

Wnt3 knockdown sensitizes human non-small cell type lung cancer (NSCLC) cells to cisplatin via regulating the cell proliferation and apoptosis

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Abstract. – OBJECTIVE: Aberrant activation of (Wingless and mouse homolog Int-1) Wnt/ β -catenin signaling pathways closely involved in the occurrence and progression of several types of human malignancies. This research was undertaken to elucidate the important role of (Wingless and mouse homolog Int-1) in lung cancer.

PATIENTS AND METHODS: Wnt3 expression in lung cancers and their respective normal tissues were examined by immunoblotting and immunohistochemistry. Then, Wnt3 was regulated with RNA interference (RNAi) technology in human lung cancer A549 cells, and the cell proliferation, cell cycle, cell invasion/metastasis, and apoptosis were evaluated.

RESULTS: In all cases, Wnt3 expression was significantly elevated in lung cancers compared with normal tissues. Knocking down Wnt3 in A549 lung cancer cells by small interfering RNAs transfection led to a distinct reduction of Wnt3 in both transcript and protein levels. Knockdown of Wnt3 expression in lung cancer cells inhibited the expression of β -catenin and cyclin D1 genes in Wnt/ β -catenin pathway. It also significantly blocked cellular proliferation, delayed cell cycle and suppressed cell invasion/metastasis, accompanied by a higher apoptosis rate.

CONCLUSIONS: We conclude that the upregulation of Wnt3 plays a crucial role in lung tumorigenesis by inducing proliferation, migration, and invasion and inhibiting apoptosis of cancer cells. Wnt3 might be a potential target for the treatment of lung cancer.

Key Words:

Lung carcinogenesis, Lung cancer, Wnt/ β -catenin, siRNA, Invasion.

Introduction

Lung cancer is the second common cancer type for both men and women worldwide¹ or is the most common one for men and the second common one for women in China². Moreover, it is markedly leading the mortality among all types of cancers both in China and worldwide^{1,2}. More than 80% lung cancers are histologically classified into non-small cell type lung cancers (NSCLC), which includes adenocarcinoma (ADC), squamous cell carcinoma (SCC), large cell carcinoma (LCC) and other types³. Multiple factors contributed to lung cancer development. Except for the family history⁴, the susceptibility locus (such as chromosome 6q23-25)⁵ and genetic polymorphisms (three main susceptibility loci, 15q25⁶, 5p15⁷, and 6p21⁸ regions) were associated with lung cancer. Moreover, the epidemiological evidence and biological plausibility unanimously support a causal association between the tobacco smoking and lung cancer risk^{9,10}. There were several factors explaining the high mortality rate of lung cancer, including late diagnosis, restriction on local eradication. More importantly, the cancer

associated biological factors and biological mechanisms underlining the aggressiveness, the tumor heterogeneity and the acquisition of resistance of lung cancer contributed to the bad outcome of lung cancer patients¹¹.

Wnt (wingless and mouse homolog Int-1)¹² gene family encodes a group of evolutionarily-conserved glycoproteins, which modulate embryonic development, tissue regeneration, and many other processes¹³. The aberrant Wnt/ β -catenin signaling has been recognized during the onset and progression of various types of cancers, such as colorectal cancer¹⁴, gastric cancer¹⁵, breast cancer¹⁶ and others. In particular, various members of Wnt family are indicated to be frequently upregulated in lung cancers¹⁷⁻¹⁹. As a member of Wnt family, Wnt3 is also relevant to the hepatic²⁰, lung^{19,21}, gastric¹⁵, and colorectal carcinogenesis²². Moreover, the upregulated promotes tumor progression of NSCLC, is significantly associated with a reduced overall survival rate of NSCLC patients¹⁹. However, the mechanism underlining the oncogenic role of Wnt3 in NSCLC was not yet clear.

In the present study, we examined the Wnt3 expression in NSCLC tissues. Then, we knocked down Wnt3 with RNA interference (RNAi) technology, and investigated the regulatory role of Wnt3 on the cell proliferation, cell cycle, and apoptosis. Our findings indicated that the over-expressed Wnt3 in NSCLC mediated (at least in part) lung carcinogenesis by promoting cellular proliferation and reducing cell apoptosis and might be a potential therapeutic NSCLC target.

Patients and Methods

Collection of Non-small Cell Type Lung Cancers (NSCLC) Specimens

The study was approved by the Ethics Committee of Jinling Hospital, Southern Medical University. The written consent form was obtained from all the participants. Human NSCLC tissue samples ($n = 30$) and paired para cancer samples (more than 1 cm away from the edge of tumor) were collected during surgery on the NSCLC patients, who underwent surgical eradication, before any chemotherapy, radiation therapy or adjunctive therapy, at our hospital between Nov 2014 and Jul 2015. The patients were selected according to clinicopathological characteristics (see details in Table I). Each sample was immediately fixed with 4% formaldehyde or was snap-frozen

in liquid nitrogen and stored at -80°C before use. Detailed clinical and pathological information about these patients were listed in Table I.

