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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).

Table 1
Real-time PCR primers.

Gene	Accession no.	Primer (5′-3′)
Wnt1	NM_005430.3	For:CGATGGTGGGGTATTGTGAAC Rev:CCGGATTTTGGCGTATCAGAC
β-catenin	NM_001012329.1	For:CCTATGCAGGGGTGGTCAAC Rev:CGACCTGGAAAACGCCATCA
CyclinD1	NM_053056.2	For:GCTGCGAAGTGGAAACCATC
GAPDH	NM_002046	Rev:CCTCCTTCTGCACACATTTGAA For:GAAGGTGAAGGTCGGAGTC Rev:GAAGATGGTGATGGGATTTC

For, forward; Rev, reverse.

Table 2
PCR reaction system.

Components	Volume per reaction
SsoAdvanced SYBR Green Super mix[a]	5 μL
Forward primer (10 μM)	0.32–0.45 μL (320–450 nM)[b]
Reverse primer (10 μM)	0.32–0.45 μL (320–450 nM)
cDNA template	75–100 ng
Nuclease-free water	Up to 10 μL

[a] SsoAdvanced SYBR Green Super mix, purchased from Bio-Rad.
[b] Primer concentrations were determined according to different gene amplification conditions.

RNA from tumor tissue was extracted according to *Taq*Man miRNA Reverse Transcription Kit manufacturer's instructions. Specific miRNA primers were used for reverse transcription, ensuring only mature miRNA reverse transcription and not miRNA precursor reaction. The transcription product cDNA was used as a template, and miR-21 expression was detected by Real-time PCR.

2.2.3. Cell invasion related experiments

Matrigel was melted at 4 °C overnight, diluted in serum-free cold DMEM medium, and added to a final concentration of 1 mg/mL. After dilution, 100 μL of Matrigel was added to the upper chamber of the Transwell, and incubated at 37 °C until it become colloidal materials. Adhesive reconstruction was carried out by adding 200 μL of DMEM medium into each well.

After cells were trypsinized, cells were centrifuged to remove the culture medium. PBS cells were washed, and cells were resuspended using serum-free medium. A549 cells were then seeded onto 24-well Transwell chambers. A medium containing 10% FBS was added at the lower chambers, and cells were cultured at 37 °C. After incubation, liquid in the upper chamber was discarded; and those in the upper chamber that did not pass through the cell membrane were removed and wiped off with a cotton swab. Then, 4% formalin-fixed for 10 min, stained with 0.1% crystal violet, and cells were observed under an inverted microscope. Three horizons were randomly selected, and perforated cells were calculated for statistical analysis.

2.2.4. Western-blotting

Cells that were collected and washed twice with PBS. After trypsin digestion, the centrifuged supernatant was discarded. Cell precipitations were lysed with RIPA lysis buffer, and protease inhibitor cocktail was simultaneously added, which was mixed by pipetting. After incubating on ice for 30 min, cells underwent sonication. An appropriate short frequency shock was applied on ice using ultrasound probes. Lysis mixture at 4 °C was centrifuged at 13 000 r/min for 20 min. Protein concentrations of the supernatant in the new centrifuge tube were determined using a Protein Assay Kit.

SDS-PAGE electrophoresis was applied on 20 μg total protein samples. After electrophoresis, the gel was immersed in a transfer buffer in equilibrium for 10 min, transferred into "sandwich", and applied 100 V for 45–60 min. After transfer membrane, PVDF membrane was rinsed with TBS for 10–15 min. The membrane was placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder, and shaken for 1 h at room temperature. An appropriate dilution of primary antibody was added [containing 1% (w/v) skim milk diluted in TBST], incubated at room temperature for 2 h, and TBST rinsed the membrane three times every 5–10 min. The film was incubated in a TBST diluted secondary antibody containing 0.05% (w/v) nonfat dry milk in (1:10 000, HRP labeled) at room temperature for 1 h. The membrane was rinsed in TBST three times every 5–10 min. Exposed, photographed, and the results were saved. The experiment was repeated three times. Quantity one v4.62 software was applied for molecular bands gray scale values (bands track quantitative method). Optical density values are plotted curve depending on the optical density of electrophoretic bands. Then, the area under the optical density curve was calculated according to quantitative electrophoretic bands for statistical analysis.

