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LncRNA ASB16-AS1 promotes proliferation and inhibits apoptosis of non small cell lung cancer cells by activating the Wnt/β catenin signaling pathway

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Abstract. – OBJECTIVE: To detect the expression of long non-coding ribonucleic acid (lncRNA) ASB16-AS1 in non-small cell lung cancer (NSCLC) tissues and cells, and to explore the effect of lncRNA ASB16-AS1 on the biological functions of NSCLC cells.

PATIENTS AND MATERIALS: The expression level of lncRNA ASB16-AS1 in NSCLC tissues and cells was detected *via* real-time fluorescence quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). The interference sequences of lncRNA ASB16-AS1 were designed and synthesized, and its transfection efficacy was detected by qRT-PCR. The knockdown of lncRNA ASB16-AS1 on the proliferation, cell cycle, and apoptosis of NSCLC cells were detected *via* cell counting kit-8 (CCK-8), colony formation assay and flow cytometry, respectively. Moreover, the expression changes in the Wnt/β catenin signaling pathway were detected *via* Western blotting.

RESULTS: LncRNA ASB16-AS1 was upregulated in NSCLC tissues and cells compared with that in normal carcinoma tissues and 16HBE cells. The results of CCK-8 assay and colony formation assay revealed that the silence of lncRNA ASB16-AS1 attenuated the proliferative ability in NSCLC cells. The results of flow cytometry manifested that the silence of lncRNA ASB16-AS1 arrested the cells in G0/1 phase, and accelerated the apoptosis rate. The key proteins in the Wnt/β-catenin signaling pathway were regulated by ASB16-AS1 in NSCLC.

CONCLUSIONS: LncRNA ASB16-AS1 is upregulated in NSCLC tissues and cells, which promotes proliferation and inhibits apoptosis of NSCLC cells through the Wnt/β-catenin signaling pathway.

Key Words:
NSCLC, LncRNA ASB16-AS1, Proliferation, Apoptosis.

Introduction

Lung cancer is a malignant tumor with the highest morbidity and mortality rates in the world. There are about 1.8 million new cases of lung cancer globally every year[1]. According to the pathological morphology and clinical features, lung cancer can be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and the latter accounts for about 80-85%[2]. Currently, surgery is still the main treatment for NSCLC. Great improvement has been made in therapeutic methods with the gradual maturity of therapeutic techniques, but the 5-year survival rate of NSCLC is still unsatisfactory (only 15%)[3,4]. Therefore, it is of great significance to explore the mechanisms underlying the occurrence and development of lung cancer for searching for effective diagnostic indexes and therapeutic targets.

Long non-coding ribonucleic acids (lncRNAs) are RNA strands with more than 200 bases in length without the protein-coding ability. Existing investigations[5-7] have demonstrated that lncRNAs are involved in the occurrence and development of various diseases, such as tumors and diabetes by regulating transcription, translation, and protein localization. Lingling et al[8] have shown that lncRNAs play a role as "oncogenes" or "tumor-suppressor genes" in the evolution of NSCLC. Hence, upregulated lncRNA SNHG20 inhibits proliferation, invasion, and migration, and promotes the apoptosis of NSCLC cells by adsorbing miR-154. Miao et al[9] found that lncRNA HAND2-AS1 inhibits the invasion and metastasis of NSCLC cells

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by regulating the transforming growth factor-β1 (TGF-β1) signaling pathway. However, the expression, biological function, and potential molecular mechanism of lncRNA ASB16-AS1 in NSCLC have not been reported yet.

LncRNA ASB16-AS1 is located on chromosome 17q21.31, with a total length of 937 bp. Its abnormal expression was first discovered by Zhang et al[10] in gliomas. Upregulated lncRNA ASB16-AS1 promotes the invasion and metastasis of glioma cells by regulating the epithelial-mesenchymal transition. Our study found for the first time that lncRNA ASB16-AS1 was upregulated in NSCLC tissues and cells, which promoted proliferation and inhibited apoptosis of NSCLC cells by regulating the Wnt/β-catenin signaling pathway. The results of the present study enrich the molecular theory of occurrence and development of NSCLC, and provide molecular targets for preventing the malignant proliferation of NSCLC.