Immunohistochemical Staining for Wnt3

NSCLC tissues were fixed with 4% formaldehyde for the preparation of histopathologic slide. For the immunohistochemical staining for Wnt3, slides were bathed in citrate buffer for 15 minutes for antigen retrieve. The Wnt3 detection was sequentially performed with the first incubation with rabbit anti-human Wnt3 antibody (Abcam, Cambridge, UK) for one hour at 37°C , with the secondary incubation with the HRP-linked anti-rabbit antibody (Cell Signaling Technology Inc., Danvers, MA, USA) for 30 minutes at room temperature and, then, with the Mayer's hematoxylin staining for the specific Wnt3-antibody binding.

Reagents, Cell Culture, and Treatment

Human NSCLC A549 cells were purchased from American Type Culture Collection (ATCC Manassas, VA, USA), and were cultured in in the Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA), adding 10% FBS (GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA) at 37°C in a humid incubator with 5% CO_2 . Cells with approximately 85% confluence were splitted and were updated with fresh medium. For the Cisplatin treatment, 2 μM Cisplatin (Sigma-Aldrich, St. Louis, MO, USA) was added into the cells' medium.

Table I. Clinico-pathological features of NSCLC patients in this study.

Features	Cases
Age at diagnosis	
< 55	9
≥ 55	21
Gender	
Male	16
Female	14
Tumor size (cm)	
<	13
$\geq$	17
Tumor stage	
I-II	17
III	13
Lymph node metastasis	
No	16
Yes	14

Quantitative Real-time Polymerase Chain Reaction (PCR) Analysis of Wnt3 mRNA Level

mRNA samples from NSCLC tissues or A549 cells were prepared with the TRIzol reagent (Life Technologies, Grand Island, NY, USA), and were supplemented with RNase inhibitor (Thermo Scientific, Waltham, MA, USA) before use. The quantitative analysis was performed with TaqMan® Fast Advanced Master Mix kit (Thermo Scientific, Waltham, MA, USA) with Wnt3 specific primers (Forward: 5'-AGAGAAGCACCGTGAGTCCCG-3', Reverse: 5'-GTGGGTGGCTTGAAGAGCG-3') and probe (5'-FAM-TGGGTGGAGACCCTCCGGC-TAMRA-3'). The reaction was performed on the LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany). Primers and probes were synthesized by Sangon Biotech (Shanghai) Co. (Shanghai, China). The Wnt3 level was presented as a fold change to the internal control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with the $\Delta\Delta C_t$ method²³.

Protein Isolation and Western Blotting Assay

Protein samples from NSCLC tissues or A549 cells were lysed with a Cell Lysis Buffer (Cell Signaling Technology Inc., Danvers, MA, USA) and were supplemented with a protease inhibitor cocktail (Roche Biochemicals, Basel, Switzerland). Protein samples were firstly separated by the electrophoresis with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and secondly were transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). Then, the membrane was firstly blocked with 2% bovine serum albumin (BSA) at 4°C overnight, secondly incubated with the rabbit anti-human Wnt3 antibody (Abcam, Cambridge, MA, USA) (with GAPDH antibody as control) for one hours at 37°C, thirdly incubated with the horse radish peroxidase (HRP)-linked anti-rabbit antibody (Cell Signaling Technology Inc., Danvers, MA, USA) for 40 minutes at room temperature. Four-time washing with PBST was performed before each incubation. Finally, the targeted blots were visualized with the enhanced chemiluminescence (ECL) detection systems (Amersham Pharmacia Biotech, Amersham, UK).

Cell Proliferation, Colony Formation Assay

The proliferation of A549 cells was curved with CCK-8 (DOJINDO, Kumamoto, Japan),

post the transfection with Wnt3-specific siRNA (siRNA 1/2-Wnt3) or with control siRNA (siRNA-Ctrl) for 0, 24, 48 or 72 hours. The 450 nm absorbance of each cell well was detected with a spectrophotometer (Crystaleye, Olympus, Tokyo, Japan). The colony formation assay of A549 cells was performed as follows. 200A549 cells were seeded in 12-well plate, and then were transfected with 50 nM siRNA 1/2-Wnt3 or siRNA-Ctrl. The cells were incubated for further 72 hours. The cell colonies were stained with 0.007% crystal violet for 10 minutes and were imaged with UVP BioSpectrum 500 imaging system (Upland, CA, USA).

