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Long non-coding RNA MIR503HG serves as a tumor suppressor in non-small cell lung cancer mediated by wnt1

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Abstract. – OBJECTIVE: The incidence and death rate of lung cancer has been rising year by year. Non-small cell lung cancer (NSCLC) seriously affects people's health and quality of life. This study was designed to explore the functional role of long-chain non-coding RNA (LncRNA) MIR503HG in the development of NSCLC.

PATIENTS AND METHODS: The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay was conducted to access the expression level of MIR503HG in NSCLC cell lines and tissues. The Cell Counting Kit-8 (CCK-8) assay, colony formation assay, and flow cytometric analysis were performed to assess the ability of MIR503HG in regulating cell proliferation and apoptosis in NSCLC. Subsequently, Western blotting was used to detect the expression level of Wnt1 in NSCLC. Besides, *in vivo* tumorigenesis assay was performed in nude mice to examine the ability of MIR503HG in tumor formation.

RESULTS: MIR503HG was downregulated in NSCLC. CCK-8 assay and colony formation assay revealed that MIR503HG negatively regulated cell proliferation in NSLCL progression. In addition, MIR503HG promoted cell apoptosis and suppressed cell cycle progression in NSCLC *in vitro*. MIR503HG inhibited tumor formation in nude mice bearing NSCLC *in vivo*. MIR503HG downregulated Wnt1 expression in NSCLC.

CONCLUSIONS: Lon non-coding RNA MIR503HG was downregulated in NSCLC. The over-expression of MIR503HG suppressed cell proliferation and promoted cell apoptosis *in vitro* and repressed tumorigenesis *in vivo*. MIR503HG suppressed NSCLC progression *via* negatively regulating Wnt1 expression.

Key Words:
LncRNA MIR503HG, NSCLC, Proliferation, Apoptosis, Wnt1.

Introduction

In the past 50 years, the incidence and death rate of lung cancer has been rising year by year. Statistical data showed that the incidence and death rate of lung cancer is the first among all malignant tumors[1]. About 80% of lung cancers pathologically belong to non-small cell lung cancer (NSCLC)[2], with poor prognosis and overall survival rate below 20%[3]. Distant metastasis is the leading cause of NSCLC-related death[4]. Therefore, it is of great practical significance to elucidate the molecular mechanisms of its occurrence and development of NSCLC.

Long non-coding RNA (LncRNA) is a kind of non-coding RNA with a length of more than 200 bp, which regulates gene expressions through various ways and plays biological functions[5]. In recent years, the relationship between lncRNAs and tumors has been gradually paid attention. Functionally, they play a pro-cancer role as proto-oncogenes, or an anti-cancer role as an anti-cancer gene[6,7]. Many lncRNAs have been shown to play important roles in the invasion and metastasis of NSCLC[8,9].

The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay was performed to examine the expression level of lncRNA MIR503HG in NSCLC, which was found to be downregulated. Through Cell Counting Kit-8 (CCK-8) assay and colony formation assay, we determined that MIR503HG suppressed cell proliferation *in vitro*. Besides, the flow cytometric analysis revealed that MIR503HG promoted cell apoptosis and suppressed cell cycle progression in NSCLC. *In vivo* assay elucidated that MIR503HG inhibited tumor formation in nude mice bearing NSCLC. Additionally, we revealed that MIR503HG exerted its physiological function by negatively regulating Wnt1 expression in NSCLC.

Patients and Methods

Tissue Samples

A total of 35 pairs of NSCLC tissues and adjacent tissues were collected. All tissue samples

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were obtained from NSCLC patients undergoing surgical treatment in Hubei Tumor Hospital from October 2016 to December 2017. The tissue samples were immediately put into liquid nitrogen. All tumor tissues were confirmed by pathological examination. This present research was approved by the Ethics Committee of Hubei Tumor Hospital. Written informed consents were signed from all participants before the study.

Cell Lines

A total of 3 NSCLC cell lines H1299, SPCA1, A549, and human bronchial epithelioid cell line 16HBE were obtained from ATCC (Manassas, VA, USA). All cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) Medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2.