Patients and Methods

Tissue Specimens

The carcinoma tissues and paracarcinoma tissues (more than 3 cm away from the carcinoma) were collected from 59 NSCLC patients undergoing excision in the First Affiliated Hospital of Harbin Medical University from January 2014 to June 2018. NSCLC was confirmed *via* pathological examination after the operation. Patients were aged 35-75 years. None received chemoradiotherapy and biotherapy before the operation, and the tissues were not combined with other tumors. The specimens were stored in the liquid nitrogen containing −80°C. The complete data of examinations, treatment, operation, and the contact information of patients were collected. This study was approved by the Ethics Committee of the hospital. The patients signed a written informed consent.

Cell Culture

The normal bronchial epithelial cell line 16HBE, human NSCLC cell lines A549, NCI-H266, NCI-H1299, and SK-MES-1 were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences (Shanghai, China). 16HBE, A549, NCI-H266, H1299, and SK-MES-1 cells were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640) or Dulbecco's Modified Eagle's Medium (DMEM; HyClone, South Logan, UT, USA) containing 100 mL/L fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in a humid environment with 50 mL/L CO_2 at 37°C.

Cell Transfection

NSCLC cells in a good growth state were inoculated into a 6-well plate (1.5×10^5 cells/well), and transfected with the negative control sequence (si-NC) or si-lncRNA ASB16-AS1 (5 μL) according to the instructions of the Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). After transfection, the cells and the supernatant were collected for corresponding detections. The sequences of the corresponding siRNA fragments were as follows: si-ASB16-AS1#1: 5'-GAAGAAGAGAAGAC[illegible]A-3'; si-ASB16-AS1#2: 5'-GATAA[illegible]GTCCACT GACA[illegible]'; si-ASB16-AS1#3: 5'-CTTGGCUAC[illegible]AACGCAUATT-3'.

Real-Time Fluorescence Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), respectively, followed by the measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative level of the target gene was expressed by the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: lncRNA ASB16-AS1 forward: 5'-ATTGAAGATGCTGCCAAGGG-3', reverse: 5'-GCCCAGTTTCAGTCCCTCTT-3'; GAPDH forward: 5'-TGCACCACCAACTGCTTAGC-3', reverse: 5'-GGCATGGACTGTGGTCATGAG-3'.

Cell Counting Kit-8 (CCK8) Assay and Colony Formation Assay

Quintuplicate wells were set in each group and cells were subjected to corresponding treatment. 100 μL of mixed medium (90 μL of medium + 10 μL of CCK-8 solution/well; Dojindo, Kumamoto, Japan) was added into each plate for incubation at 37°C for 2 h. The absorbance was detected at 450 nm and analyzed using a SpectraMax 190 light absorption microplate reader (Molecular Devices,

San Jose, CA, USA). In the experimental group and control group, the cells were inoculated into the 6-well plate (1×10^3 cells/well) and cultured for about 14 d until the formation of visible colonies. The cells were washed with Phosphate-Buffered Saline (PBS) for several times, fixed with paraformaldehyde (10 g/L) for 30 min, stained with crystal violet dye for 10 min, air dried, and photographed. The assay was repeated for 3 times in each group.

Cell Cycle

In the experimental group and control group, the cells were collected, fixed with cold ethanol (700 mL/L) at 4°C overnight, and stained with propidium iodide (PI, 0.05 mg/mL) and RNase (2 mg/mL) at room temperature for 30 min. Then, the cell cycle was analyzed *via* flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA), and the cell ratio in each phase (G0/G1, S and G2/M phase) was evaluated using the Cell Lab Quanta SC software.

Apoptosis Rate

In the experimental group and control g[illegible] the cells were collected and washed twi[illegible] pre-cooled PBS. According to the instructi[illegible] of the apoptosis kit, the cells were resuspended [illegible] 1×binding buffer (1×10^6 cells/mL). Then, 100 [illegible] of the suspension was added wi[illegible] of Anne[illegible] in V-FITC (fluorescein isoth[illegible]anat[illegible]nd 5 μL of PI, incubated in a dark [illegible]e at roo[illegible]empera-ture for 15 min, and mixe[illegible]ly w[illegible]f binding buffer, follow[illegible]by [illegible] determina-tion using flow cyt[illegible]try.