FACScan Flow Analysis for Cell Cycle and Cell Apoptosis

For cell cycle assay, A549 cells post-treatment were digested with 0.25% EDTA-free trypsin and were resuspended in 700 μ L binding buffer supplemented with 5 μ L 7-amino-actinomycin (7AAD) for incubation at 37°C for 10 minutes. Then, another 300 μ L binding buffer supplemented with 2 μ L annexin-V-phycoerythrin were added. The stained A549 cells were fixed with 70% ethanol at 4°C for two hours, and then were incubated with 0.5 mg/mL RNase A at 37°C for 10 minutes, and then with 5 μ L propidium iodide at room temperature for 30 minutes. Cell cycle status was analyzed with a FACScan flow cytometer (Bio-Rad, Hercules, CA, USA) under the guidance of the manufacturer's recommendations. For the apoptosis analysis, A549 cells were resuspended in 500 μ L binding buffer supplemented with 5 μ L 7AAD for incubation at room temperature for 15 minutes. Before the analysis, another 450 μ L binding buffer and 1 μ L annexin-V-phycoerythrin were added in sequence. After 15 minutes, cell apoptosis was analyzed using flowcytometry, and data analysis was performed by using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical Analysis

GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA) was utilized for the statistical analysis. Quantitative data were presented as mean $\pm$ SE. Statistical difference was evaluated with Student's *t*-test or ANOVA test, which was followed by Bonferroni test for a post-hoc test. A *p*-value < 0.05 or less was considered statistically significant.