Cell Transfection

MIR503HG overexpression in selected cell lines was conducted through the transfection of pcDNA3-MIR503HG with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). All transfections were conducted following the manufacturer's instructions. The transfection efficiency was detected by the qRT-PCR assay.

Isolation of Total RNA and qRT-PCR

The total RNA of tissue specimens and cell lines were extracted through TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following standard protocols. All complementary deoxyribose nucleic acids (cDNAs) were synthesized *via* Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) according to standard protocols. The lncRNA-MIR503HG expression level was assessed through SYBR Green real time-PCR, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as normalization. The primers were as following: MIR503HG F: 5'-CAG CCT TCC TGA AAG ACC A-3', R: 5'-TGT TGA TGT AGT GTT CCT GGG T-3'; GAPDH F: 5'-CCA AAA CCA GAT GGG GCA ATG CTG G-3', R: 5'-TGA TGG CAT GGA CTG TGG CCA TCC A-3'.

Colony Formation

The cells (1.0×10^3) were plated into the culture plates (60 mm) and cultured for 2 weeks. The cells were then washed by phosphate-buffer saline (PBS; Gibco, Rockville, MD, USA) twice and fixed in ice-cold 70% methanol for 15 min. Crystal Violet Staining Solution (Beyotime, Shanghai, China) was used to stain the cell colonies. All the colonies were subsequently captured and counted.

CCK-8 Assay

The CCK-8 assay was conducted to examine cell proliferation. The transfected cells were planted into 96-wells plates (6×10^3/well) and incubated with CCK-8 solution (Beyotime, Shanghai, China) (10 μL/well) for 2 hours at 37°C. The optical density (OD) value (450 nm) was then evaluated.

Flow Cytometric Analysis

For detecting the apoptotic cells, the Annexin V-FITC (fluorescein isothiocyanate)/Propidium Iodide (PI) Apoptosis Detection Kit (Vazyme, Nanjing, China) was used according to the instruction. To examine the cell cycle, the transfected cells were immersed in 70% ethanol at -20°C overnight before staining with propidium iodide (PI; Vazyme, Nanjing, China). The flow cytometric analysis was taken place at BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blot

The total protein was isolated by RIPA buffer (Thermo, Waltham, MA, USA) and phenylmethanesulfonyl fluoride (PMSF). Protein lysates isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were then transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Then, the membrane was immunostained at 4°C by primary antibodies overnight. Primary rabbit antibodies used in the current study included anti-Wnt1 (CST, Danvers, MA, USA), and rabbit anti-GAPDH (CST, Danvers, MA, USA) as a loading control. The protein level was determined by Image Lab software.

Xenograft Model

This study was approved by the Animal Ethics Committee of Experimental Animal Center of Tongji Medical College. The transfected SPCA1 or A549 cells (7×10^5/ml) were injected into two flanks of nude mice (6 weeks old) subcutaneously. Tumor growth was monitored and recorded every week. The formula (volume = length × width^2 × 1/2) was used to calculate tumor volume. The tumors were extracted after 4 weeks.

Statistics Analysis

All experiments in this study were performed three times independently, at least. All data re-

corded were exhibited as mean ± standard deviation (SD). The Student's unpaired *t*-test was used to analyze the differences between the two groups. $p<0.05$ was considered as statistically significant.

Results

Long Non-Coding RNA MIR503HG Was Downregulated in NSCLC

QRT-PCR assay was used to determine the expression level of MIR503HG. The expression level of MIR503HG was significantly downregulated in NSCLC tissues than that of adjacent ones (Figure 1A). Similarly, we examined the expression level of MIR503HG in NSCLC cell lines. It turned out that NSCLC cell lines had a relatively lower expression level of MIR503HG (Figure 1B). The transfection efficiency of pcDNA3-MIR503HG was accessed by qRT-PCR. As shown in Figure 1C, the transfection of pcDNA3-MIR503HG significantly upregulated MIR503HG.