Western Bl[illegible]g

In the e[illegible]erimental g[illegible] and control group, the cells[illegible]re collected [illegible]ysed with radioimmu[illegible]ecipita[illegible]ion assay (RIPA) lysis buffer (Be[illegible]ne, Sh[illegible]hai, China) on ice for 30 min, follo[illegible]by [illegible]asonic [illegible]cillation at 20 W for 2 min an[illegible]ifugati[illegible] 12000 g and 4°C for 10 [illegible] The [illegible]na[illegible] was collected, and the protei[illegible]ncentr[illegible]as measured. Then, 50 μg of pr[illegible]s were s[illegible]parated *via* sodium dodecyl sul-f[illegible]mide gel electrophoresis (100 g/L; [illegible]acer gel at 80 V for 40 min, and separation gel [illegible]120 V for 90 min), transferred onto the mem-[illegible] at 100 V for 90 min, and sealed at room tem[illegible]erature for 1 h. The membranes were incubated with primary antibodies of p21, β-catenin, CyclinD1 (1:500), and GAPDH (1:1000) at 4°C overnight. After the membrane was washed, the proteins were incubated with secondary antibodies at 37°C for 1 h, and the membrane was washed again, followed by image development.

Statistical Analysis

The Statistical Product and Servic[illegible]tatis[illegible] (SPSS) 18.0 software (SPSS Inc.[illegible]cago, IL, USA) was used for statistical an[illegible] The data were expressed as mean ± standa[illegible]viation (`χ±s). The *t*-test was used [illegible]analyze [illegible]ea-surement data. The diffe[illegible]es between t[illegible] groups were analyzed [illegible]e Stud[illegible] *t*-test. [illegible] comparison between [illegible]ple [illegible]ups wa[illegible] done using One-way A[illegible]VA [illegible]owed [illegible] post-hoc test (Leas[illegible]gnificant [illegible]feren[illegible] $p<0.05$ suggested th[illegible]ifference w[illegible]tically significant.

Results

LncRNA ASB1[illegible]S1 Was Highly E[illegible]essed in [illegible]CLC Tissues and Cells

[illegible]sults [illegible]T-PCR showed that among 59 pairs [illegible] tissues and paracarcinoma tissues, the expression of lncRNA ASB16-AS1 was [illegible]lated in 46 cases (78%) and downregulat-[illegible] cases (22%) (Figure 1A). Compared with that in human normal bronchial epithelial cells (16HBE), the expression of lncRNA ASB16-AS1 was upregulated in NSCLC cells (Figure 1B). To study the biological function of lncRNA ASB16-AS1 in NSCLC cells, si-lncRNA ASB16-AS1 was designed. The transfection efficacy of si-lncRNA ASB16-AS1 in NSCLC cells was detected *via* qRT-PCR (Figures 1C and 1D).

Effect of LncRNA ASB16-AS1 on Proliferation of NSCLC Cells

To study the effect of lncRNA ASB16-AS1 on the proliferation of NSCLC cells, the cells were transfected with si-lncRNA ASB16-AS1 or si-NC, respectively. The results of CCK8 assay revealed that the proliferation ability of NSCLC cells was weakened after the silence of lncRNA ASB16-AS1 (Figures 2A and 2B). Then, the colony formation assay was performed, and the same results were obtained (Figures 2C and 2D).

Effects of LncRNA ASB16-AS1 on Cell Cycle and Apoptosis of NSCLC Cells

The effects of lncRNA ASB16-AS1 on cell cycle progression and apoptosis of NSCLC cell cycle were detected *via* flow cytometry. It is found that