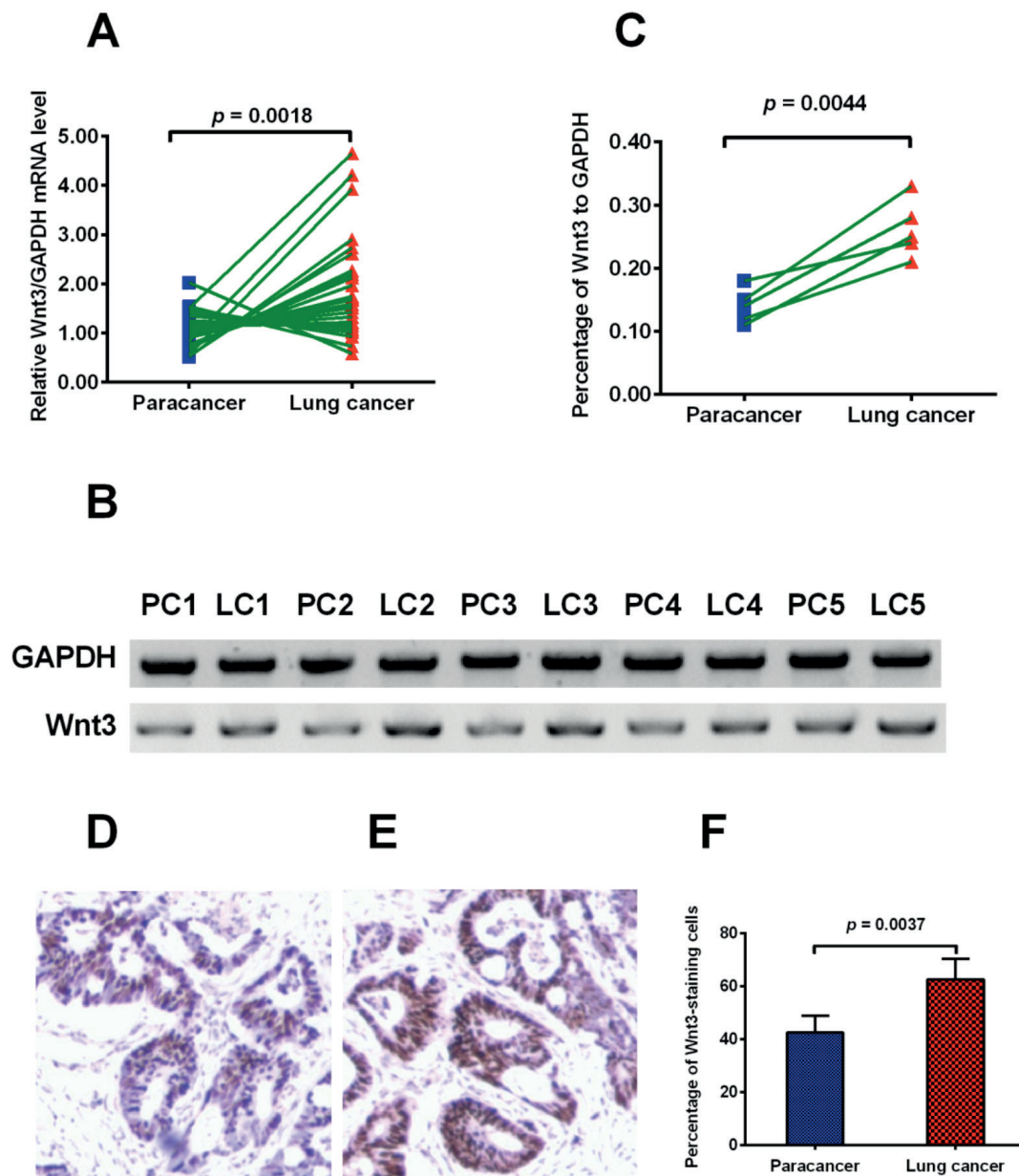


Figure 1. Upregulated Wnt3 expression in non-small cell type lung cancer (NSCLC) patients. **A**, Relative mRNA level of Wnt3 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in Non-small cell type lung cancer (NSCLC) tissues (n = 30), with para cancer tissues as control (n = 30). **B** and **C**, Western blot analysis (B) and relative protein level (C) of Wnt3 in NSCLC or para cancer tissues (n = 5 respectively). **D** and **E**, Immunohistochemistry staining of Wnt3 in para cancer (D) or in NSCLC (E) tissues (n = 15 respectively). Statistical significance was calculated with paired Student *t*-test and was indicated in each graph.

Results

Wnt3 is Overexpressed in Non-small Cell Type Lung Cancers (NSCLC) Tissues

To determine whether Wnt3 signaling is deregulated in the microenvironment of NSCLC, we examined the expression of Wnt3 in both mRNA

and protein levels in NSCLC specimens (n = 30). Figure 1A demonstrated that the relative Wnt3 mRNA level (to GAPDH) was significantly higher in the 30 NSCLC specimens than in the 30 para cancer specimens ($p = 0.0018$). The Western blotting results (Figure 1B and 1C) indicated that the protein level of Wnt3 was also markedly higher in

NSCLC tissues than in the para cancer tissues ($n = 5$ respectively, $p = 0.0044$). In addition, the immunohistochemical analysis was also performed to confirm the protein level of Wnt3 in NSCLC tissues. It was shown in Figure 1D and 1E that the Wnt3 was found to be overexpressed in NSCLC samples, compared to the lower levels in the para cancer tissues with a statistical significance ($n = 15$ respectively, $p = 0.0037$, Figure 1F). Taken together, Wnt3 is overexpressed in NSCLC, probably exerting an oncogenic effect in the onset and development of NSCLC.

Downregulated Wnt3 by Wnt3-siRNA Inhibits the Proliferation of NSCLC A549 Cells

To investigate a possible oncogenic or tumor suppressive role of Wnt3 in NSCLC cells, we manipulated the Wnt3 expression with Wnt3-specific siRNA transfection. As indicated in Figure 2A, the transfection with 30 or 60 nM Wnt3-specific siRNA (siRNA 1/2-Wnt3) markedly downregulated the Wnt3 mRNA level than the transfection with control siRNA (siRNA-Ctrl) ($p < 0.01$ respectively). And the downregulation of Wnt3 was also confirmed in the siRNA 1/2-Wnt3-transfected (60 nM) A549 cells ($p < 0.05$ or $p < 0.01$, Figure 2B). We then curved the growth of the siRNA 1/2-Wnt3- or siRNA-Ctrl-transfected A549 cells. Figure 2C demonstrated that the siRNA 1-Wnt3- or the siRNA 2-Wnt3-transfected A549 cells grew less efficiently than the siRNA-Ctrl-transfected A549 cells ($p < 0.01$ or $p < 0.001$). To reconfirm the inhibition by the Wnt3 knockdown on the proliferation of A549 cells, we then performed a colony-forming assay for the three groups of A549 cells. Figure 2D indicated that there were less colonies formed by the A549 cells, post the transfection with 60 nM siRNA 1-Wnt3 ($p < 0.01$) or siRNA 2-Wnt3 ($p < 0.05$).

Downregulated Wnt3 by Wnt3-siRNA Deregulates the Cell Cycle and Induces the Apoptosis of NSCLC A549 Cells

Further experiments were performed to examine the regulation by the Wnt3 knockdown on the cell cycle distribution, in the presence of low level of Cisplatin (2 μ M). Flow cytometry showed that in contrast to the siRNA-Ctrl-transfected A549 cells (Figure 3A), less proportion of cells in S phase was observed in the groups of A549 cells, post the siRNA 1-Wnt3 (Figure 3B) or siRNA 2-Wnt3 (Figure 3C) transfection, with a significant difference ($p < 0.01$ respectively, Figure 3D).

