DUAL SILENCING OF NET-1 AND VEGF GENES AFFECT BIOLOGICAL BEHAVIOR OF LUNG CANCER IN VITRO EXPERIMENT

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ABSTRACT

Aims: *Dual gene targeting siRNA (DGT siRNA) composed of Neuroepithelial transforming gene-1 (NET-1) and Vascular endothelial growth factor (VEGF) siRNA sequences was used to silence NET-1 and VEGF gene in lung cancer (LC) cell. And then the biological characteristics of LC cell were investigated.*

Materials and methods: *DGT siRNA, NET-1 and VEGF was transfected to lung adenocarcinoma cells lines A549. Quantitative real-time PCR (RT-qPCR), Western Blot immunofluorescence was used to detect the transcription and expression of NET-1 and VEGF gene. Cell proliferation was detected by MTT (methyl thiazolyl tetrazolium) assay. Apoptosis were evaluated using Annexin V-FITC (Annexin V-Fluorescein Isothiocyanate) assay and Hoechst staining. The migration invasion potential of A549 cells were tested by Wound healing and Transwell invasion experiment. Supernatant of A549 with or without treated by siRNA was applied to incubate the HUVECs (human umbilical vein endothelial cells). The tube formation ability of HUVECs was investigated.*

Results: *Single targeting siRNA and DGT siRNA could down-regulate the expressions of NET-1 and VEGF mRNA and protein expression. In DGT siRNA transfected group, the down-regulated gene expression of NET-1 and VEGF was more obvious. The apoptosis rate was increased and the ability of migration and invasion was decreased after transfection by single or DGT siRNA. The effects in DGT siRNA transfected group were more obvious, as well. Concentration of VEGF in the supernatant obtained from A549 cells transfected by single and dual targeting siRNA decreased and HUVECs tube formation ability was decreased compared with the control group.*

Conclusions: *Biological behavior and angiogenesis of lung cancer can be inhibited by NET-1 and VEGF silencing technology. Simultaneous silencing of NET-1 and VEGF using DGT siRNA achieved better inhibiting effects, which may provide an alternative therapeutics for LC.*

Key words: *RNAi, Dual gene targeting siRNA; NET-1, VEGF, Lung cancer.*

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Introduction

Lung cancer (LC) is the main cause of cancer deaths worldwide, the highest in men and the second in women[(1)]. Non-small cell lung cancer (NSCLC) is one of the most common human cancers with a poor prognosis and accounts for 80% of all diagnosed lung cancers[(2)]. Advances in molecular and cell biology have led to elucidation of the molecular mechanism underlying malignant transformation in LC. Because mutations and abnormal expression of various genes are involved in tumorigenesis, gene modulation is being explored as a very promising approach to correct those abnormalities.

The present study aims at suppressing two up-regulated genes simultaneously, NET-1 and VEGF, in LC.

NET-1 is a member of the NET-x family, and is a new member of the molecules of the Tetraspan Superfamily (TM4SF)[(3)], which is involved in a range of biological processes including cell proliferation, apoptosis, differentiation and cytoskeletal reorganization, through their regulation of Rho A activity[(4)]. Some studies provided evidences that up-regulation of NET-1 proteins is clearly associated with carcinogenesis[(5)]. And it was found to be over-expressed in some tumors, such as gastric cancer, cervical carcinoma, hepatocellular carcinoma, colorectal adenocarcinoma and ovarian carci-

noma[6-9]. In addition, over-expression of NET-1 protein may be associated with the biological behavior indicating a high degree of malignancy. Therefore, NET-1 may be a logical target for NSCLC therapy.

VEGF is one of the most potent regulators of angiogenesis. VEGF produced by tumor cells has various biological functions, perhaps promoting the proliferation and survival of tumor cells[10-13]. Since 1971, when Judah Folkman published his innovative hypothesis on tumoral vascularization, angiogenesis has been progressively considered responsible for the evolution of many cancer types. Besides, the angiogenic process is a paramount feature of NSCLC and the introduction of targeted therapies directed at key molecules of this process. Bevacizumab, a monoclonal antibody with an anti-angiogenic effects that specifically antagonizes and blocks VEGF, have shown great benefits in NSCLC patient treatment, especially when the malignancy is in advanced stages[14, 15]. Therefore, anti-angiogenic therapies based on inhibition of VEGF signaling were powerful clinical strategies in oncology[16]. VEGF is an attractive therapeutic target for cancer therapy.