2.2.5. Statistical analysis

SPSS 11.5 statistical software was used on experimental data, and results were expressed as mean ± SD. *t*-test was used to compare the two groups and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Overexpression or inhibition of miR-21 in A549 cells and MTT assay detection of the effect of miR-21 on A549 cell proliferation

miRNAs mimics can simulate endogenous miRNA; thus, increasing the amount of intracellular expression of miRNAs. After A549 cells were transfected with miR-21 mimic for 24 h (control group is control mimic), miR-21 expression levels were detected by Real-time PCR, as shown in Figure 1. Compared to control cells, miR-21 expression levels were significantly increased ($P < 0.01$). In addition, miR-21 inhibitors could

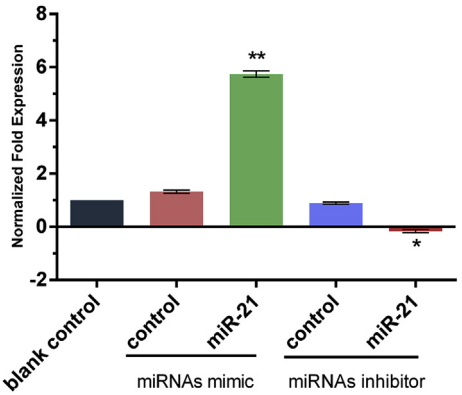

Figure 1. Overexpression and inhibition effect of miR-21.
*$P < 0.05$, **$P < 0.01$.

inhibit miR-21 expression levels ($P < 0.05$). The effect of miR-21 on A549 cell proliferation was detected by MTT assay, as shown in Figure 2. In the cell growth curve, time-periods are shown in the horizontal axis, while A570 absorbance values are shown in the vertical axis. As seen from the results, A549 absorbance and control cells slightly changed within 0–12 h of transfection. Within 12–48 h, miR-21 overexpression could significantly enhance the absorbance value of cells. After miR-21 inhibition, A570 value showed a downward trend, compared with the control group.

3.2. Key molecules in the Wnt/β-catenin signaling pathway: correlation among Wnt1, β-catenin, CyclinD1 and miR-21

After A549 cells were transfected with miR-21 mimics for 24 h, *Wnt1*, *β-catenin* and *CyclinD1* gene expressions were detected by Real-time PCR. Figure 3 shows that *β-catenin* and *CyclinD1* expression levels significantly increased in cells transfected with miR-21 mimics, compare with the control group

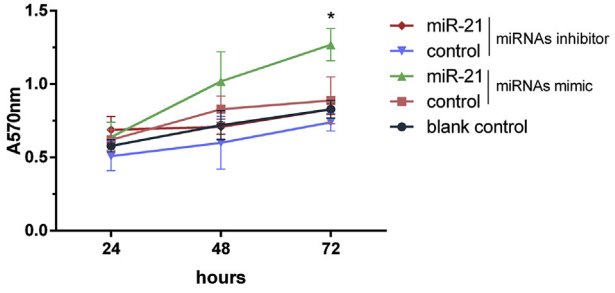

Figure 2. Proliferation of A549 cells detected by MTT assay.
*$P < 0.05$.

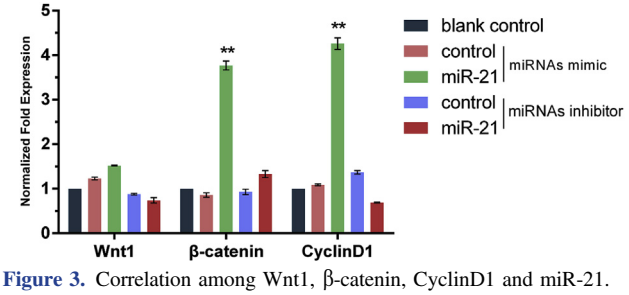

Figure 3. Correlation among Wnt1, β-catenin, CyclinD1 and miR-21.
*$P < 0.05$, **$P < 0.01$.

