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Original Article

Neuroleukin regulates the metastasis of non-small cell lung cancer cells through the Wnt/β-catenin signaling pathway

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Abstract: Purpose: We investigate the mechanisms of neuroleukin (NLK) in the metastasis of non-small cell lung cancer (NSCLC). Methods: A549 cells were separated into an si-NLK group, an NC group, and a control group. The expression of NLK was measured by qRT-PCR. The proliferation of A549 cells was measured using cell cloning and an MTT assay, and the migration ability of the A549 cells was measured using a scratch test, and the invasiveness of A549 cells was tested isomg a Transwell invasion assay. Additionally, immunofluorescence was used to observe the distribution of β-catenin in the cells, and western blot was applied in measuring the expressions of β-catenin, cyclin D1, and c-myc. Results: After transfection with NLK-siRNA, the expression of NLK was distinctly decreased compared to the control and NC group ($P < 0.05$). The proliferation in the si-NLK group was prominently slower than it was in the other two groups ($P < 0.05$). The cell cloning, migration, and invasiveness abilities in the si-NLK group was markedly decreased compared to the control and NC groups ($P < 0.05$). A high positive expression of β-catenin in the nuclei was detected in the control and NC groups. Moreover, in the si-NLK group, it was mainly expressed in the membranes, and the expression showed a downward trend. By comparison with the control and NC groups, the expressions of c-myc, β-catenin, and cyclin D1 in the si-NLK group were markedly down-regulated ($P < 0.05$). Conclusions: NLK significantly promotes the metastasis of NSCLC cells by adjusting the Wnt/β-catenin signaling pathway, which could provide a new target for controlling the metastasis of NSCLC at the gene level.

Keywords: NLK, non-small cell lung cancer, cell proliferation, metastasis, Wnt/β-catenin pathway

Introduction

Non-small cell lung cancer (NSCLC), including squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma, makes up more than 85% of all lung cancer cases [1]. NSCLC is considered the main subtype of lung cancer, with a high incidence and poor prognosis [2]. The common causes of NSCLC include smoking, occupational and environmental exposure, ionizing radiation, chronic lung infections, genetic factors, and air pollution. At present, chemotherapy, radiotherapy, and surgical resection are the main treatments for NSCLC [3, 4]. However, the current treatment techniques are involve a long treatment cycle, and the cancer is prone to recurrence. In order to alleviate the suffering of patients, a new method is urgently needed to diagnose and treat NSCLC quickly and effectively.

Molecular targeted therapy was introduced because it could directly and quickly reach the lesion site for treatment. miR-449a can inhibit the proliferation, migration and invasion of NSCLC cells by mediating the NF-κB signal transduction pathway [5]. In addition, the overexpression of miR-584 restrains the cloning and invasion of NSCLC cells by directly targeting MTDH [6]. In fact, researchers also found that Nimo-like kinase (NLK) involves tumor proliferation and invasion other than miRNA. Moreover, NLK, an evolutionarily conserved serine/threonine kinase, could be used to treat

NSCLC [7]. The inhibition of NLK expression can significantly promote the proliferation of NSCLC cells [8]. Along with extensive research on the treatment of NSCLC targets, a number of studies have been done on the relevant pathways for the treatment of cancer. The Wnt pathway is an important signal transduction system, and mutations in the Wnt/β-catenin pathway-associated protein expression lead to a variety of growth-related pathologies and cancers [9]. Vilchez's research has shown that the up-regulation of the Wnt/β-catenin pathway takes part in maintaining tumor initiation cells, drug resistance, tumor progression, and metastasis [10]. Importantly, NSCLC cell proliferation can be promoted by regulating the Wnt/β-catenin pathway [11, 12].

Specifically, NLK is recognized as a key regulator of many cancers. However, the mechanism of NLK and the related signaling pathways in NSCLC are still unclear. In this study, we examined the effects of NLK on NSCLC metastasis through the Wnt/β-catenin pathway from cell multiplication, migration, and invasion in A549 cells, which may provide new ideas for the clinical treatment of NSCLC.

