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Mechanism of miR-21 via Wnt/β-catenin signaling pathway in human A549 lung cancer cells and Lewis lung carcinoma in mice

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ABSTRACT

Objective: To study the mechanism of effect of miR-21 via Wnt/β-catenin signaling pathway in human A549 lung cancer cells and Lewis lung carcinoma in mice.

Methods: The effect of miR-21 on A549 cells were detected by MTT method. MiR-21 expression levels were overexpressed or inhibited in A549 cells by transfecting with miR-21 mimics or inhibitors. Correlation among key molecules (Wnt1, β-catenin, CyclinD1 and miR-21) of mRNA and protein levels in Wnt/β-catenin signaling pathway were studied by Real-time PCR and Western blot hybridization assay. Invasive ability of A549 cells was determined via Transwell chamber cell invasion assay; the role of miR-21 in A549 cells was explored via the Wnt/β-catenin signaling pathway. A Lewis lung carcinoma animal model was established to detect miR-21 expressions in tumor animals and controlled animal tissues, and verify expression changes of the above molecules in the Wnt/β-catenin signaling pathway was determined in the animal level.

Results: MTT assay results showed that miR-21 overexpression could markedly enhance cell absorbance value; that is, miR-21 could increase the ability proliferation of A549 cells. β-catenin and CyclinD1 expression levels were significantly higher in miR-21 mimic transfected cells ($P < 0.05$), and *Wnt1* gene had no significant change. *Wnt1*, *β-catenin* and *CyclinD1* gene expression showed no significant change when miR-21 expression was suppressed, compared with controls. After cells were transfected with miR-21 mimics, cell invasion assay revealed that the perforated cells was significantly higher than the perforated cells in the control group ($P < 0.01$). Lewis lung assay revealed that miR-21 expression levels in the Lewis lung carcinoma were significantly higher; and at the same time, *Wnt1*, *β-catenin* and *CyclinD1* gene expression levels were significantly increased, compared to controls.

Conclusions: In A549 human lung cancer cells and Lewis lung carcinoma in mice, key molecules β-catenin and CyclinD1of miR-21 expressions and the Wnt/β-catenin signaling pathway are positively correlated.

1. Introduction

Lung cancer is disease with the highest morbidity in malignant tumors worldwide; and is one of the cancers that has the greatest threat to human health and life [1,2]. Although studies on the pathogenesis of lung cancer have made great progress, at present, there is still a lack of effective treatment and early diagnosis due to the complex biological processes of lung cancer's occurrence and development. In a research performed by Nusse *et al*. in 1982, *Wnt* genes were found in the course of papillomavirus-induce breast cancer in mice [3]. Wnt signaling pathway in multicellular eukaryotes is highly conserved, and is closely associated with tumor development. The β-catenin-mediated pathway is a classical signaling pathway in the Wnt signaling pathway. In normal somatic cells, β-catenin is only used as a cytoskeletal protein in the membrane area and forms a complex with E-cadherin to maintain the same type of cell adhesion, and plays a role in

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preventing cell movement. Extracellular Wnt signaling molecules combined with specific receptors in frizzled membrane proteins, phosphorylation-mediated inhibition of cytoplasmic β-catenin is degraded, and through inhibiting glycogen synthase kinase-3β (GSK3β); allowing β-catenin to accumulate in the cytoplasm. Accumulated β-catenin transferred in the nucleus, combined with the transcription factor Tcf/Lef in family, could activate *cyclinD1*, *cmyc* and other proto-oncogenes; causing cancer to occur. Therefore, activation of the Wnt/β-catenin signaling pathway has an important role in cancer occurrence and development [4–8].

miRNAs are a class of small RNA molecules, which are approximately 17–25 nucleotides in length; and play a very important role in the gene regulation process [9,10]. Even though the number of miRNAs in the human genome is less than protein-coding genes, miRNAs are considered to regulate more than half of human mRNAs [11,12]. miRNAs pair with its miRNA-target mRNA base, degrade mRNAs or hinder their translation, which have regulatory roles in cells. Each miRNA can have multiple target genes and several miRNAs can also jointly regulate with a gene. This is also a reason why miRNAs have very complex biological effects. Recent studies have shown that miRNAs play an important regulatory role in tumorigenesis and development [13–15]. Recent studies have shown the prognostic value of miR-21 abnormal expressions in lung cancer could be an effective prognostic indicator in patients with lung cancer. Therefore, in the overexpression/inhibition of miR-21 expression in human non-small cell lung cancer cell line, A549, at the mRNA level and protein levels, this study investigates the effect of miR-21 via the Wnt/β-catenin signaling pathway on A549 cells. A Lewis lung carcinoma animal model was established to detect the expression of miR-21 in tumor animals and animal tissue controls, and verify expression changes of the above molecules in the Wnt/β-catenin signaling pathway in the animal level.