In this study, we designed a dual gene targeting siRNA for NET-1 and VEGF, featured with a gap in either sense strand or antisense strand. We intended to evaluate the effect of dual silencing of NET-1 and VEGF genes on lung cancer cell proliferation, apoptosis, migration, invasion and anti-angiogenic activity, to explore the advantages of dual gene silencing, and thus to provide the basis for the application of dual gene silencing.

Matherials and methods

Cell culture

A549 and HUVECs were purchased from the Institute of Cell Biology, Chinese Academy of Sciences and were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (Gibco), 100 U/ml of penicillin and 100 μg/ml of streptomycin. These cells lines were maintained at 37°C in a humidified incubator with 5% CO_2.

siRNA design, construct and transfection of dsRNA

According to the recommendation of the manufacturer (an optimization principle of siRNA), a 19 nt sequence-specific siRNA targeting either NET-1 (NET-1 siRNA) or VEGF (VEGF siRNA), and a dual gene targeting siRNA (DGT siRNA, patent: Liang D., Sweedler D., Cui K., WO 2009/035539, March 19, 2009, Nantong Biomics Biotechnology Corporation) for NET-1 and VEGF were designed in vitro. All chemically synthesized oligonucleotides were obtained from Biomics Biotechnology Corporation and identified by PCR and DNA sequencing. Sequences of all the siRNAs are shown in Table 1.

Name	Sequences
NET-1 siRNA	5'-CCACAAUGGCUGAGCACUUdTdT-3'(sense)
	3'-TdTdGGUGUUACCGUCACGUGAA-5'(antisense))
VEGF siRNA	5'-GGAGUACCCUGAUGAGAUCdTdT-3'(sense)
	3'-TdTdCCUCAUGGGACUACUCUAG-5'(antisense)
DGT siRNA	5'-GGAGUACCCUGAUGAGAUCUGACCACAAUGGCUGAGCACUUdTdT-3'(sense)
	3'-TdTdCCUCAUGGGACUACUCUAG-5'
	3'-TdTdGGUGUUACCGUCACGUGAA-5'(antisense)

Table 1: Sequences of VEGF, NET-1 and DGT siRNAs.

In addition , suspension of 1×105 of cells was plated into each well of multi-well plates (Corning Costar Corp., Cambridge, MA), so that they could become about 70% confluence next day at the time of transfection. Transfection of dsRNA was performed with commercial reagent Lipofectamine 2000 (Invitrogen, USA) following manufacturer's instructions.

Real-time quantitative polymerase chain reaction

Total RNA was isolated from A549 cells after transfection for 24h using RISO reagent (Biomics), and then was submitted to a 25μl PCR reaction in the presence of 12.5μl of 2×Master Mix, 0.5μl of each primer mix (10μM), 0.5μl of 50×SYBR-Green I and 5μl RNA. The PCR mixtures were first subjected to 30 min at 42°Cfor reverse transcription and initially denatured for 10 min at 94°C and then to 40 cycles of amplification with the following cycling parameters: 20s at 95°C, 30s at 55°C and 30s at 72°C. The primer pairs for each gene were designed with Primer Premier 5.0 software. All samples were normalized to a glyceraldehyde-

3-phosphate dehydrogenase (GAPDH) control. Sequences of all the primers were seen in Table 2.

Name	Sequences
VEGF Primers	5'-GACATCTTCCAGGAGTACC-3' (Forward)
	5'-TGCTGTAGGAAGCTCATCTC-3' (Reverse)
NET-1 Primers	5'-GTGGCTTCACCAACTATACG-3' (Forward)
	5'-GACTGCATTAGTTCGGATGT-3' (Reverse)
GAPDH Primers	5'-GAAGGTGAAGGTCGGAGTC-3' (Forward)
	5'-GAAGATGGTGATGGGATTTC-3' (Reverse)

Table 2: Sequences of VEGF, NET-1 and GAPDH primers for RT-qPCR.