On the other side, the G0/G1 phase was upregulated, however, not yet significantly. We also investigated the impact of Wnt3 knockdown on the cell apoptosis of A549 cells. Figure 4A demonstrated that Cisplatin (2 μ M) induced more apoptotic A549 cells, with the Wnt3 knockdown by either siRNA 1-Wnt3 or siRNA 2-Wnt3, in comparison with the A549 cells without a Wnt3 knockdown ($p < 0.01$ respectively, Figure 4B). In addition, we analyzed the expression of apoptosis-associated markers in the A549 cells, with or without Wnt3 knockdown. The Western blotting results (Figure 5A) demonstrated that more caspase 9 and caspase 3 were activated (cleaved form, Cleaved CASP 9 or Cleaved CASP 3) (Figure 5B and 5C). Taken together, these results revealed that downregulation of Wnt3 deregulates cell cycle and promotes apoptosis in NSCLC A549 cells.

Discussion

Previous studies demonstrated that various members of Wnt family are frequently upregulated in multiple types of human cancers. The Wnt signaling has been recognized to associate with the NSCLC prognosis²⁴. The overexpression of Wnt1 and Wnt5a have been identified to pose effects in the tumor biology in NSCLCs^{25,26}. In the present study, the overexpression of Wnt3 was confirmed in both mRNA and protein levels by three types of methods (immunohistochemistry, Western blotting, and real-time quantitative PCR). Wnt3 was indicated as an important biomarker for NSCLC patients. As one member of Wnt family, Wnt3 gene is located on human chromosome 17q21²⁷, and its product stimulates the canonical Wnt/-catenin pathway^{27,28}. The activated Wnt/-catenin complex was accumulated in the cytoplasm²⁹, was translocated into the nucleus and then activates the transcription of the Wnt-target genes. However, the mechanism underlining the oncogenic role of Wnt3 remains not clear.

Wnt3 has also been recognized to promote the tumor progression in NSCLC³⁰. Our followed results also confirmed the promotive role of Wnt3 in the progression of NSCLC cells. The marked knockdown of Wnt3 with RNAi technology posed a significant regulation on the proliferation of NSCLC A549 cells. The growth curve of either siRNA 1-Wnt3- or siRNA 2-Wnt3-transfected A549 cells was much lower than the siRNA-Ctrl-transfected A549 cells and the colonies formed by either siRNA 1-Wnt3- or siRNA