MIR503HG Suppressed Cell Proliferation In Vitro

Subsequently, we conducted the CCK-8 assay and colony formation assay to examine cell proliferation. As Figure 2A showed, the overexpression MIR503HG markedly decreased the viability compared with the control group. Similarly, the relative colony number was lower in NSCLC cells overexpressing MIR503HG (Figure 2B). All data elucidated that upregulated MIR503HG inhibited cell proliferation *in vitro.*

Upregulated MIR503HG Repressed Cell Cycle Progression and Promoted Cell Apoptosis In Vitro

Flow cytometric analysis was performed to examine the effect of MIR503HG on cell cycle and apoptosis. As shown in Figure 3A,

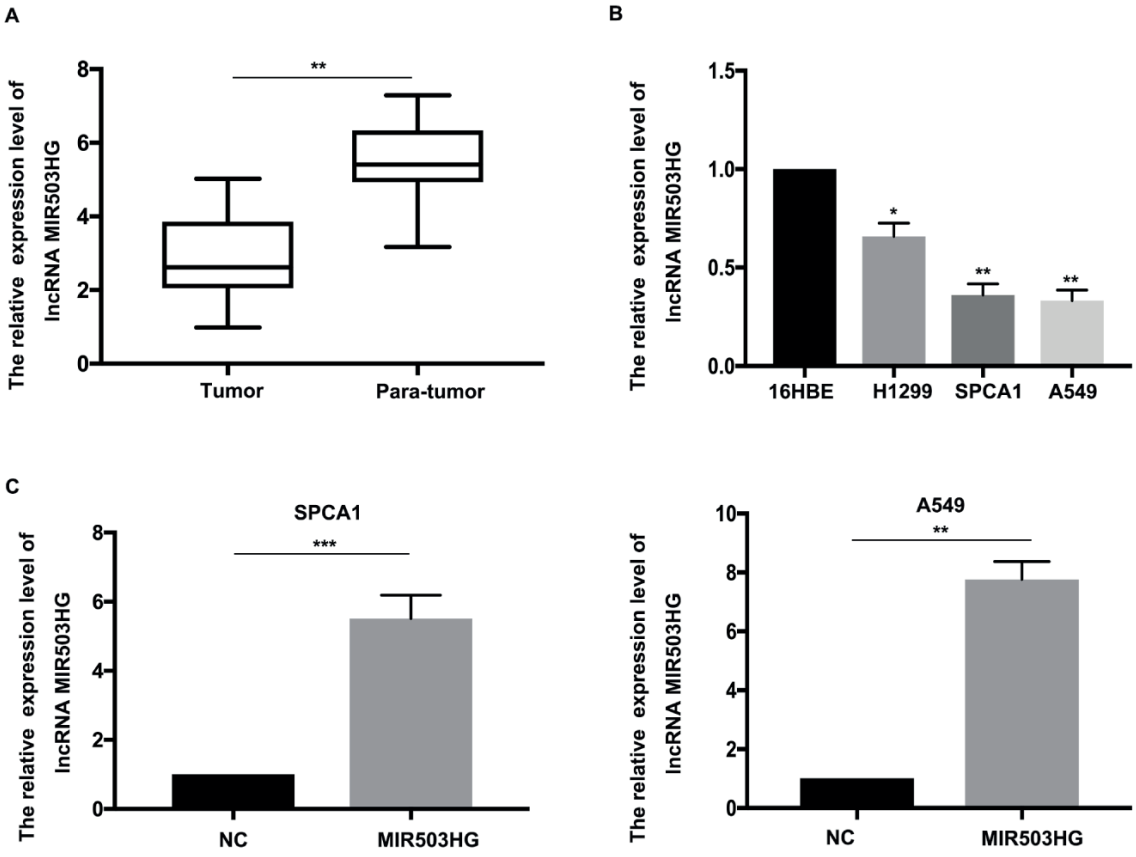

Figure 1. Long non-coding RNA MIR503HG was downregulated in NSCLC. **A,** The expression level of MIR503HG in NSCLC tissues and para-tumor tissues. **B,** Analysis of MIR503HG expression level in NSCLC cell lines. **C,** Transfection efficiency was evaluated by qRT-PCR. Data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

MIR503HG overexpression arrested cell cycle progression in the G1/0 phase. Moreover, the upregulated MIR503HG facilitated cell apoptosis *in vitro* (Figure 3B). Taken together, we considered that MIR503HG overexpression led to arrested cell cycle progression and promotion in cell apoptosis.