Western blot analysis

After siRNA transfection for 48h, A549 cells in 6-well plates were lysed in a RIPA buffer (Beyotime Institute of Biotechnology, China), which 10μl of the samples were separated using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide). Then the separated proteins diverted onto PVDF membranes (Pharrnacai, USA), which were washed with TBST (Tris Buffer Solution Tween) and then blocked for 2h with a TBST solution containing 5% skim milk. Blocked membranes were rinsed twice with TBST and incubated with rabbit-anti-NET-1 polyclonal antibody (1:200 dilution), rabbit-anti-VEGF monoclonal antibody (1:1000 dilution, Santa Cruz) at 4°C overnight. After the membranes were washed with TBST, membranes were incubated (30min, 37°C. 1h room temperature) with the secondary peroxidase-labeled goat anti-rabbit Ig whole antibody (1:6000 dilution, GE Healthcare Corp.). All the membranes were probed with a monoclonal mouse anti-β-actin antibody (1:500 dilution, Boster, China). Membranes were washed with TBST and then an ECL reagent was used to incubate the immune complex and signals were collected by Bio-Rad ChemiDoc XRS. Developed membranes were semi-quantitatively analyzed by scanning gray scale using an Image J densitometer (National Institutes of Health). Results were expressed as optical volume density corrected by β-actin for loading.

The size of target protein was measured by a comparison with protein molecular weight markers (Bio-Rad Laboratories Ltd.).

Immunofluorescence test

Cells were grown on glass covers lips. After siRNA transfection for 24h, the cells were fixed cells with 4% paraformaldehyde, penetrated with 0.5% TritionX-100%, Blocked with 1% BSA (Bovine Serum Albumin) in PBS (phosphate buffer saline). Sections were incubated with rabbit-anti-NET-1 polyclonal antibody (1:100 dilution), rabbit-anti-VEGF monoclonal antibody (1:200) at 4°C overnight. Staining patterns were visualized with Goat Anti-Rabbit IgG, TRITC-Conjugated (Beyotime Institute of Biotechnology, China) (1:200 dilution) for 1 hour at room temperature. The nuclei and cytoskeleton were counterstained with DAPI (4,6-diamino-2-phenyl indole) (Beyotime) and Phalloidin (Invitrogen). The cells were observed by fluorescent microscopy (400×).

Cells proliferation assay

Cell proliferation was detected by MTT assay. MTT (Sigma Aldrich) was dissolved in PBS at a concentration of 5mg/ml. A549 cells were seeded in 96-well plates at a density of 5×103 cells/well. Culture media was replaced with 90μl DMEM (Dulbecco's minimum essential medium) and 10μl of MTT solution (1:10 dilution), added to each well on the 1st to 5th days after transfection. Followed by 4h incubation at 37°C, cells were lysed with 150μl DMSO (dimethyl sulfoxide) for 10 min. The absorbance at 490 nm was determined in Microplate Reader (Bio-Rad 680). All samples were tested in triplicates and differences with the test groups were analyzed.

Annexin V-FITC apoptosis assay and Hoechst staining

The levels of apoptosis were evaluated using an annexin V-FITC apoptosis detection kit (Beyotime Institute of Biotechnology, China) as described by the manufacture's instruction. Briefly, after siRNA transfection for 48h, the cells were harvested, washed with PBS, resuspended in an annexin V binding buffer, incubated with annexin V-FITC/PI in dark for 10 min and analyzed by flow-cytometry using cell quest software (BD Biosciences, USA). The annexin V-positive cells indicated early and late apoptotic cells. In addition, after siRNA transfection for 24h, the cells were

treated with hoechst33285 for 30 min and evaluated by fluorescence microscopy. Apoptotic cells were defined as those containing nuclear fragmentation and condensed chromatin. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total counted cells (400×).

Wound healing assay

The migration potential of A549 cells was detected by wound healing assay. Briefly, the A549 cells were seeded in a 24-well plate with the same numbers in complete medium, respectively, and incubated until the cells grown to 80% confluence. After transfection, a sterile pipette tip was used to scratch wounds on each monolayer with the same width, then the plates were washed with PBS to remove the detached cells and the remaining cells were cultured in serum-free DMEM medium. Pictures were subsequently taken at 0, 24, and 48, 72 h. The closure of the wounds was enumerated by the distance of cells moved into the wounded area. The experiment was repeated twice with triplicate measurements in each experiment.