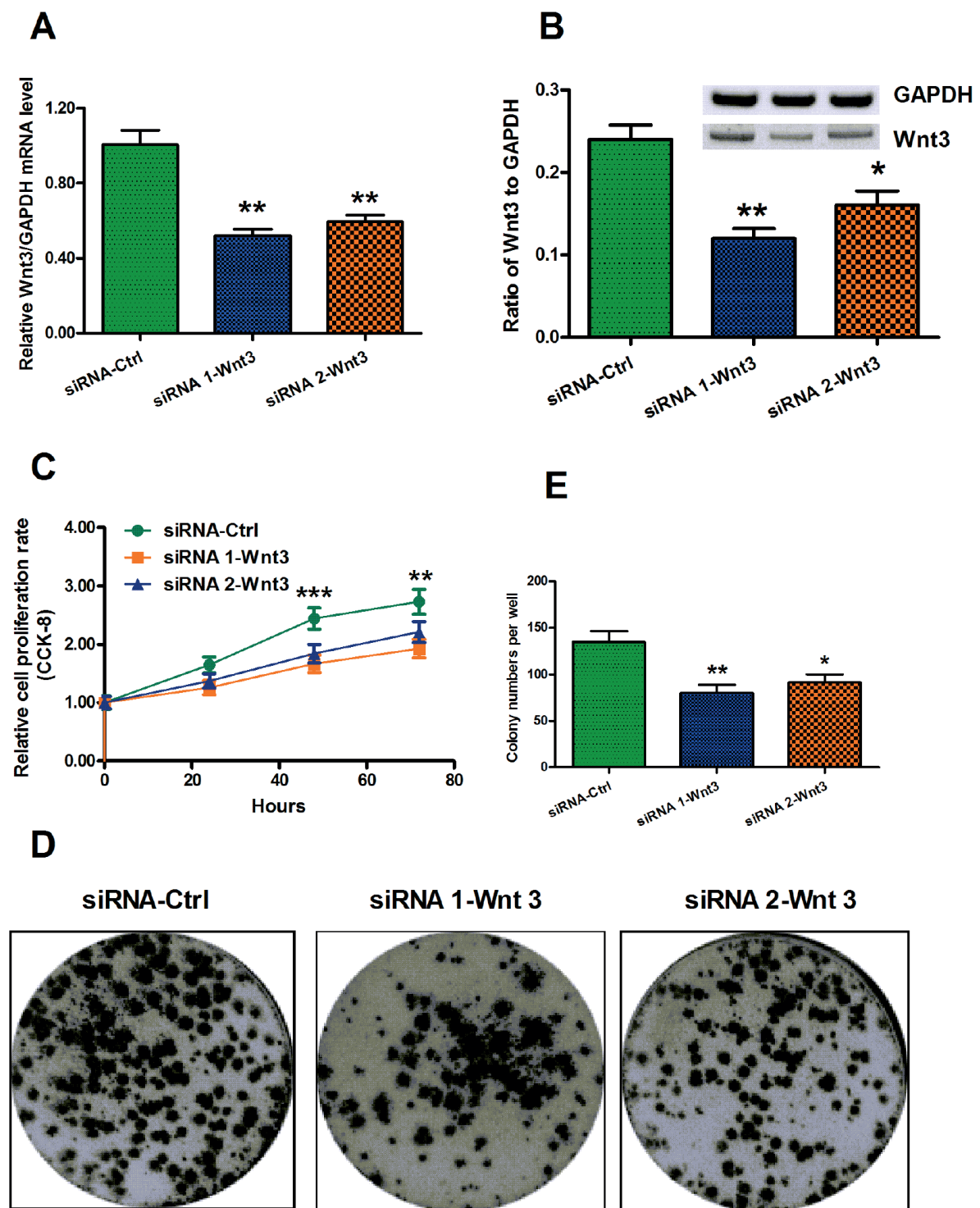


Figure 2. Proliferation of NSCLC A549 cells post the knockdown of Wnt3 with siRNAs. **A**, Relative mRNA level of Wnt3 to GAPDH in NSCLC A549 cells, which were transfected with 50 nM Wnt3-specific siRNAs (siRNA 1/2-Wnt3) or control siRNA (siRNA-Ctrl) for 24 hours. **B**, Western blot analysis of Wnt3 expression in protein level in A549 cells, which were transfected with 50 nM siRNA 1/2-Wnt3 or siRNA-Ctrl for 24 hours, with GAPDH as an internal control. **C**, CCK-8 analysis of the proliferation of siRNA 1/2-Wnt3- or siRNA-Ctrl-transfected (50 nM) A549 cells. **D**, Colony formation assay of siRNA 1/2-Wnt3- or siRNA-Ctrl-transfected (50 nM) A549 cells. Experiments were performed independently in triplicate. Statistical significance was shown as * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