Methods

Cell culture

A549 cells (cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were cultured in an RPMI 1640 medium containing 100 IU/mL penicillin/streptomycin and 10% fetal calf serum at 37°C and 5% CO_2. Single cell suspension was obtained by trypsin digestion, then the cells were washed with 0.01 mol/L PBS and suspended at about 1×10^6 cells/mL. The cells were passaged once every other day and in the rapid growth phase were used for subsequent experiments.

Cell transfection

The A549 cells were divided into a control group (conventional culture), an NC group (transfected by siRNA-NC), and an si-NLK group (transfected by siRNA-NLK). After digestion, centrifugation, and rinsing with sterile saline, the cells were added to a six-well plate. After cell fusion reached 60%, DMEM-f12 complete medium (Hyclone Company) was replaced by serum-free DMEM-f12 medium for 1 h. Then 250 μL Opti-MEM with 5 μL (100 pmol) siRNA reagents (Shanghai Jima Pharmaceutical Technology Company, Shanghai, China) and 250 μL Opti-MEM with 5 μL lipo2000 reagent were mixed and incubated at 25°C for 20 min. Finally, the mixed droplets were added to the cells in the six-well plate and transfected in a 37°C incubator for 6-8 h. Then the culture medium was converted to a DMEM-f12 complete medium with 10% FBS.

QRT-PCR assay

Trizol reagent was used to extract total RNA. The ratio of OD260/OD280 was determined by nucleic acid quantitative proteinometer to identify the purity of RNA. The reverse transcription reaction was used to synthesize the template of cDNA using a PCR amplifier, and the qRT-PCR was performed using an ABI 7500 quantitative PCR (Thermo Fisher, Singapore). QRT-PCR was processed based on the following conditions: pre-treatment at 95°C for 10 min, following by 40 cycles of 95°C for 5 s, 60°C for 34 s and 60°C for 30 s. The $2^{-\Delta\Delta Ct}$ method was used to analyze the data. β-actin was used as the internal reference. The special primers used were as follows: NLK-F, 5'-CAGATTTTGCGAGGTTTG-3'; NLK-R, 5'-AGGAGATTCCCTGGCTTA-3'; β-actin-F, 5'-GCAAGGTCATCCCTGAGCTGA-3'; β-actin-R, 5'-ACGCCTGCTTCACCACCTTC-3'.

MTT assay

After transfection for 48 h, the A549 cells of each experimental group were suspended in complete medium by trypsinization and inoculated at 2,000 cells per well on a 96-well plate. Then the cells were cultured in an incubator at 37°C and 5% CO_2. A total of 10 μL of 5 mg/mL MTT (Roche, Shanghai, China) was added to each well for 4 h, and then 100 μL of DMSO (TEDIA, USA) was added to terminate the reaction. The absorbance (A) at 490 nm was measured with a microplate reader (SpectraMax® M5/M5e, California, USA), and the growth curve was plotted.

Cell cloning assay

After transfection, the A549 cells were digested by trypsin, suspended in a complete medium and counted, and then they were inoculated into a 6-well plate. At the end of the experiment, the cells were fixed with paraformaldehyde for

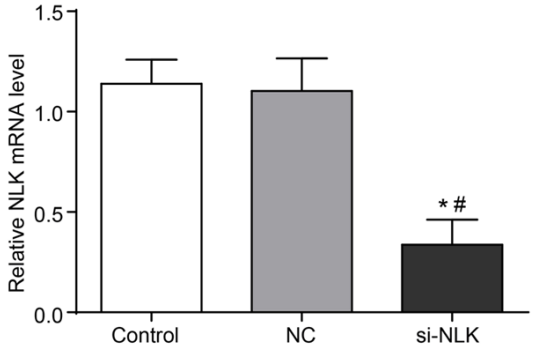

Figure 1. The effect of NLK knockdown was verified by qRT-PCR. $^{*}P < 0.05$ (versus control group), $^{\#}P < 0.05$ (versus NC group).