Transwell invasion assay

The invasion assay was conducted in a 24-well transwell cell culture apparatus fitted with multi-porous polycarbonate membrane insert (8-μm pore size. Corning). The upper side of the filter was coated with a 60μl of gelled solution of matrigel. Briefly, after siRNA transfection for 24h, cells were collected and resuspended in serum-free media at a density of 1×105cells/ml. The top chamber of transwell was loaded with 100μl of cell suspension, and the lower chamber was filled with 0.6 ml of media supplemented with 10% FBS as a chemoattractant. After incubation at 37°C with 5% CO2 for 24h, the apparatus were removed, rinsed two times with PBS, fixed with 4% paraformaldehyde, and stained with 0.5% crystal violet. Cells on the upper side of the filter were wiped off with cotton swabs. Cells on the lower side of the filter were determined by counting with a microscope (200×).

Endothelial cell tube formation assay

The ability of VEGF or NET-1 to induce endothelial cells to proliferate and organize into capillary-like sprouts was examined by HUVECs. Briefly, 1.5×105 cells/ml were collected and resuspended in culture media which was the supernatant of A549 transfected with DGT siRNA, VEGF siRNA, NET-1 siRNA or untreated one for 48h, and seeded in 24-well plates that were coated with a 200μl of gelled solution of matrigel. The usually used complete DMEM medium was taken as negative control. At daily intervals, the number of nodes in 10 random fields (40×) was observed after 12 h. The data were obtained from wells under each experimental condition at each time point.

Statistical analysis

All experiments were performed independently at least three times. Data were expressed as means ± SD (n=number of experiments). One-way ANOVA and t-test analyses were utilized to identify differences between groups. Statistical analysis was performed with SPSS software 19.0. $P < 0.05$ was considered to be significant.

Results

Effects of VEGF siRNA, NET-1 siRNA and DGT siRNA on VEGF and NET-1 expression in lung cancer cells

The levels of mRNA of NET-1 and VEGF genes in A549 cells were determined by RT-qPCR technique and the protein was determined by western blot (Fig. 1).

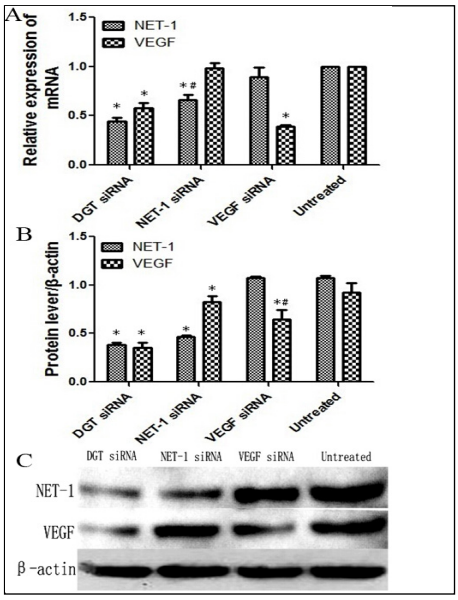

Fig. 1: Expressions of NET-1 and VEGF on mRNA and protein levels were detected. NET-1 mRNA and VEGF mRNA expressions in A549 cells were measured by RT-qPCR after siRNA transfection. (Fig. 1A). Expressions of NET-1 and VEGF in A549 cells were measured by western blot after siRNA transfection. Densitometric analysis of the two proteins was made relative to β-actin. (Fig. 1B, C). All data are expressed as the percentage of untreated control volume density. Each bar represents the mean ± SE. *p<0.05, compared with untreated group; #p<0.05, compared with DGT siRNA group.

We examined DGT siRNA on NET-1 and VEGF expressions respectively. The results showed that NET-1 mRNA in NET-1 siRNA group and VEGF mRNA in VEGF siRNA group were down-regulated by 48.1 ±3.9 % and 42.9±5.6% respectively compared with untreated group (Fig. 1A, $p < 0.05$). NET-1 protein in NET-1 siRNA group and VEGF protein in VEGF siRNA group levels were down-regulated by 48.3±5.0 % and 45.7±2.2% respectively compared with untreated group (Fig. 1B, 1C, $p < 0.05$). When compared to NET-1 siRNA or VEGF siRNA alone group, the DGT siRNA showed higher inhibition on both NET-1 mRNA expression and protein level, and only inhibition on VEGF protein but mRNA expression. All calculations are based on the mean value of PCR reactions performed in triplicate.