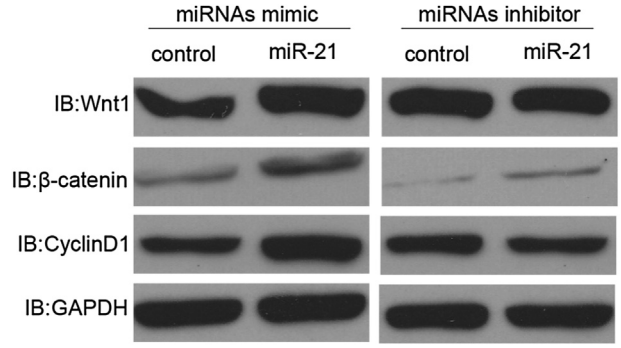

Figure 4. Western blot results.

($P < 0.01$); while the *Wnt1* genes had no significant change. After *miR-21* expressions were inhibited, *Wnt1*, *β-catenin* and *CyclinD1* gene expression levels had no significant changes, compared with control group. Further, we detected *Wnt1*, *β-catenin* and *CyclinD1* gene level changes by Western blot, which was consistent with Real-time PCR results. That is, *miR-21* could significantly increase *β-catenin* and *CyclinD1* expression levels ($P < 0.05$) (Figures 3 & 4).

3.3. miR-21 affects invasive ability of A549 cells

A549 cells were seeded into 24-well Transwell chambers. A medium containing 10% FBS was added in the lower chamber. Cells were cultured at 37 °C for 24 h. Then, liquid in the upper chamber was discarded and the upper chamber was removed. Those that did not pass through the cell membrane were wiped off with a cotton swab. Then, 4% formalin fixed for 10 min, stained with 0.1% crystal violet, and cells were observed under an inverted microscope. Three horizons were randomly selected and perforated cells were calculated for statistical analysis. The results in Table 3 show that after cells were transfected with miR-21 mimics, the number of perforated cells (193.3 ± 19.8) was significantly higher than the control group (123.3 ± 12.8) ($P < 0.01$).

3.4. miR-21 expression level in Lewis lung cancer mice and correlation among Wnt1, β-catenin, CyclinD1 and miR-21

Mice were sacrificed in 14 days after tumor was received, and tumor tissues were separated. Among the 20 mice, two prematurely died due to infection, three had unpaired tumors, and a total of 15 tumors tissue samples were taken. Corresponding normal tissues were taken from 20 mice in the control group. Obtained tissue proteins were extracted from all tissues and grinded in liquid nitrogen. Wnt1, β-catenin, CyclinD1 expression levels were detected by Western blot. Quantity one v4.62 software molecular bands gray value (Trace Tracking) was applied to calculate optical density values, and optical density curve was plotted according to the different electrophoretic bands. Then, area under the optical density curve was calculated as quantitative electrophoretic bands for statistical analysis. Results have shown that Wnt1, β-catenin and CyclinD1 expression levels were significantly higher in Lewis lung cancer ($P < 0.01$), compared with control group.

Total RNA was extracted from tissue samples by a total RNA extraction kit, and miR-21 expression was detected by Real-time

Table 3
Cell invasion assay results.

		miRNAs mimic		miRNAs inhibitor	
	Blank control	Control	miR-21	Control	miR-21
V1[a]	97[b]	140	221	184	127
V2	121	121	183	119	107
V3	139	109	176	127	99
	119.0 ± 17.2	123.3 ± 12.8	193.3 ± 19.8**	143.3 ± 28.9	111.0 ± 11.8

[a]: Field of vision;[b]: the number of perforated cells; compared to control, *$P < 0.05$,**$P < 0.01$.