MIR503HG Overexpression Inhibited Tumor Formation In Vivo

The tumorigenicity assay was performed to analyze the ability of MIR503HG in tumor formation in nude mice. As Figure 4A showed, the tumor volumes in mice administrated with pcDNA3-MIR503HG were relatively smaller than the

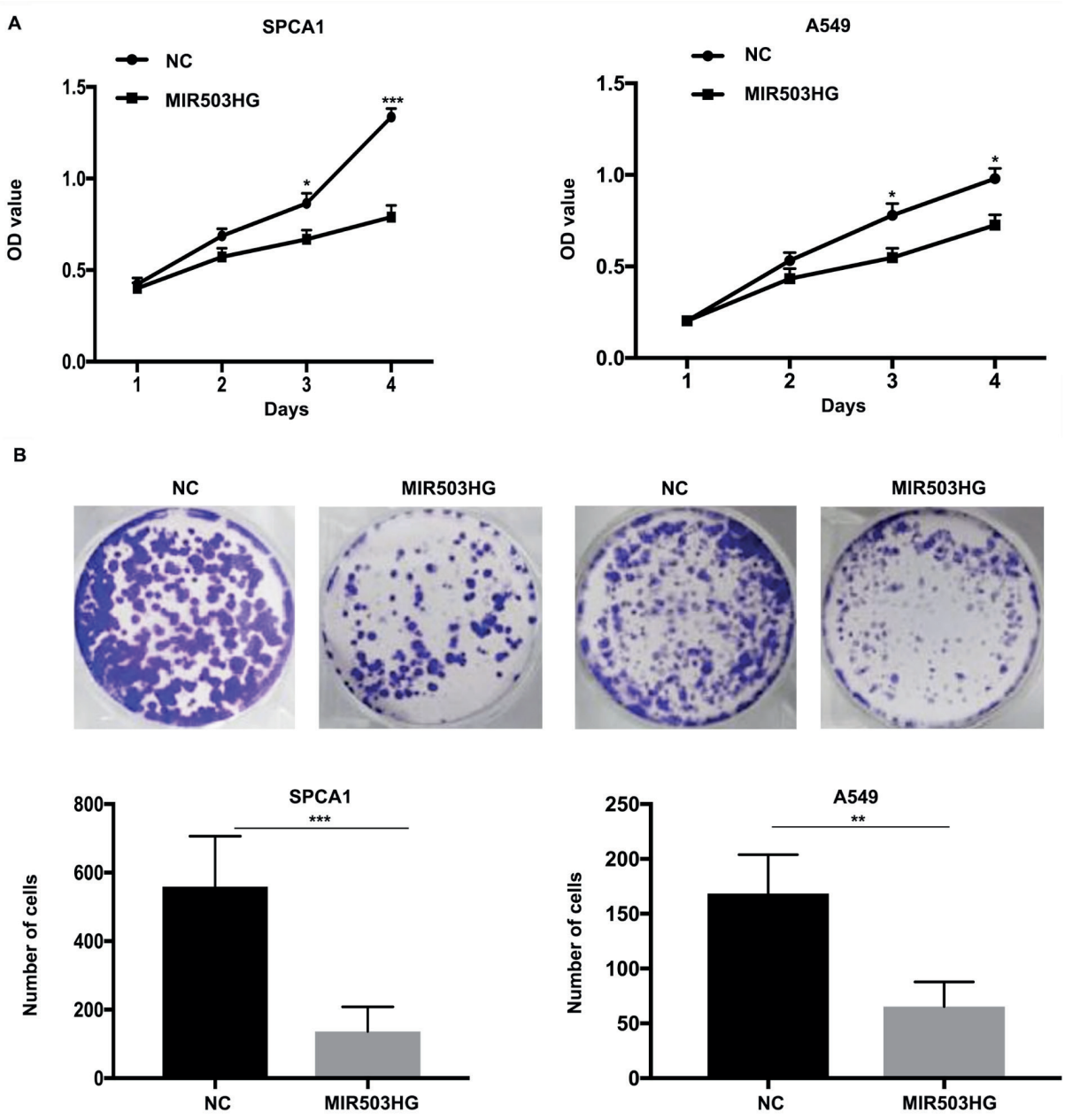

Figure 2. MIR503HG suppressed cell proliferation *in vitro*. **A,** The cell proliferation ability was determined by CCK-8 assay. **B,** Colony formation assay was recruited for detecting cell proliferation (magnification: 10x). Data are presented as the mean ± SD of three independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001.

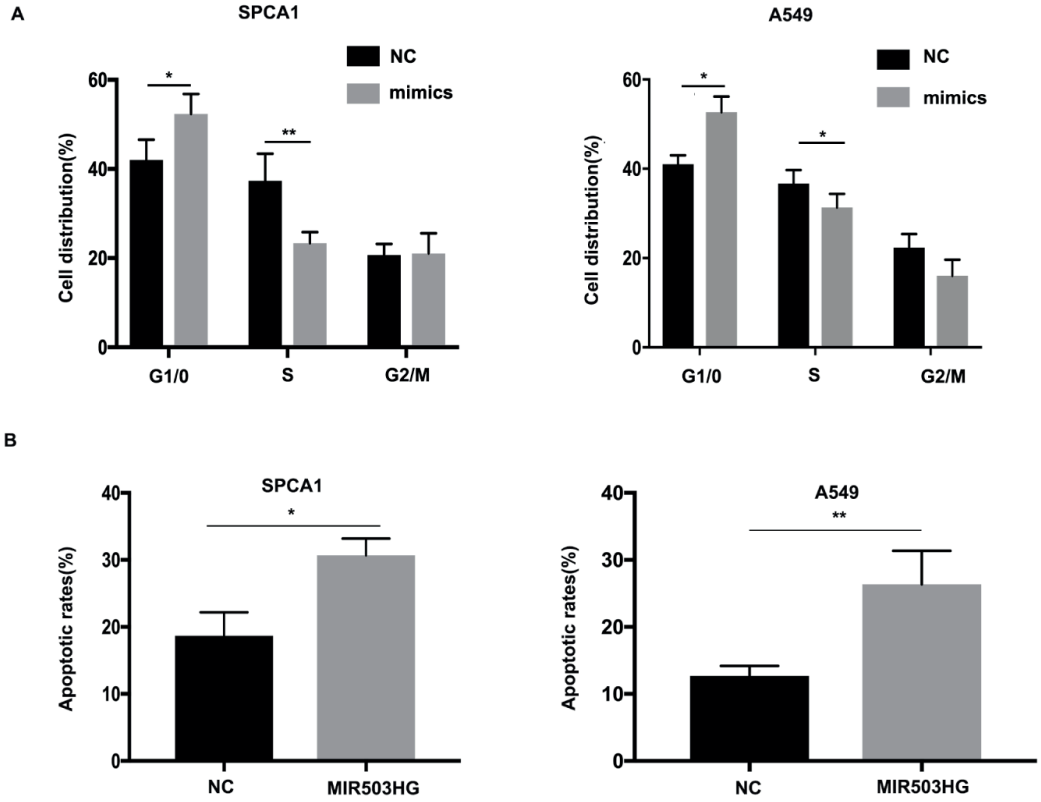

Figure 3. Upregulated MIR503HG repressed cell cycle progression and promoted cell apoptosis *in vitro*. **A,** Cell cycle progression was detected by flow cytometric analysis in the transfected groups. **B,** Flow cytometric analysis was performed to detect the apoptotic rates in the transfected cells. Data are presented as the mean ± SD of three independent experiments. **p*<0.05, ***p*<0.01.

control group. Consistently, tumor weight was lighter in mice overexpressing MIR503HG as well (Figure 4B). Besides, we examined the expression level of MIR503HG in the generated tumors. The results showed that mice overexpressing MIR503HG had a relatively higher expression level of MIR503HG (Figure 4C). In sum, the results indicated that MIR503HG suppressed tumor formation *in vivo*.