Immunofluorescence and Fluorescence Microscopy

Expression of NET-1 and VEGF protein were also examined by immunofluorescence. The fluorescence microscope showed that NET-1 and VEGF proteins were mainly distributed in the cytoplasm (Figure 2).

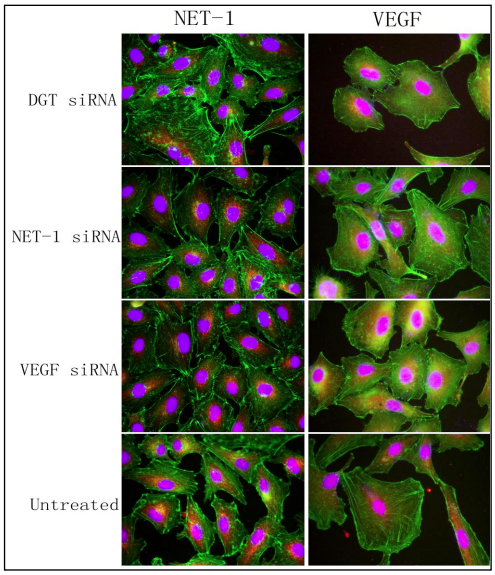

Fig. 2: Immunofluorescence analysis reveals that NET-1and VEGF protein is detectable in the cytoplasm of A549 cells in both all samples. Phalloidin for β-actin and DAPI were used to counterstain the cells which could show the cell outline much more clearly. The cells were observed by fluorescent microscopy (400×). NET-1 and VEGF were all distributed in the cytoplasm (green indicating the cytoskeleton, blue indicating the nucleus, red representing the target protein). The fluorescence intensity of both NET-1 siRNA group and VEGF siRNA group was significantly lower than untreated group but much higher than DGT siRNA group.

The fluorescence intensity of DGT siRNA group was significantly lower than other groups. The fluorescence intensity of both NET-1 siRNA group and VEGF siRNA group was significantly lower than untreated group but much higher than DGT siRNA group.

Effect of DGT siRNA, NET-1 siRNA and VEGF siRNA on A549 cell proliferation

We examined the effect of silencing of NET-1 and VEGF on cell proliferation of A549 cells (Fig. 3). The absorbance values of A549 cells with either VEGF siRNA or NET-1 siRNA were significantly lower than those of untreated cells at 48, 72 and 96h after transfection, respectively. Furthermore, the absorbance values of the A549 cells treated with DGT siRNA showed a significant decrease in cell proliferation compared with the cells treated with either NET-1 siRNA or VEGF siRNA at 48, 72 and 96h, respectively. There was no significant difference between the growth of cells treated with NET-1 siRNA and that of VEGF siRNA.

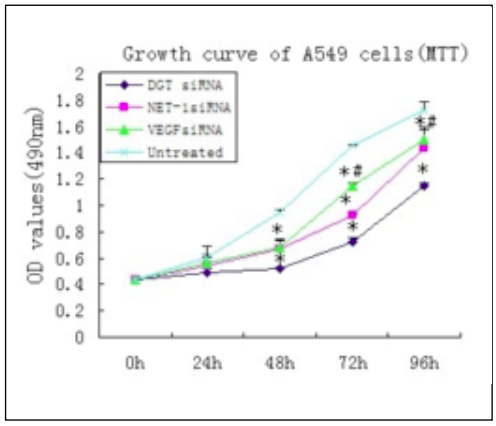

Fig. 3: Cell proliferation was detected by MTT assay on the 1st to 5th days after transfection. Growth curve of A549 cells was shown for each group. The proliferation was assayed in triplicates at 24, 48, 72 and 96 h post-transfection of siRNAs.