30 min, stained with Giemsa for 20 min, and the number of cloned cells was finally counted. Each group was repeated 3 times.

Scratching test

About 5×10^5 cells were added to each well of the 6-well plate, and serum medium was added for culture. When cell fusion reached 80%, a 10 μL tip was used to draw a trace perpendicular to the horizontal line on the cell surface. Then, a serum-free medium was added into the delineated cells. Subsequently, the cells were observed and photographed with an optical microscope at 0 h and 24 h after scratching, and then the cell migration distance was calculated. Finally, the scratch healing rate was reckoned using Image Tool (Bechtel Nevada, California, USA).

Transwell invasive assay

After transfection for 48 h, approximately 1×10^5 A549 cells were added to the Transwell upper chamber with the inner membrane coated with Matrigel, and 200 μL of FBS-free cell culture medium was added. A total of 500 μL of complete medium was added to the lower chamber, the upper chamber was placed, and the entire Transwell chamber was cultured in an incubator for 24 h. Then, the cells and the medium inside the upper chamber were wiped off with a cotton ball, and then the upper chamber was immersed in 4% formaldehyde at 4°C for 8 min. Subsequently, the Transwell upper chamber was inverted, and the outer membrane was stained with a drop of crystal violet solution for 10 min. Next, the crystal violet solution was washed away by PBS solution, and the membrane was dried. The number of invasive cells was calculated using an inverted microscope.

Immunofluorescence

After transfection for 24 h, the cells were digested with 0.25% trypsin, and incubated in a 37°C incubator for 48 h. Then, the cells were rinsed thrice in PBS, fixed at 25°C for 20 min with 4% paraformaldehyde and permeabilized with 0.1% Triton followed by blocking with 1% BSA for 30 min at 25°C. The cells were incubated overnight with a 1:100 diluted monoclonal antibody against β-catenin. Next, the cells were incubated for 1 h at 37°C with an FITC-labeled secondary antibody. Subsequently, DAPI was used to stain the nucleus for 10 min under light-proof conditions. The staining results were observed using a confocal microscope (LeiCa microsystem Heidelberg GmbH, Germany).

Western blotting

After transfection, the cells were treated with a RIPA cell lysate (Beijing Beyotime Biotechnology Co., Ltd., China), and the total protein was extracted. The equivalent protein was subjected to SDS-PAGE and transferred onto the membranes. Subsequently, it was blocked with 5% skim milk powder for 1 h. These membranes were separately incubated overnight at 4°C with the following primary antibodies: 5% BSA-containing β-catenin antibody (Cell Signaling Technology, Massachusetts, USA), cyclin D1 antibody (Santa Cruz, California, USA), or c-myc antibody (Santa Cruz, California, USA). The membranes were incubated with an HRP-labeled anti rabbit secondary antibody (Zhongshan, Beijing, China) for 1 h at 25°C. This experiment used β-actin as an internal reference, and the protein was quantified using luminescence image analyzer software.

Statistical analysis

All data were represented by mean values ± SD. SPSS 21.0 (SPSS Inc., Chicago, USA) was used for inter-group comparisons with one-way analysis of variance and post-hoc comparisons. Statistical significance was accepted at $P < 0.05$.