2. Materials and methods

2.1. Materials

Human non-small cell lung cancer cell line, A549, was purchased from ATCC, and stored in liquid nitrogen at our laboratory. LLC lung cancer cells in mice were purchased from the Chinese Academy of Sciences committee Type Culture Collection cell bank, and stored in liquid nitrogen at our laboratory. C57BL/6 male mice, weighing (20 ± 5) g, were purchased from Shanghai SLAC laboratory Animal Co., Ltd.

RNA extraction kit was purchased from TIANGEN -DP430; reverse transcription kit (TaqMan MicroRNA Reverse Transcription Kit) was purchased from Applied Biosystems-4366597; Real-time PCR fluorescence quantification kit (SsoAdvanced SYBR Green Supermix) was purchased from Bio-Rad-172-5264; reverse transcription kit (*Taq*Man MicroRNA Reverse Transcription Kit) was purchased from Applied Biosystems- 4366597; hsa-mir-21 mimic was purchased from Life Technologies; hsa-mir-21inhibitor was purchased from Life Technologies; Lipofectamine 3000 Transfection Reagent was purchased from invitrogen-L3000-00; Transwell was purchased from Corning-4395; Matrigel Matrigel (5 mg/mL) was purchased from BD; horseradish peroxidase (HRP) labeled secondary antibody was purchased from Beijing ZSGB biotechnology Co., Ltd; ECL Chemiluminescent Substrate Reagent Kit was purchased from Life Technologies-WP20005; PVDF membrane (Polyvinylidene fluoride) was purchased from Millpore; Wnt1, β-catenin, CyclinD1 monoclonal antibodies was purchased from CST; MTT cell proliferation and cytotoxicity detection kit was purchased from Beyotime Biotechnology.

Optical Microscope, Olympus BX53; CO_2 incubator, Thermo Scientific Series 8000; nucleic acid quantification analyzer, Qubit Fluorometer; fluorescence quantitative PCR detection system, CFX96 Touch.

2.2. Methods

2.2.1. Establishing cell culture and animal model

A549 and LLC cells were preserved in liquid nitrogen. After reviving with DMEM medium (GIBIC), 15% fetal bovine serum (Sino-US joint venture, Lanzhou MinHai Bio-Engineering Co., Ltd.) was added, and cultured in 5% CO_2 at 37 °C.

C57BL/6 mice were housed in standard animal feeding cages (five per cage). During the experiment, mice were free to eat and drink. The room was kept ventilated with natural day and night lighting, and temperature was maintained at 18–25 °C. The logarithmic growth phase of LLC cells were obtained and washed with sterile PBS to remove residual serum and media; then, cell suspension was prepared and cell concentration was adjusted to 10^7 cells/mL. All 40 male C57BL/6 mice were randomly divided into two groups: lung cancer model group and control group. Mice were anesthetized with pentobarbital sodium by 0.5 mg/10 g body weight dosages. A 5-mm incision was made on the left anterior costal arch on the front line with an approximately 2 cm cut, 100 μL cell suspension was injected in the left lung. After orthotopic implantation, the control group was injected with the same volume of PBS, and treated with antibiotics after operation. Tumors were observed and measured. Mice were sacrificed 14 days after reunification, and tumor tissues were isolated and preserved at −80 °C.

2.2.2. Real-time PCR

After cells were washed with PBS, total RNA was extracted by RNA extraction kit, RNA concentration and purity was detected by Qubit Fluorometer, total RNA was reverse transcribed to cDNA by reverse transcription kit based on manufacturer's instructions, and relevant genes were detected by Real-time PCR. *Wnt1*, *β-catenin* and *CyclinD1* gene mRNA sequence were queried from the NCBI database. Real-time PCR primers were designed. All primers were synthesized by Shanghai Generay Biotech Co., Ltd., and specific sequences are shown in Table 1. Calculation of relative expression levels of target genes by double ΔCt value method: mean value of experiments that were repeated in triplicate and used as Ct values of each sample, ΔCt = Ct (Target gene) -Ct (internal control), ΔΔCt = ΔCt (sample) - ΔCt (contro1); therefore, relative expression levels of the target gene = $2^{-\Delta\Delta Ct}$, and the relative expression of the control group would be $2^0 = 1$ (Table 2).