Annexin V-FITC apoptosis assay and flow cytometry

A549 cells grown were examined for apoptosis by Annexin V-FITC staining and flow cytometry analysis. As Fig.4 A and B illustrated, compared with untreated cells (2.37±0.47%), the apoptotic cells were greatly increased by DGT siRNA treatment (4.57±0.44%, $p<0.05$) and NET-1 siRNA treated (3.49 ±0.27%, $p<0.05$), but apoptotic cells are not increased by VEGF siRNA treatment (2.57 ± 0.38%, $p=0.597$). In addition, there were significant differences in apoptosis rates between DGT

siRNA treated cells and VEGF siRNA or NET-1 siRNA treated cells (both $p < 0.05$). We also performed Hoechst nuclear staining in order to determine whether apoptosis occurred. We found that cells treated with siRNA for 24 h resulted in decreased cell viability and increased cell apoptosis, especially in the DGT siRNA group. The results of Hoechst nuclear staining were showed on Fig. 4 C.

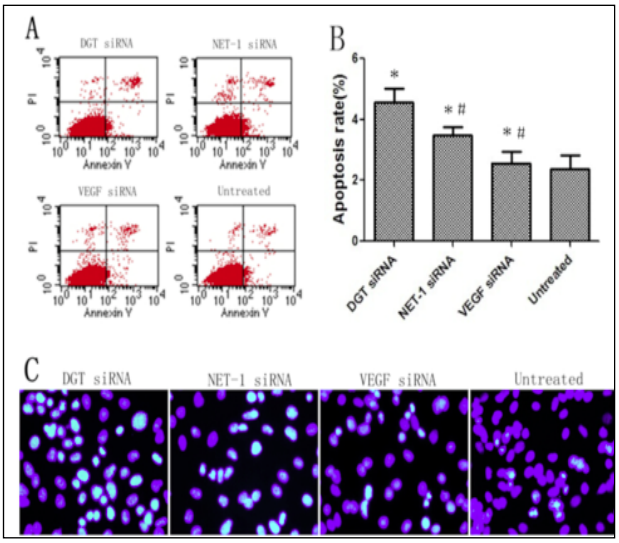

Fig. 4: (A). Cell apoptosis in A549 cells after 48h transfection was detected by flow cytometry assay after Annexin V-propidium iodide (PI) staining. (B). Percentage of apoptotic cells included both early- and late-stage apoptosis (AV + /PI - and AV + /PI +). Each bar represents the mean ± SE (n=3). (*p<0.05, compared with untreated group; #p<0.05, compared with DGT siRNA group). (C). The results of Hoechst nuclear staining (40×).

Wound healing assay

The association between NET-1 and VEGF protein and migration potential of A549 cells was explored by wound healing assay. The results demonstrated that the healing ability of A549 cells in DGT siRNA, NET-1 siRNA or VEGF siRNA group was significantly weaker than that of A549 cells in untreated group at 24 and 48 h after wound scratched (P<0.05, respectively). While no statistically significant differences of healing ability were detected among DGT siRNA, NET-1 siRNA or VEGF siRNA groups at 24 and 48 h after wound scratched (Fig. 5).

Transwell migration and invasion assay

The association between NET-1 and VEGF protein and invasion potential of A549 cells was explored by transwell invasion assay. The results demonstrated that the number of A549 cells penetrated to the lower side of the polycarbonate membrane in DGT siRNA (25.63±7.27), NET-1 siRNA (35.7±7.15) or VEGF siRNA (28±4.90) group was significantly smaller than that of A549 cells (104.88±23.68, p<0.001) in untreated group. Statistically significant difference of the number of penetrated cells was detected between DGT siRNA and NET-1 siRNA groups (p<0.05), but not happened between DGT siRNA and VEGF siRNA groups (p=0.386). (Fig. 6)

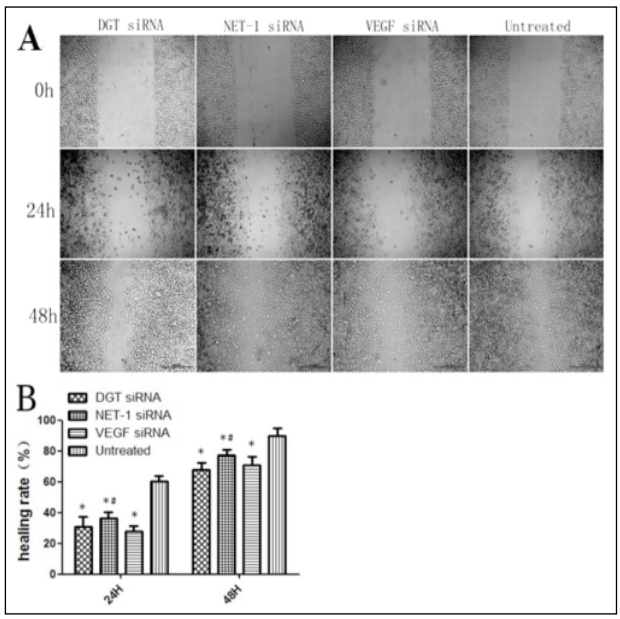

Fig. 5: The migration potential of A549 cells was detected by wound healing assay.
(A) The representative pictures of wound healing assay (40×).
(B) The quantitative analysis of the migration potential of A549 cells. Each bar represents the mean ± SE (n=3).