MIR503HG Negatively Regulated Wnt1 in NSCLC Progression

To reveal the underlying molecular mechanism of MIR503HG in NSCLC progression, we validated the involvement of Wnt1 in NSCLC progression. As shown in Figures 5A and 5B, the overexpression of MIR503HG lowers the expression level of Wnt1 *in vitro*. However, the expression level of MIR503HG was unchangeable in the cells overexpressing Wnt1 (Figure 5C). Hence, all data indicated that MIR503HG may exert its physiological function in NSCLC progression *via* negatively regulating Wnt1 expression.

Discussion

Lung cancer has one of the highest mortality rates in the world. The incidence of non-small cell lung cancer (NSCLC) accounts for 4/5 of the total cases of lung cancers[10,11], and the prognosis is not optimistic. The survival rate of NSCLC patients over 5 years is less than 15%[12,13]. In recent years, surgery, chemoradiotherapy, and other treatment methods have been improved, but the overall efficacy of lung cancer is not ideal[4]. In the past few decades, the carcinogenesis and pathophysiology of NSCLC have been widely studied, but the molecular mechanism of its occurrence and development remain unclear. It is of great significance to study the molecular mechanism of the develop-

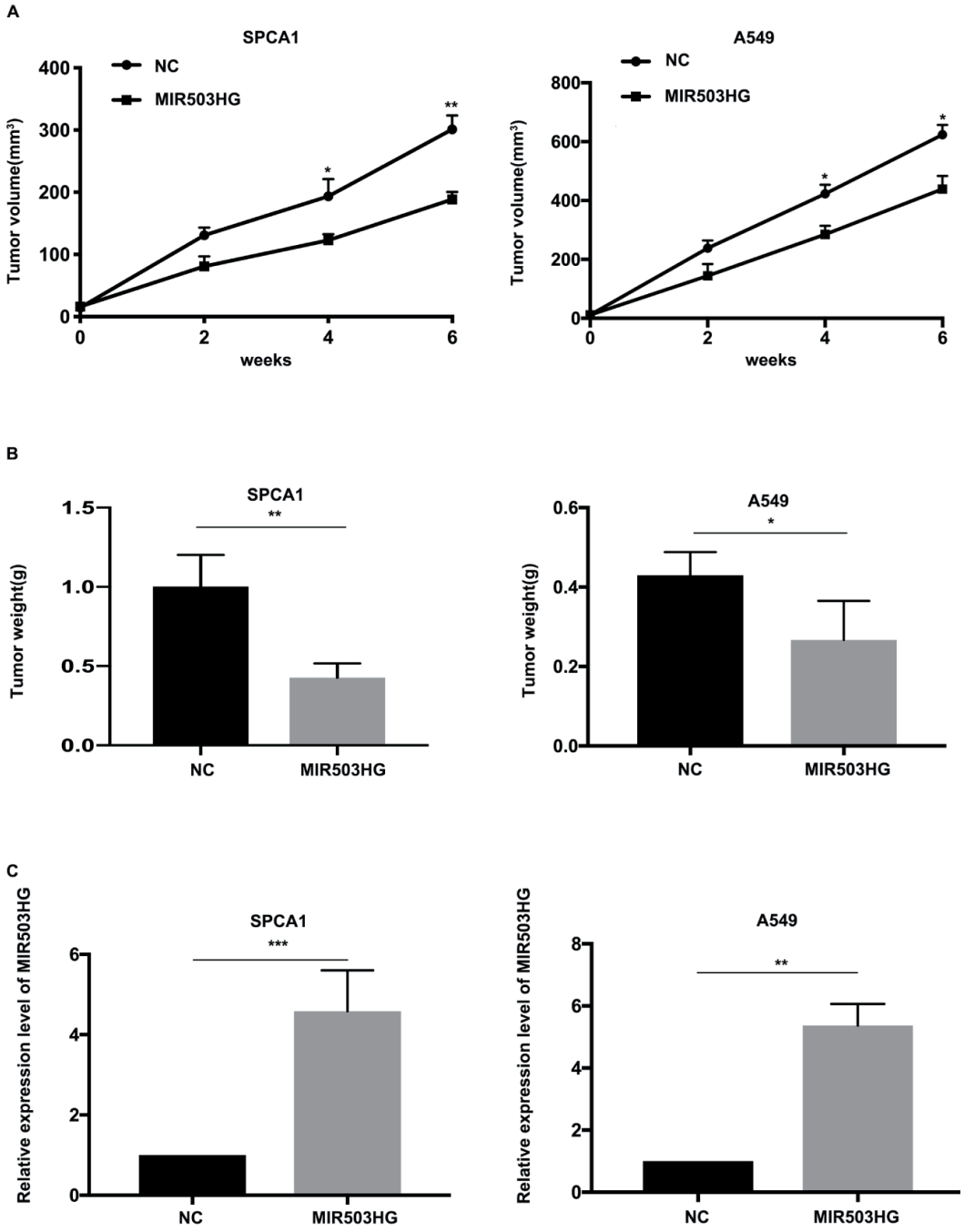

Figure 4. Overexpressed MIR503HG inhibited tumor formation *in vivo.* **A,** After tumor extraction, the tumor volume was calculated. **B,** Tumor weight was recorded. **C,** The relative expression of MIR503HG in tumors was examined by qRT-PCR. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

ment of lung cancer to improve the prognosis of patients with advanced lung cancer.

Long non-coding RNA is believed to regulate the growth, apoptosis, and metastasis of cancer cells[14]. Recent studies have shown that long non-coding RNA plays an important role in tumor development. It regulates gene expressions at the transcriptional or post-transcriptional level and plays an important role in the development of tumor[15]. It was found that the long non-coding RNA is correlated with the development of NSCLC[16], glioma[17], and thyroid cancer[18]. A large