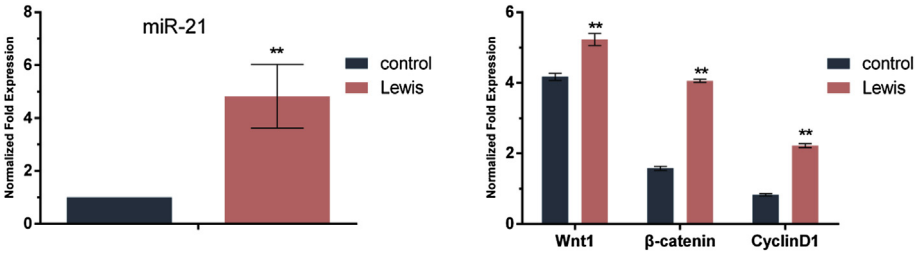

Figure 5. Analysis of miR-21 expression levels in Lewis lung cancer in mice and correlation among Wnt1, β-catenin, CyclinD1 and miR-21. *$P < 0.05$, **$P < 0.01$.

PCR. Results have shown that miR-21 expression levels were significantly higher in Lewis lung cancer ($P < 0.01$), compared with control group (Figure 5).

4. Discussion

4.1. miRNAs could be widely found in vivo

With in-depth research on miRNAs, a variety of biological functions have been found; and its mechanism of action is also very complex. Each miRNA could regulate multiple target genes and several miRNAs could regulate together with a gene, miRNAs and their target genes or other miRNAs form a complex regulatory network that is extensively involved in cell proliferation, apoptosis and other life processes; and abnormalities in this regulatory network often lead to serious consequences [16–18]. Studies in recent years have shown that miRNAs and tumor development have very important roles. These miRNAs not only plays a role in general cancer-promoting genes or tumor suppressor genes, but also plays a different role in different tumors, and even in different stages of the tumor. It is precisely due to the important features of these miRNAs that miRNA research has become a focus in tumor marker, cancer biotherapy and other areas of research.

miR-21 is a kind of miRNA which has been extensively and comparatively studied. Studies have found that miR-21 was upregulated in a variety of tumor tissues [19–21]. For example, Ribas and other studies found that miR-21 expression levels in prostate cancer tissues increased 3.52 times more, compared with normal tissues [22]. In addition, miR-21 expression levels in cancer tissues were significantly higher than adjacent tissues in non-small cell lung cancer; wherein, miR-21 was associated with its prognosis. In various types of lung cancer, expression of miR-21 in lung adenocarcinoma is higher than in squamous cell carcinoma [23–25]. This also reminds us that miR-21 plays different roles in different tumors and even in different types of tumors, but with high expression levels in tumors; thus, playing the role as a "cancer-promoting gene", which is a broad consensus on miR-21.

The Wnt signaling pathway is so named due to its Wnt. In normal somatic cells, β-catenin, as a kind of cytoskeletal protein forms a complex with E-cadherin in the cell membrane to maintain the same type of cell adhesion; and plays a role in preventing cell movement. When extracellular Wnt signaling molecules and specific receptors frizzled in membrane proteins bind, mediated-phosphorylation of GSK3β is inhibited; and further inhibits cytoplasmic β-catenin degradation. β-catenin could not be degraded and accumulated in the cytoplasm, and enters the nucleus. CyclinD1, cmyc and other proto-oncogenes could be activated in the nucleus combined with the transcription factor Tcf/Lefs family. The process involves cell differentiation, proliferation, apoptosis, cycle and cellular adhesion, and angiogenesis; leading to tumorigenesis [4,5]. Thus, Wnt/β-catenin signaling pathway activation has an important role in tumor development. Previous studies have showed that in non-small cell lung cancer development, abnormal expressions of β-catenin promote lung cancer metastasis in the active state of the Wnt signaling pathway. Experimental studies have found that after β-catenin was inhibited, A549 cell proliferation and migration could be inhibited. Therefore, this Wnt pathway study provided candidate target molecules for lung cancer and other cancers and in clinical [26].