Endothelial cell tube formation assay

The concentration of VEGF protein was tested by ELISA. VEGF in supernate treated by DGT siRNA and VEGF siRNA was significant lower than that in NET-1 siRNA and untreated group, although the VEGF concentration in NET-1 siRNA was lower than that in untreated group. HUVECs was incubated in supernate treated by DGT siRNA, NET-1 siRNA, VEGF siRNA or untreated. The Tube formation of HUVECs was explored by tube formation assay. The results demonstrated that the number of tube nodes induced by the conditioned supernate of DGT siRNA and VEGF siRNA group was significantly lower than that in NET-1 siRNA and untreated group (P<0.01) in 6h, while the number of tube nodes induced by the conditioned

supernate of DGT siRNA, NET-1 siRNA and VEGF siRNA group was significantly lower than that in untreated group (P<0.01) in 12h and 24h. (Fig.7.)

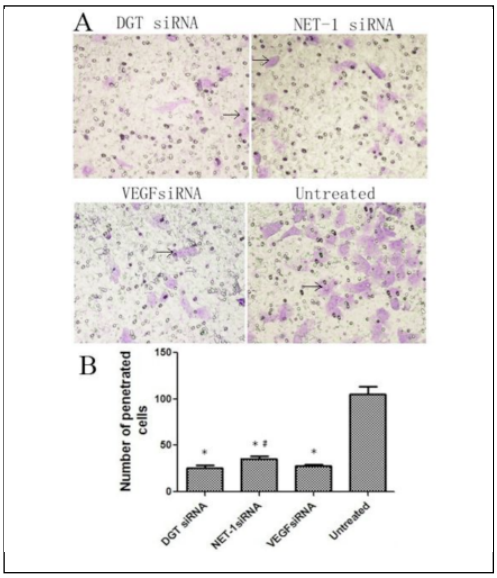

Fig. 6: The invasion potential of A549 cells was detected by transwell invasion assay. (A) Cells that penetrated to the lower side of the polycarbonate membrane were stained and photographs were taken under a microscope (200×, the cell was stained purple and showed by).

(B) The quantitative analysis of the invasion potential of A549 cells. Each bar represents the mean ± SE (n=3). *p<0.05, compared with untreated group; #p<0.05, compared with DGT siRNA group.

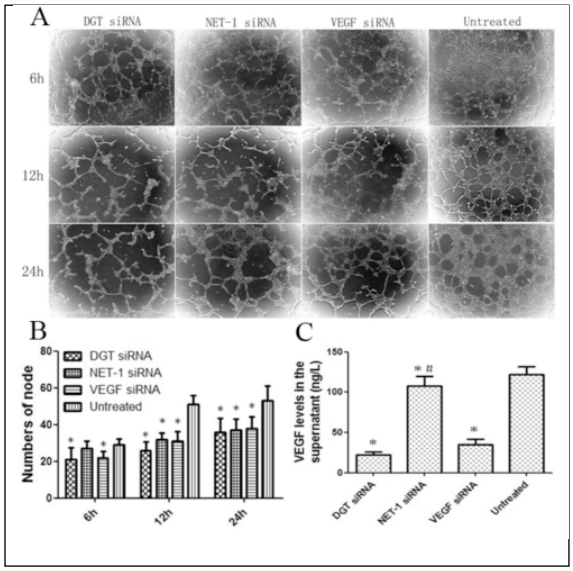

Fig. 7: Influence of NET-1 and VEGF on angiogenesis was detected by HUVEC tube formation assay. B. Each bar represents the mean ± SE (n=3). (*p<0.05, compared with untreated group; #p<0.05, compared with DGT siRNA and VEGF siRNA group).