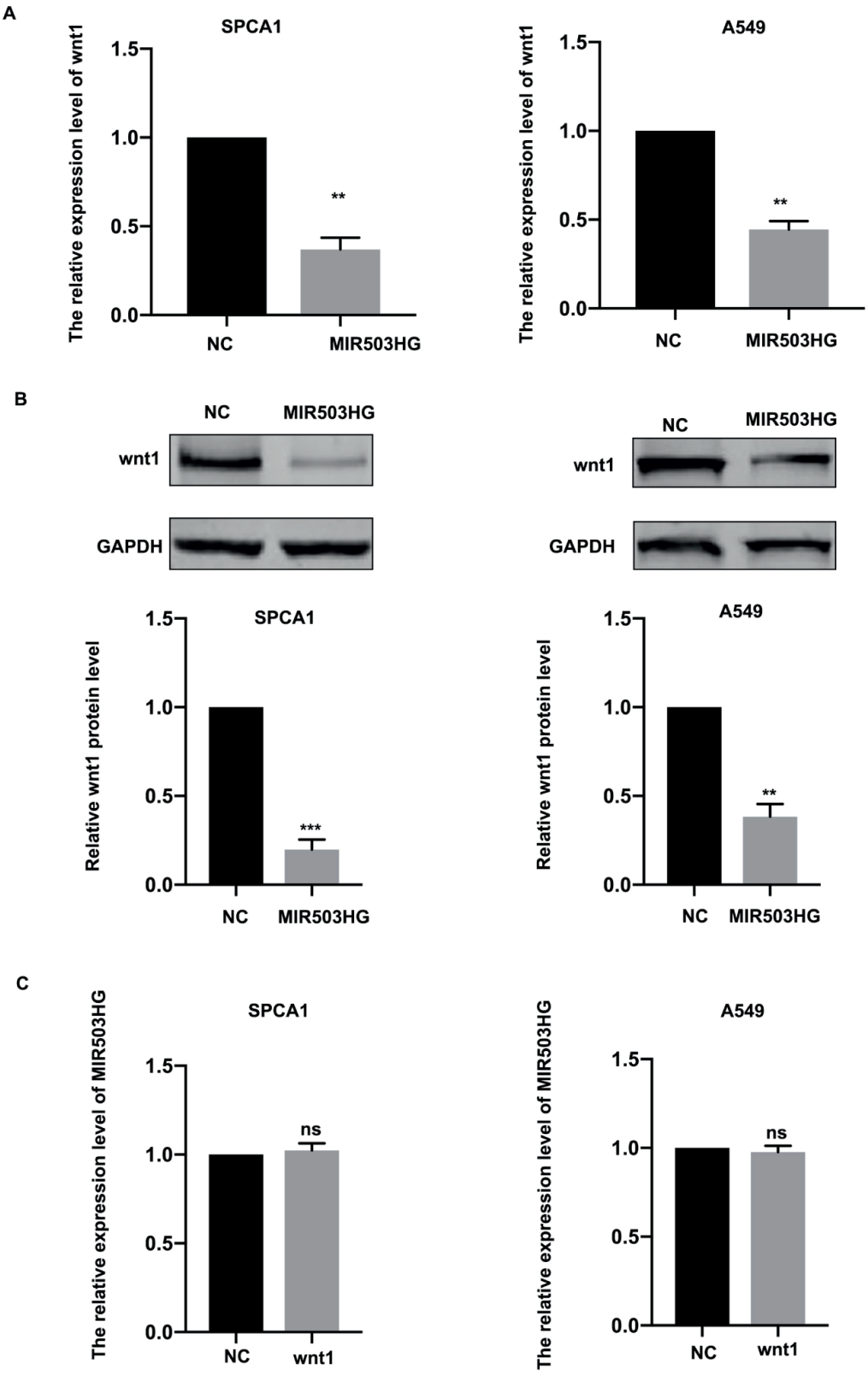

Figure 5. MIR503HG negatively regulated Wnt1 in NSCLC progression. **A,** Wnt1 expression level was determined in the transfected cell lines. **B,** Protein level of Wnt1 was examined in transfected cell lines. **C,** MIR503HG expression level was detected in Wnt1 over-expression cell lines. Data are presented as the mean ± SD of three independent experiments. $^{**}p<0.01$, $^{***}p<0.001$.

number of lncRNAs have been identified to be involved in the development of NSCLC, such as PCAT6[13], AFAP1-AS1[19], SNHG16[20], etc. These lncRNAs influence the occurrence and development of NSCLC by regulating autophagy, proliferation, apoptosis, invasion, migration, or EMT of NSCLC cells.

In the current study, we determined that MIR503HG was downregulated in NSCLC. Through CCK-8 assay and colony formation assay, we revealed that MIR503HG negatively regulated cell proliferation in NSLCL progression. In addition, flow cytometric analysis indicated that MIR503HG was also involved in promoting cell apoptosis and suppressing cell cycle progression *in vitro*. In tumor formation assay, it turned out that MIR503HG inhibited tumor formation *in vivo*. To figure out the underlying mechanism, we examined the expression level of Wnt1 in the transfected cells, and it suggested that MIR503HG downregulated Wnt1 expression, thereafter regulating the development of NSCLC.

Conclusions

Long non-coding RNA MIR503HG was downregulated in NSCLC. The overexpression of MIR503HG suppressed cell proliferation and promoted cell apoptosis *in vitro* and repressed tumor formation *in vivo*. We revealed that MIR503HG suppressed NSCLC progression *via* negatively regulating Wnt1 expression.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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