. Thus, inhibition of *KSP* Can inhibit cell mitosis, the cells stop proliferating. In this study, the use of siRNA to inhibit *KSP* expression, apoptosis was significantly increased, suggesting that reduced expression of *KSP* can inhibit cell proliferation and induce apoptosis.

In most solid malignant tumors, including breast expression, neuroblastoma and lung cancer, *VEGF* and *KSP* were raised, related to the malignant behavior of tumor. Therefore, more and more research will regard *VEGF* and *KSP* as a target site for cancer treatment[17]. There is no simultaneous siRNAs targeting *VEGF* and *KSP in* non-small cell lung cancer cell proliferation, apoptosis, invasion of research reports. Therefore, this study show that synthesis siRNAs and transfection of A549 cells to inhibiting the expression of *VEGF* and *KSP* genes. Results of this study showed that double interference group apoptosis rate was significantly higher than the single interference group.Similarly,VEGF and KSP expression in a co-interference were significantly lower than KSP or VEGF groups.

In summary, siRNA tramsfection reduced expression of *VEGF* and *KSP* gene, the effect of inhibiting the growth of lung cancer cells was significantly higher than any of the individual down-regulated genes; *VEGF* and *KSP* can be used as both an important target for cancer therapy. Joint downregulation of *KSP* and *VEGF* can be used as a reference for breast cancer treatment. This research results provides an experimental basis to multiple gene silencing to inhibition of tumor cell proliferation.

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