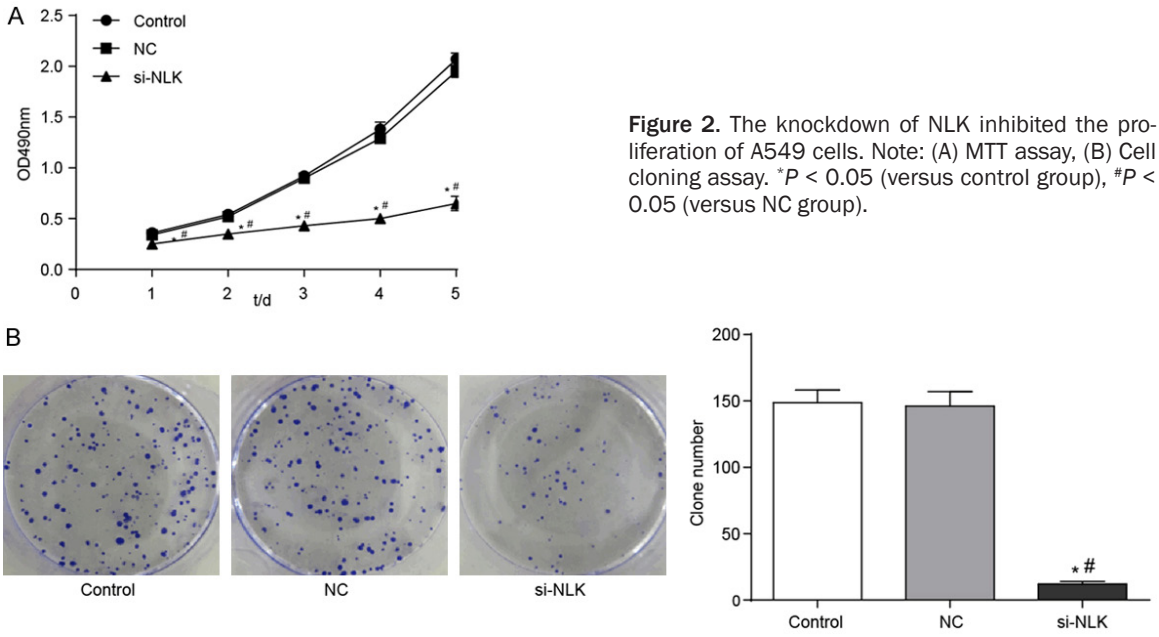

Figure 2. The knockdown of NLK inhibited the proliferation of A549 cells. Note: (A) MTT assay, (B) Cell cloning assay. $^{*}P < 0.05$ (versus control group), $^{\#}P < 0.05$ (versus NC group).

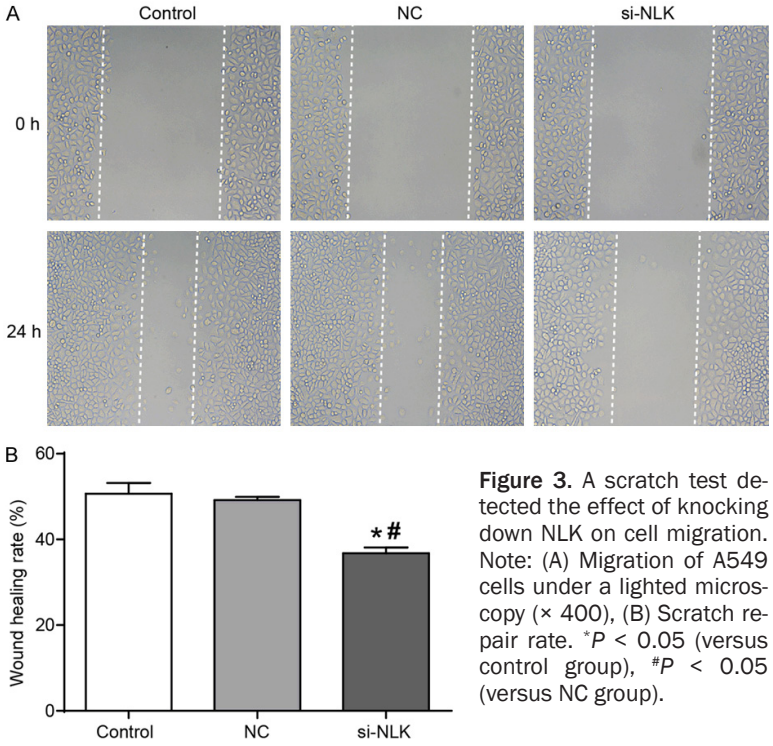

Figure 3. A scratch test detected the effect of knocking down NLK on cell migration. Note: (A) Migration of A549 cells under a lighted microscopy (× 400), (B) Scratch repair rate. $^{*}P < 0.05$ (versus control group), $^{\#}P < 0.05$ (versus NC group).