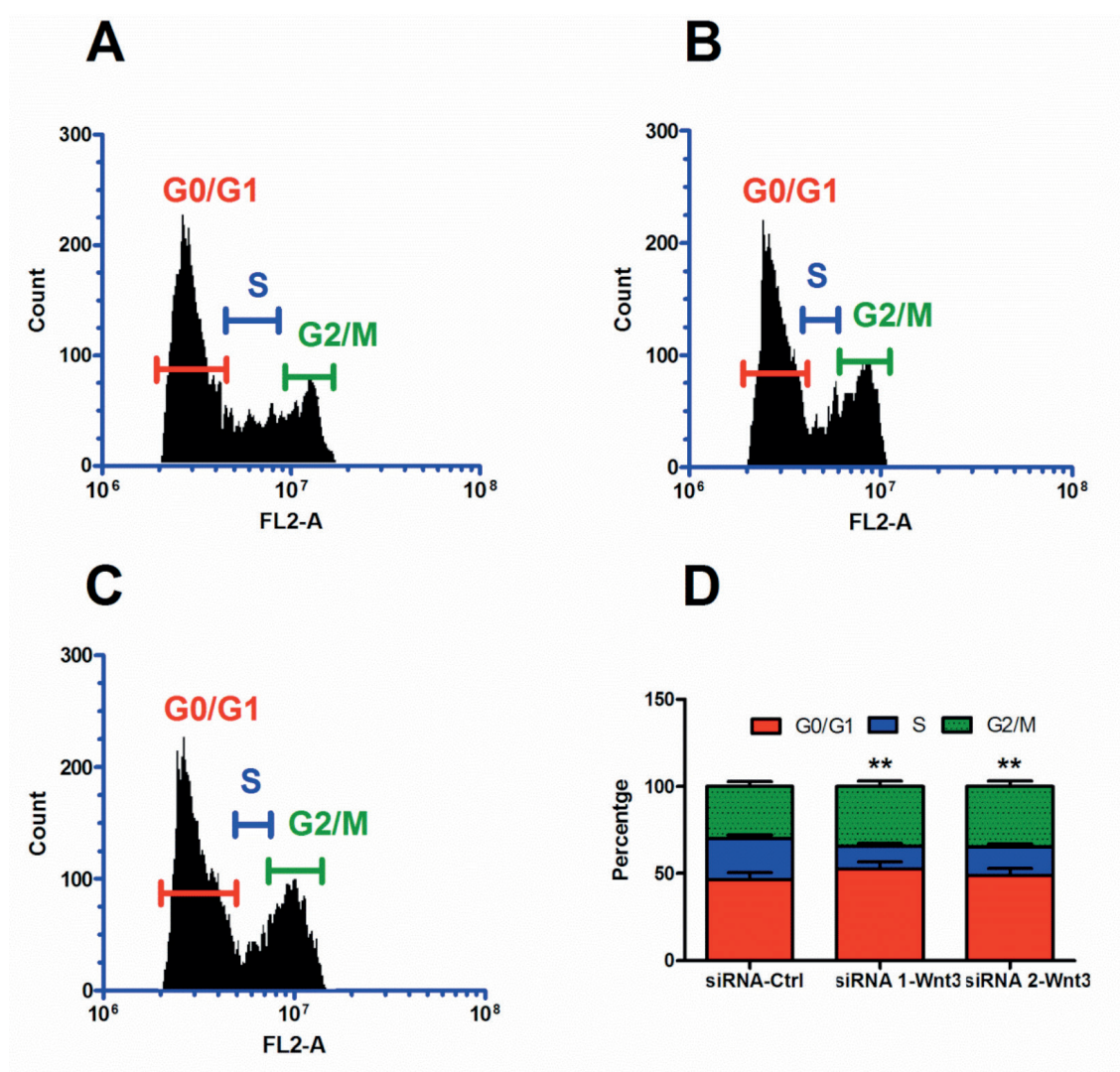


Figure 3. Cell cycles of the A549 cells post the knockdown of Wnt3 with siRNAs. **A-C**, Flow cytometry analysis of the cell cycle distribution in the A549 cells, which were transfected with 50 nM Wnt3-specific siRNAs (siRNA 1/2-Wnt3) or control siRNA (siRNA-Ctrl) for 24 hours, in the presence of 2 μ M Cisplatin. **D**, The percentages of cells in the G0/G1, S, G2/M phases of the cell cycle in each group were measured by using the cellQuest Pro software.

1-Wnt3-transfected A549 cells were also less than the colonies formed by siRNA-Ctrl-transfected A549 cells. Such role of Wnt signaling has also been confirmed in other studies in which inhibition of Wnt/ β -catenin signaling with Wnt inhibitor or Wnt depletion attenuated the proliferation and motility of NSCLC cells and increased their apoptosis³¹.

The Wnt signaling has been recognized to associate with the resistance of metastatic NSCLC to chemotherapy^{24,32}. Our study indicated a sensitization effect by the Wnt3 knockdown. Cispla-

tin-induced more apoptotic A549 cells, with the Wnt3 knockdown, in comparison with the A549 cells without Wnt3 knockdown. And our Western blotting results presented more activated caspase 9 and caspase 3 in the Wnt3 knocked-down A549 cells. Therefore, Wnt3 knockdown sensitized human non-small cell type lung cancer cells to cisplatin via regulating the cell proliferation and apoptosis. More importantly, our results demonstrated that the Wnt3 knockdown also deregulated the cell cycle of A549 cells. Flow cytometry results demonstrated a less proportion of cells in