In conclusion, human non-small cell lung cancer cell line, A549, and Lewis lung cancer in mice were used as research objects in this study. Further, the role of miR-21 was explored via the Wnt/β-catenin signaling pathway in A549 cells. We first transfected cells within 0–12 h; wherein, miR-21 cells overexpressed and absorbance values of control cells slightly changed. Within 12–48 h, overexpression of miR-21 could significantly enhance cell absorbance values. After inhibition by miR-21, the rate of cell proliferation was reduced. This is consistent with the biological function of miR-21. At present, multiple miR-21 target genes have been confirmed. Most of these genes are tumor suppressor genes that can control cell cycle. For example, miR-21 could inhibit the expression of PTEN; thereby causing the downstream of the Akt signaling pathway, and enhancing cancer cell proliferation and metastasis. In addition, TPM1, Maspin, MARCKS and Cdc25A have also been proven to be

miRNA-21 target genes [27,28]. We inhibited the expression of miR-21 via inhibitor, which reduced the proliferation of A549 cells. This shows that miR-21 did play a similar "cancer-promoting gene" role. Experiments via overexpression or inhibition of miR-21 expression were conducted to study the correlation between miR-21 via Wnt/β-catenin signaling pathway. In this study, key molecules of mRNA and protein levels in the Wnt/β-catenin signaling pathway were studied to observe the expression changes of Wnt1, β-catenin and CyclinD1. From the results, we can see that miR-21 could significantly increase *β-catenin* and *CyclinD1* gene expressions. As described, miR-21 is indeed involved in the Wnt/β-catenin signaling pathway through its upstream target genes and positive regulation of *Wnt*; thereby, activating the Wnt/β-catenin signaling pathway, and causing *β-catenin* and *CyclinD* expressions to increase. Many downstream genes such as *CyclinD* belong to the proto-oncogene. These genes regulate cell proliferation by controlling the cell cycle. Thus, when the proto-oncogene is activated, cell cycle loses control and transforms into cancer cells; enhancing cell metastasis invasion abilities. Based on the above analysis, we obtained there is a positive correlation among the following key (β-catenin and CyclinD1) of miR-21 and the Wnt/β-catenin signaling pathway in A549 human lung cancer cells and Lewis lung cancer in mice; Cell migration and invasion ability would be enhanced by changing the expression of above key molecules.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. *Nat Rev Cancer* 2006; **6**(4): 259-269.

[2] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**(2): 74-108.

[3] Clevers H, Loh KM, Nusse R. Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. *Science* 2014; **346**(6205): 1248012.

[4] Moon RT, Bowerman B, Boutros M, Perrimon N. The promise and perils of Wnt signaling through beta-catenin. *Science* 2002; **296**(5573): 1644-1646.

[5] Lecarpentier Y, Claes V, Duthoit G, Hébert JL. [Circadian rhythms, Wnt/beta-catenin pathway and PPAR alpha/gamma profiles in diseases with primary or secondary cardiac dysfunction]. *Front Physiol* 2014; **5**: 429.

[6] LiuY Zhang CG, Zhou CY. Classic Wnt/beta-catenin signaling pathway in two-way regulation. *J Beijing Univ (Medi Ed)* 2010; **2**: 238-242.

[7] Klaus A, Müller M, Schulz H, Saga Y, Martin JF, Birchmeier W. [Wnt/β-catenin and Bmp signals control distinct sets of transcription factors in cardiac progenitor cells]. *Proc Natl Acad Sci U. S. A* 2012; **109**(27): 10921-11096.

[8] Kypta RM, Waxman J. Wnt/β-catenin signalling in prostate cancer. *Nat Rev Urol* 2012; **9**(8): 418-428.