Results

Effects of NLK knock down

After the transfection of NLK-siRNA, the expression of NLK was observably less than the expressions of the control and NC groups ($P < 0.05$) (**Figure 1**). It was confirmed that knocking down NLK was successful.

Knockdown of NLK inhibited the proliferation of A549 cells

To further study the potential effects of NLK on A549 cell proliferation, we knocked-down NLK by siRNA. As shown in **Figure 2A**, with the prolongation of culture time, the proliferation of A549 cells in the si-NLK group was observably slower when it was compared to the control and NC groups. In the first 5 days, the A490 values of the si-NLK group were remarkably lower than those in the other two groups ($P < 0.05$).

Compared to the control and NC groups, the amount of cell clone formation and the ability of cell cloning in the si-NLK group were obviously decreased ($P < 0.05$, **Figure 2B**), suggesting that the knockdown of NLK inhibited the proliferation of A549 cells.

Knockdown NLK inhibited migration and invasion of A549 cell lines

The fusion rate of scratch cells was observed 24 h after transfection. Compared with the other two groups, the cell fusion rate of the si-

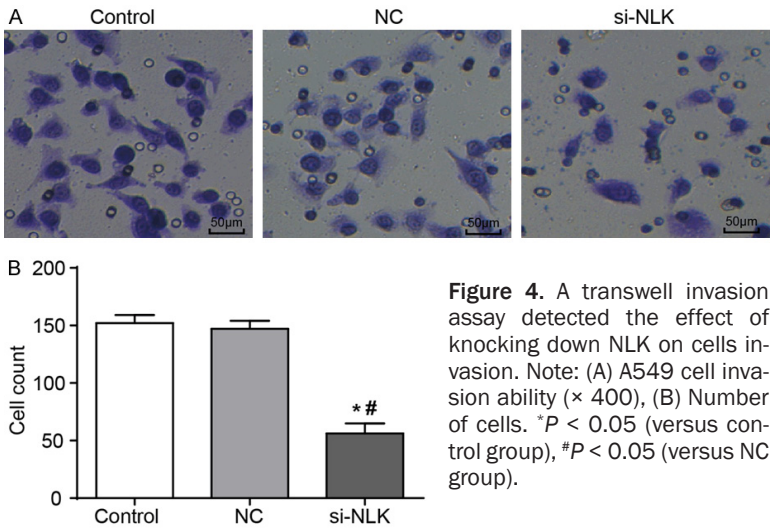

Figure 4. A transwell invasion assay detected the effect of knocking down NLK on cells invasion. Note: (A) A549 cell invasion ability (× 400), (B) Number of cells. $^{*}P < 0.05$ (versus control group), $^{\#}P < 0.05$ (versus NC group).

NLK group was prominently slower. After the si-NLK transfection, the scratch repair rate in the si-NLK group was clearly lower than it was in the control and NC groups ($P < 0.05$, **Figure 3**). The above experimental findings showed that the knockdown of NLK expression inhibited the migration of A549 cells.

After knocking down NLK, the invasive ability of the A549 cells in the si-NLK group was distinctly lower than it was in the control and NC groups. Moreover, the number of cells passing through the polycarbonate membrane descended markedly ($P < 0.05$, **Figure 4**), which suggested that the knockdown of NLK expression inhibited the invasion of A549 cells.

The knockdown of NLK inhibited the Wnt/β-catenin signaling pathway

An immunofluorescence staining analysis indicated that a high level of β-catenin in the nuclei was found in the control and NC groups. Conversely, β-catenin in the si-NLK group was mainly expressed in the cytoplasms, and the level of β-catenin was reduced after transfection (**Figure 5A**). A Western blot analysis showed that the expressions of c-myc, β-catenin and cyclin D1 were down-regulated in the si-NLK group more so than in the control and NC groups ($P < 0.05$) (**Figure 5B**). These results suggested that the knockdown of NLK expression inhibited the Wnt/β-catenin pathway activity.

