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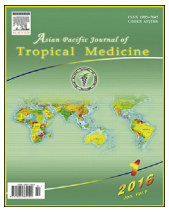

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Mechanism of Wnt/β-catenin signaling pathway in enhanced malignant phenotype of non-small cell lung cancer induced by anti-angiogenesis therapy

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ABSTRACT

Objective: To study the mechanism of Wnt/β-catenin signaling pathway in the enhanced malignant phenotype of A549 cells of human non-small cell lung cancer induced by the anti-angiogenesis therapy.

Methods: The siRNA technique was employed to inhibit the expression of vascular endothelial growth factor (VEGF) in A549 cells and simulate the clinical course of anti-angiogenesis therapy. Real-time PCR and western blot were used to study the change in the expression of Wnt/β-catenin signaling molecules at the mRNA and protein level respectively, as well as the effect on the epithelial mesenchymal transition in A549 cells. The proliferation and invasion abilities of tumor cells were detected to discuss the mechanism of Wnt/β-catenin signaling pathway in the enhanced malignant phenotype of non-small cell lung cancer induced by the anti-angiogenesis therapy.

Results: The specific siRNA could significantly inhibit the expression of VEGF in cells to simulate the anti-angiogenesis therapy. Under the action of 50 nM VEGF siRNA, the proliferation ability of A549 significantly increased ($P < 0.05$). After being treated with VEGF siRNA, the invasion ability of cells increased. Twenty-four hours after the transcription of 50 nM siRNA into cells, the number of cells that come through the membrane was 278.3 ± 12.9. Compared with the Ctrl siRNA group, when VEGF was inhibited, the expression of *β-catenin* and *Cyclin D1* increased by 86% and 55% respectively. Meanwhile, the expression of E-cadherin decreased, while the one of vimentin increased.

Conclusions: siRNA can significantly inhibit the expression of VEGF. For the anti-angiogenesis therapy, the inhibited expression of VEGF can activate the Wnt/β-catenin signaling pathway to cause the epithelial mesenchymal transition and then the enhanced malignant phenotype of non-small cell lung cancer.

1. Introduction

The metabolism of tumor cells is more active than that of the normal cells, especially the malignant cancer. The new blood vessels can provide more oxygen and nutrients for the tumor cells to offer the pathway for the hematogenous metastasis of tumor cells. Meanwhile, the endothelial cells of new blood vessels can express and secrete many growth factors, such as the insulin-like growth factor and platelet-derived growth factor, which can stimulate the growth of adjacent tumor cells [1–4]. Therefore, the angiogenesis plays a key role in the infiltration and metabasis of tumor growth. The angiogenesis that can inhibit the tumor would lead to the insufficient nutrients for the growth of tumor and thus cause the inhibition against the growth of tumor cells [5]. In recent years, the research on the anti-angiogenesis therapy of tumor has been a hot topic in the field of tumor biotherapy and a great number of chemical small molecule drugs and monoclonal antibody agents for the anti-angiogenesis have been sold on the market [6–8]. These drugs mainly affect the angiogenesis in the tumor for the therapy. However, the previous researches reported that these drugs might reduce the efficiency of some other anti-cancer drugs and enhance the invasion of tumors. For instance, it's found that the angiogenesis inhibitor could reduce the delivery of cytotoxic drugs to the tumor location and thus affect the therapeutic effect of drugs [9,10]. With

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the in-depth research and development of anti-angiogenesis drugs, more and more research data indicated that these treatments had the certain limitations [11,12].

In this study, by using siRNA to inhibit the expression of vascular endothelial growth factor (VEGF) in A549 cells, it was to study the effect of change in the expression of VEGF on Wnt/β-catenin signaling pathway. Besides, by analyzing the correlation between the change in the proliferation and invasion of A549 cells and the expression of VEGF, it was to discuss the mechanism of Wnt/β-catenin signaling pathway in the enhanced malignant phenotype of non-small cell lung cancer induced by anti-angiogenesis therapy.

2. Materials and methods

2.1. Main materials and reagents

The human non-small cell lung cancer A549 cells were purchased from Committee on Type Culture Collection of Chinese Academy of Sciences and the cells conserved in the liquid nitrogen in this laboratory.

VEGF siRNA was purchased from Santa Cruz Biotechnology (America, item No. sc-29520); the total RNA extraction kit from TIANGEN Biotech (China, item No. DP430); miRNA isolation kit from mirVana (America, item No. AM1561); the reverse transcription kit from Applied Biosystems (America, item No. 4366597); Real-time PCR fluorescent quantitative kit from Bio-Rad (America, item No. 172–5264); ReadyPrep protein extraction kit from Bio-Rad (America); BCA protein quantitative kit from Vazyme Biotech (China, item No. E112-01); Gsk3β, p-Gsk3β and VEGF monoclonal antibodies from Santa Cruz Biotechnology (America, item No. 377213, 81496 and 390741); Transwell chamber from Corning (America, item No. 4395); Matrigel from BD (America); horseradish peroxidase-labeled secondary antibody from Beijing Zhongshan Jinqiao Biotechnology; ECL Chemiluminescent Substrate Reagent Kit from Life Technologies (America, item No. WP20005).