[9] Chen B, Li H, Zeng X, Yang P, Liu X, Zhao X, et al. Roles of miRNA on cancer cell metabolism. *J Transl Med* 2012; **10**: 228.

[10] Zhu L, Liu J, Cheng G. Role of miRNAs in schistosomes and schistosomiasis. *Front Cell Infect Microbiol* 2014; **4**: 165.

[11] Lee RC, Feinbaum RL, Ambros V. The C. Elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993; **75**(5): 843-854.

[12] Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, et al. Microarray analysis shows that some miRNAs downregulate large numbers of target mRNAs. *Nature* 2005; **433**(7027): 769-773. Epub 2005 Jan 30.

[13] Li LC, Okino ST, Zhao H, Pookot D, Place RF, Urakami S, et al. Small dsRNAs induce transcriptional activation in human cells. *Proc Natl Acad Sci U. S. A* 2006; **103**(46): 17337-17342.

[14] Kim DH, Saetrom P, Snøve O Jr, Rossi JJ. [miRNA-directed transcriptional gene silencing in mammalian cells]. *Proc Natl Acad Sci U. S. A* 2008; **105**(42): 16230-16235.

[15] Place RF, Li LC, Pookot D, Noonan EJ, Dahiya R. miRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U. S. A* 2008; **105**(5): 1608-1613.

[16] Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 2006; **34**: 140-144.

[17] Wagner A, Mayr C, Bach D, Illig R, Plaetzer K, Berr F, et al. MicroRNAs associated with the efficacy of photodynamic therapy in biliary tract cancer cell lines. *Int J Mol Sci* 2014; **15**(11): 20134-20157.

[18] Liu L, Chen L, Xu Y, Li R, Du X. microRNA-195 promotes apoptosis and suppresses tumorigenicity of human colorectal cancer cells. *Biochem Biophys Res Commun* 2010; **400**(2): 236-240.

[19] Kang W, Lee J, Oh S, Lee S, Jung C. Stromal expression of miR-21 in T3-4a colorectal cancer is an independent predictor of early tumor relapse. *BMC Gastroenterol* 2015; **15**(1): 2.

[20] ONE Staff PLOS. Correction: upregulation of miR-21 in cisplatin resistant ovarian cancer via JNK-1/c-Jun pathway. *PLoS One* 2014; **9**(12): e116447.

[21] Toiyama Y, Takahashi M, Hur K, Nagasaka T, Tanaka K, Inoue Y, et al. Serum miR-21 as a diagnostic and prognostic biomarker in colorectal cancer. *J Natl Cancer Inst* 2013; **105**(12): 849-859.

[22] Ribas J, Lupold SE. The transcriptional regulation of miR-21, its multiple transcripts, and their implication in prostate cancer. *Cell Cycle* 2010; **9**(5): 923-929.

[23] Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 2005; **6**(5): 376-385.

[24] Yang Y, Meng H, Peng Q, Yang X, Gan R, Zhao L, et al. Downregulation of microRNA-21 expression restrains non-small cell lung cancer cell proliferation and migration through upregulation of programmed cell death 4. *Cancer Gene Ther* 2015; **22**(1): 23-29.

[25] Xu LF, Wu ZP, Chen Y, Zhu QS, Hamidi S, Navab R. MicroRNA-21 (miR-21) regulates cellular proliferation, invasion, migration, and apoptosis by targeting PTEN, RECK and Bcl-2 in lung squamous carcinoma, Gejiu City, China. *PLoS One* 2014; **9**(8): e103698.

[26] Yan S, Liu G, Pei C, Chen W, Li P, Wang Q, et al. Inhibition of NADPH oxidase protects against metastasis of human lung cancer by decreasing microRNA-21. *Anticancer Drugs* 2015; **26**(4); http://dx.doi.org/10.1097/CAD.0000000000000198.

[27] Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res* 2008; **18**(3): 350-359.

[28] Zhu S, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem* 2007; **282**(19): 14328-14336.