Discussion

In this research, we verified that the knockdown of NLK inhibited the proliferation, migration and invasion of NSCLC cells by mediating the Wnt/β-catenin pathway. Remarkably, NLK is involved in the development and progression of tumors by phosphorylating multiple transcription factors, which is extremely important in a variety of signal transduction pathways [13]. Silencing NLK could significantly inhibit cell proliferation and tumorigenicity in vitro and in vivo [14]. In this study, the knockdown of NLK inhibited the proliferation, migration, and invasion of A549 cells. Similarly, Li *et al.* [15] have demonstrated that the absence of NLK impedes the proliferation and development of colorectal tumors. The knockdown of NLK could also inhibit SCLC cell growth and metastasis [16]. Moreover, xenograft tumor mice show that knocking out NLK reduces the ability of cells to form tumors [12]. These findings indicate that the knockdown of NLK is beneficial to inhibiting the development of NSCLC.

In particular, the sensitization of the Wnt/β-catenin pathway is associated with tumorigenesis, development, and metastasis. For instance, the activation of the Wnt/β-catenin signaling leads to an increase in glioma cells [17]. The negative regulation of β-catenin-associated complexes could reduce transcriptional activation of downstream target genes and inhibit tumorigenesis [18]. Additionally, β-catenin plays a key role in epithelial cell migration. It has been proved that Hsp27 promotes β-catenin nuclear transport induced prostate cancer metastasis [19]. Masoumi and Sumihito *et al.* [20, 21] state that NLK can negatively regulate the Wnt signaling pathway.

Remarkably, c-myc is a common proto-oncogene which encodes proteins involved in normal cell differentiation and proliferation. In addition, the expression of c-myc is induced by the Wnt/β-catenin signaling pathway [22]. Under certain conditions, the overexpression of c-myc could induce cell transformation and tumor formation [23]. Moreover, the upregulation and targeting of c-myc in NSCLC results in increased cell activity and invasiveness [24]. A recent study also has verified that the content of c-myc in NSCLC tissues is distinctly higher