CO_2 cell culture incubator (Thermo Scientific Series 8000), DNA/RNA analyzer (Qubit Fluorometer); the cell nuclear transfer system (Amaxa Nucleofector) and the fluorescent quantitative PCR system (Bio-Rad-CFX96 Touch) were also used in this study.

2.2. Methods

2.2.1. Cell culture and transfection

A594 cells were conserved in the liquid nitrogen. DMEM culture medium was used after the recovery of cells; MRC-5 was cultured in EMEM culture medium that contained 15% fetal bovine serum (GIBIC) at 37 °C and 5% CO_2.

The cell nuclear transfer system of Amaxa Nucleofector was used for transfection of siRNA. By regulating and optimizing the electroporation parameters, the exogenous siRNA was then transfected into the cell nucleus directly.

To verify the inhibition efficiency of siRNA and confirm its concentration range, A549 cells were seeded on 6-well plate. When cells grown up by about 70%, the cell nuclear transfer system of Amaxa Nucleofector was employed to transfect VEGF siRNA and Control siRNA with the different concentrations. Real-time PCR and western blot were adopted to detect the inhibition efficiency of siRNA.

2.2.2. Real-time PCR

The collected cells were washed with PBS (RNase free). miRNA isolation kit and total RNA extraction kit were used to extract miRNAs and total RNA. Qubit Fluorometer system was used to detect the concentration and purity of RNA. The total RNA was reversely transcripted to cDNA following the instruction manual of reverse transcription kit (it was guaranteed that only the mature miRNAs were transcripted, but no precursor reaction on miRNA. The transcription product cDNA was used as the template). The Real-time PCR was employed to detect the expression of related genes. The mRNA sequence of *E-cadherin*, *Vimentin*, *β-catenin* and *Cyclin D1* genes could be referred to NCBI database and then the Real-time PCR primers could be designed.All primers were synthesized by Shanghai Generay Biotech Co, Ltd.

2.2.3. Cell invasion assay

To study the effect of inhibited expression of VEGF on the growth of A549 cells, A549 cells were transfected with 0, 5, 10 and 50 nM VEGF siRNA respectively, while the control was Ctrl siRNA with the random sequence. MTT method was employed to detect the effect of inhibited expression of VEGF on the proliferation of A549 cells. Matrigel was taken out and then melted at 4 °C over night. The pre-cooled serum-free DMEM medium was used to dilute Matrigel to the final concentration of 1 mg/mL 100 μL digested Matrigel was added in the center of bottom of upper chamber of Transwell and then it was incubated at 37 °C to be gelatinous. 200 μL DMEM medium was added in each well for the reconstruction.

After being trypsinized and centrifuged, the culture medium was removed and cells were washed with sterile PBS. The serum-free culture medium was used to resuspend the cells. A549 cells was added in the upper chamber of Transwell, while the lower chamber contained DEME medium with 10% FBS for the further 24 h of culture. The cells were cultured at 37 °C. After the culture, the liquid in the upper chamber was removed. Then the upper chamber was taken out and the cells that were not transferred were wiped by the cotton swab. 4% Paraformaldehyde was used for the fixation at room temperature for 10 min and it was then stained with the crystal violet. The number of membrane-penetrating cells in 3 fields was counted as mean ± SD under the inverted optical microscope and the statistical analysis was performed.

2.2.4. Western blotting

The collected cells were washed with PBS twice. After being trypsinized and centrifuged, the supernatant was removed. ReadyPrep protein extraction kit was used to extract the total protein (After being put on the ice for 30 min, the probe-type ultrasound was used to produce the short impact with the appropriate frequency on the ice. The lysis mixture was centrifuged at 4 °C and 13 000 r/min for 20 min). The supernatant was transferred to the new centrifuge tube. BCA kit was employed to detect the protein concentration.

SDS-PAGE electrophoresis was performed on 20 μg protein samples. The gel was soaked in the transfer buffer for 10 min of equilibrium. It was installed with the transfer 'sandwich' with 100 V and 45–60 min. After the transfer, PVDF film was washed with TBS for 10–15 min. The film was placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shaken at the room temperature for 1 h. Then the primary antibody with the appropriate degree of dilution was added [diluted with TBST containing 1% (w/v) skimmed milk powder]. It was incubated at the room temperature for 2 h and then the film was washed with TBST for 3 times, 5–10 min every time. The film was incubated with the secondary antibody (1:10 000, horseradish peroxidase-labeled) that was diluted with