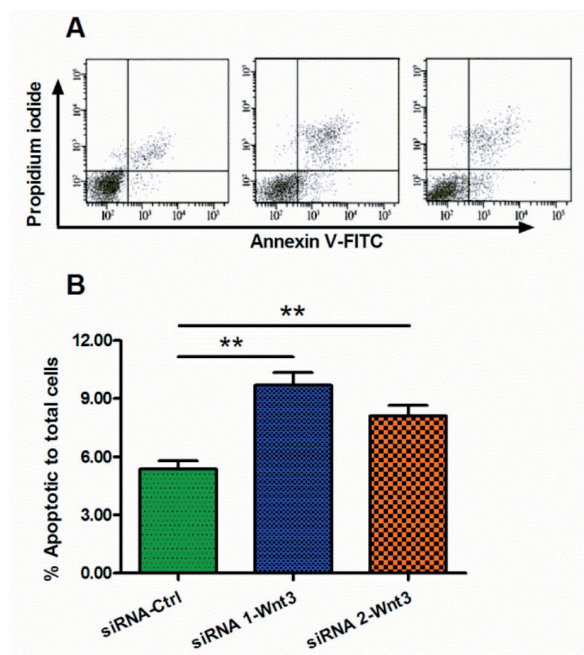


Figure 4. Apoptosis in the A549 cells post the knockdown of Wnt3 with siRNAs. A549 cells were transfected with 50 nM siRNA 1/2-Wnt3 or siRNA-Ctrl, and then were incubated with 2 μ M Cisplatin for 24 hours; then, the cell apoptosis was assessed by flow cytometry using Annexin V-FITC/PI Apoptosis Detection Kit. **A** and **B**, Representative images (**A**) and the percentage (**B**) for apoptotic A549 cells, post the transfection with siRNA 1/2-Wnt3 or siRNA-Ctrl (Later apoptotic cells were denoted in the upper right quadrant, and the early apoptotic cells were denoted in the lower right quadrant). ** $p < 0.01$.

S phase while a more proportion of cells in G0/G1 phase in the Wnt3 knockdown group than in the control group of cells. We speculated that the regulatory role of Wnt3 in the sensitization of NSCLC cells to cisplatin might be associated with the deregulation by it on the cell cycle.

Conclusions

Wnt3 is overexpressed in non-small cell type lung cancer tissues, Wnt3 knockdown sensitizes NSCLC cells to chemotherapy, via inhibiting the proliferation, inducing the apoptosis and deregulating the cell cycle of NSCLC cells. Our study suggests that Wnt3 signaling might be effective target for the chemotherapy of NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

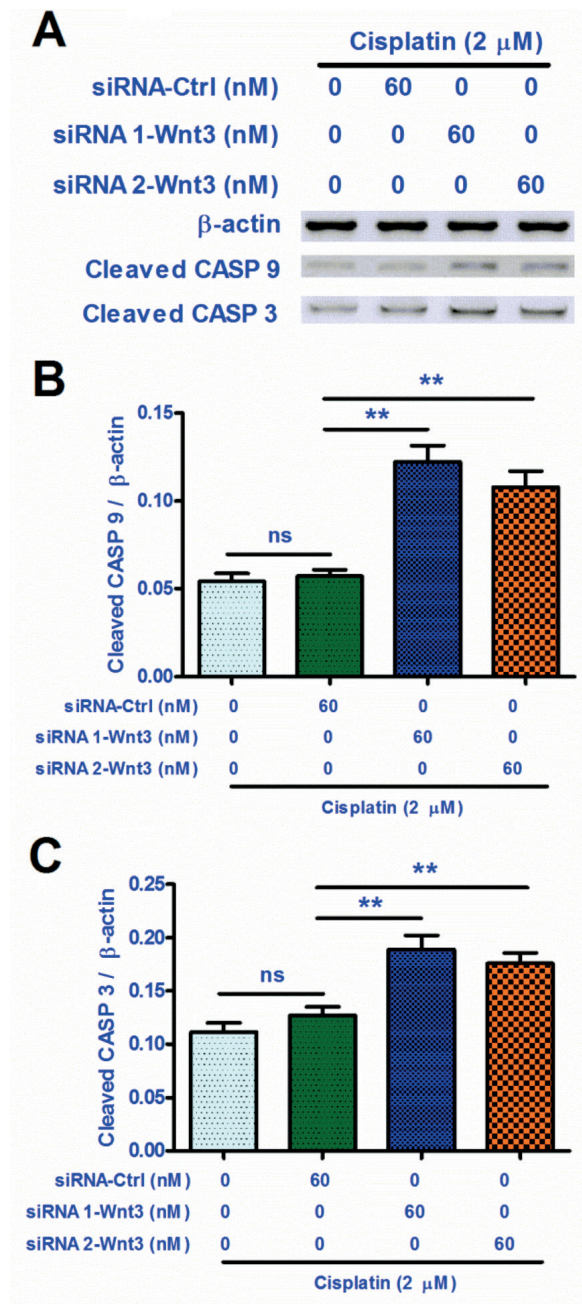


Figure 5. Western blotting assay for apoptosis-associated caspases in the A549 cells post the Wnt3 knockdown. A549 cells were transfected with 50 nM siRNA 1/2-Wnt3 or siRNA-Ctrl, and then were incubated with 2 μ M Cisplatin for 24 hours; then, Western blotting assay was performed to examine the levels of active caspase 9 and active caspase 3 (Cleaved CASP 9 or Cleaved CASP 3). **A**, Representative Western blot analysis (**A**) and the relative levels of Cleaved CASP 9 (**B**) or Cleaved CASP 3 (**C**) in the siRNA 1/2-Wnt3- or siRNA-Ctrl-transfected A549 cells, post the Cisplatin treatment (2 μ M for 24 hours). Experiments were performed independently in triplicate. ** $p < 0.01$, ns: no significance.

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