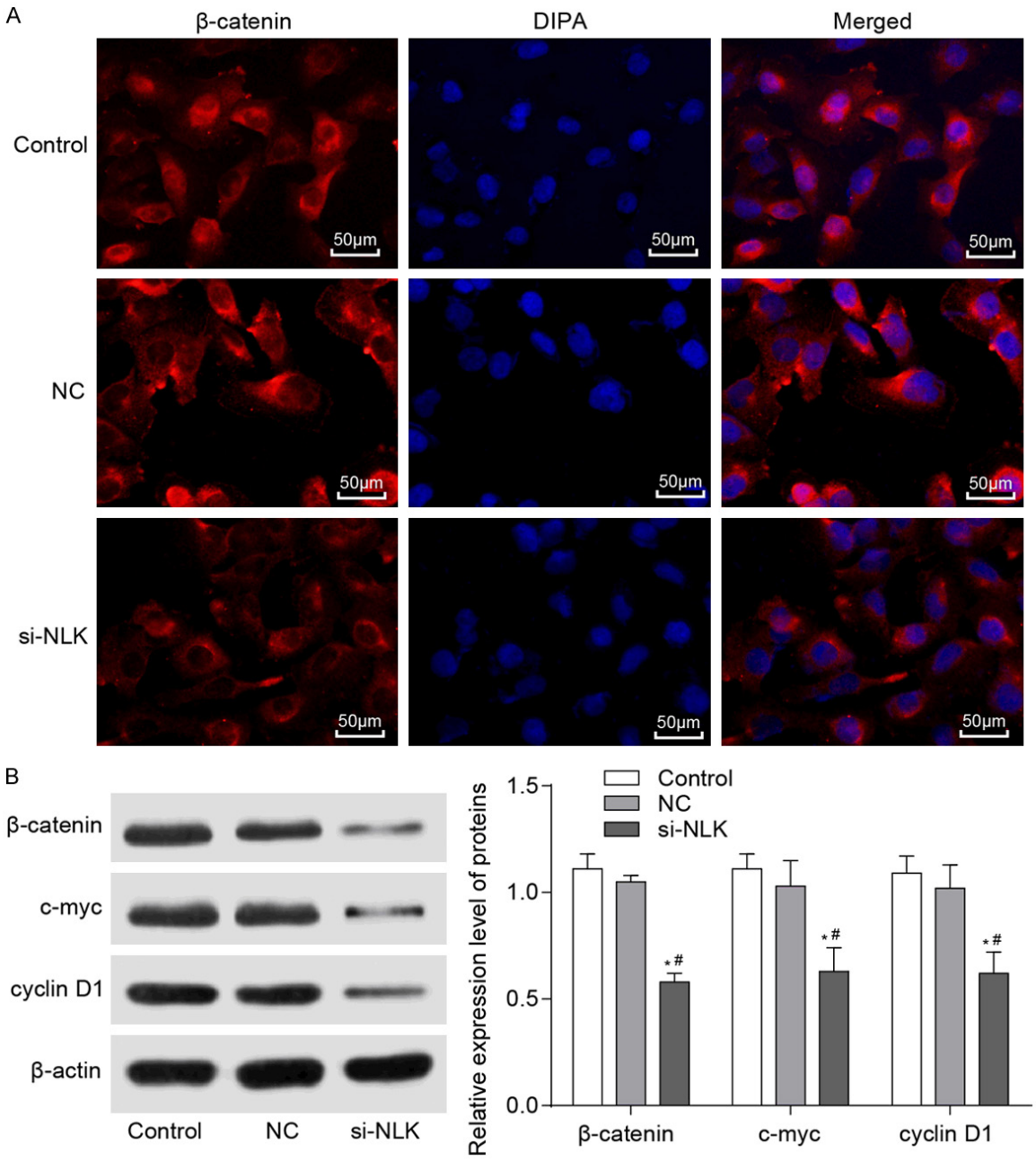

Figure 5. The effect of NLK knockdown on the Wnt/β-catenin pathway. Note: (A) Immunofluorescence results (× 200), (B) Western blot detection. $^{*}P < 0.05$ (versus control group), $^{\#}P < 0.05$ (versus NC group).

than it is in healthy tissues, and the positive expression rate of c-myc proteins is clearly correlated with the grade of tumor differentiation [25]. It was further shown that reducing the level of c-myc is beneficial to inhibit the development of NSCLC.

Moreover, cyclin D1 gene is a highly conserved member of the cell cycle family, and its abnormal expression could be used as an indicator for cancer diagnosis. Cyclin D1 strengthens the invasion and metastasis of cancer cells through cytoplasmic mechanisms [26]. Malusecka *et al.* [27] found that in 57% patients with cyclin D1-positive tumors, the tumors were associated with an overexpression of cyclin D1. Researchers also illustrate that the expression of cyclin D1 in NSCLC tissues is obviously higher than it is in healthy tissues [28]. The progression of the NSCLC cell cycle is inhibited by

down-regulating cyclin D1 [29]. In this work, the expressions of c-myc and cyclin D1 were down-regulated more in the si-NLK group than they were in the control and NC groups, suggesting that the knockdown of NLK expression inhibits Wnt/β-catenin pathway activity in NSCLC cells.

In conclusion, the current study provides new insights into the role of NLK in A549 cells. It reveals that the knockdown of NLK can inhibit A549 cell proliferation, migration and invasion through the Wnt/β-catenin pathway, which could provide a new target for controlling the metastasis of NSCLC at the gene level.

Disclosure of conflict of interest

None.

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