Discussion

With the development of biotechnology, gene therapy has become a new strategy for treating cancer[17]. RNA interference (RNAi) is a post-transcriptional gene-silencing event in which short double-stranded RNA (dsRNA) degrades target mRNA[18]. The functional unit of the pathway is small interfering RNA[19]. Because of its potent and highly specific gene-silencing effect, RNAi is expected to be used in the treatment of various diseases. Cancer is one of the major targets of RNAi therapy, because silencing oncogenes or other genes contributing to tumor progression can be target genes for RNAi[20]. Due to incidence of tumor being a multi-step, multi-factor in biological processes, intervention of single gene has some limitations[21]. Therefore, use of multi-targeting siRNAs could be an advantageous strategy, by which expression of multiple genes can be inhibited simultaneously, and thus this may lead to more effective anticancer therapies than what single-targeting siRNA could achieve[22].

In this study, we used a DGT siRNA to target NET-1 and VEGF expression in NSCLC cells. The mRNA and protein levels of NET-1 in A549 cells were down regulated by 48.1 % and 32.7 % and those of VEGF were 57.9% and 38.6% compared to untreated cells, respectively. We demonstrated that DGT siRNA silencing NET-1 and VEGF effectively reduced the expression of NET-1 and VEGF simultaneously with high potency compared with the corresponding single-target siRNAs.

Cell homeostasis depends on the balance between proliferation and programmed cell death. Excessive cell accumulation during carcinogenesis can result not only from increased cellular proliferation, but also from diminished cell death[5]. The data revealed that inhibition of NET-1 or VEGF slowed cell proliferation and increased cell apoptosis, and these effects were more pronounced by co-inhibition of both genes. Inhibition of cell proliferation could result from apoptosis. Therefore, the effects of DGT siRNA inhibiting cancer cell growth could be attributed to the increased apoptosis. Additionally, the results revealed a fact that NET-1 siRNA presented similar VEGF siRNA effects proliferation and apoptosis on A549 cells.

As we know, invasion and migration are typical features of malignant tumors. Metastasis and invasion of the tumor cells to the neighboring organs is the major cause of treatment failure in

patients with NSCLC. Cancer metastasis is a major cause for cancer-related death and inhibiting cancer metastasis is an alternative way to treat cancer[23]. In the process of invasion and migration, it is necessary for tumor cells to possess the capacity of degrading the extracellular matrix and breaking through organizational barriers. The degradation of the extracellular matrix mainly depends on the roles of proteolytic enzymes. Burton and colleagues found that the VEGF-C/VEGFR-3 axis was one growth factor-receptor set that regulates tumor invasion and metastasis (24). Its molecular mechanisms are still not clear and under intensive investigations. In this study, cell migration and invasion were inhibited in three experimental groups. The migration and invasion power of A549 cells transfected with DGT siRNA, VEGF siRNA and NET-1 siRNA declined significantly than that of untreated cells. These results showed that knocking down VEGF could obtain similar inhibitive effect as dual silence, but silence NET-1 gene alone could obtain less effect than that of DGT siRNA or VEGF siRNA.

Angiogenesis is essential for tissue repair, inflammatory diseases, and tumor growth, metastasis and so on. The angiogenesis process includes the breakdown of the basement membrane, migration and proliferation of endothelial cells, formation of new vessels by the recruitment of pericytes[25]. Angiogenesis is essential for tumor growth and metastasis, as the tumor reaches 1-2mm in diameter, it becomes starved due to the limited supply of nutrients and oxygen[26]. VEGF binds to VEGF receptors on the surface of endothelial cells, inducing a variety of signal transductions that lead to the formation of new blood vessels.

In this study, HUVECs cultured in the medium of A549 cells treated with DGT siRNA, VEGF siRNA and NET-1 siRNA showed less power to form capillary-like spouts because the concentration of VEGF was significantly lower than untreated group.

Conclusions

Dual silence of NET-1 and VEGF gene can inhibit proliferation and angiogenesis, reduce invasion and migration, and promote apoptosis of LC cell, which can improve the curative effect especially for LC patients in the advanced stage. In the further work, nude mice models that bore human lung cancer xenografts will be built, and the effects of NET-1 and VEGF gene DGT siRNA on biological characteristics of LC will be investigated.

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