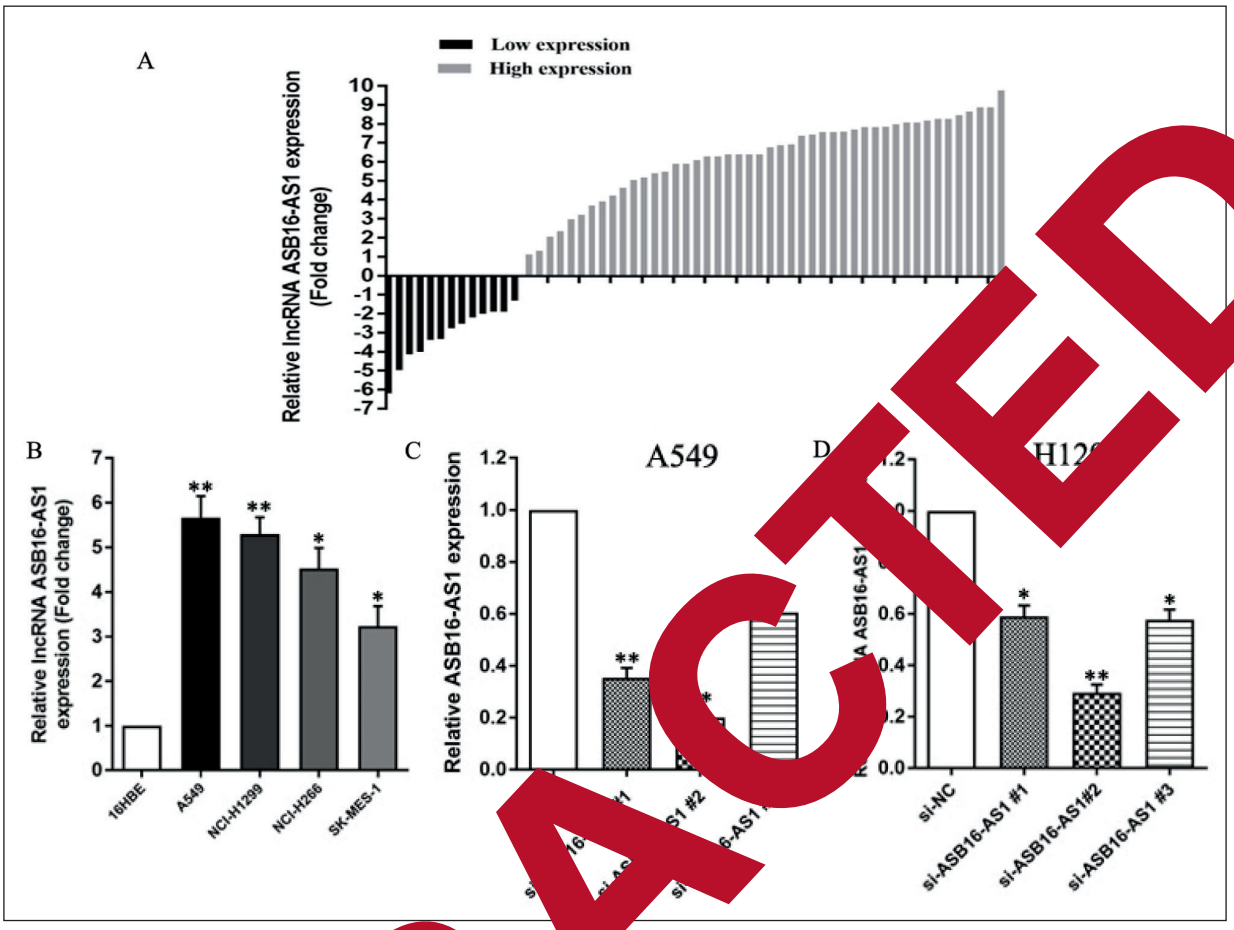

Figure 1. Expression of lncRNA AS[illegible] 59 pairs [illegible]SCLC tissues and paracarcinoma tissues are detected *via* qRT-PCR. LncRNA ASB16-AS1 is upr[illegible]ed in [illegible]ases and [illegible]regulated in 13 cases. **B,** The relative expression of lncRNA ASB16-AS1 is detected *via* qRT[illegible] in NSC[illegible]and 16HBE[illegible]lls. LncRNA ASB16-AS1 is upregulated in NSCLC cells. **C, D,** Interference efficiency of si[illegible]A ASB[illegible]detected *via* qRT-PCR in A549 and H1299 cells (**p<0.01, *p<0.05).

cell cycle was n[illegible]rrested in [illegible]phase after the knockd[illegible]of [illegible]NA ASB16-[illegible]S1 in NSCLC (Figu[illegible]3A and 3E[illegible]reover, an increased apoptosi[illegible]e was identifie[illegible]NSCLC cells with lncRN[illegible]SB16-AS1 knockdo[illegible](Figures 3C and 3D). [illegible]sequen[illegible]the potential involvement of the Wnt[illegible]en[illegible]ignalin[illegible]athway in NSCLC progressio[illegible]ated b[illegible]cRNA ASB16-AS1 was [illegible]med[illegible]We[illegible] blotting (Figures 3E and 3F). [illegible]herefo[illegible]NA AGB16-AS1 promoted the pr[illegible]ration an[illegible]inhibited apoptosis of NSCLC [illegible]e Wnt/β-catenin pathway.

Discussion

NSCLC is the most common malignant tumor in the world, which has extremely high morbidity and mortality rates, ranking first in all malignant tumors[11,12]. In recent years, both morbidity and mortality rates of NSCLC in China have shown an increasing trend, and its treatment faces severe challenges[13]. The pathogenesis of NSCLC involves multiple genes, steps, and stages, in which the genetic and environmental factors are extensive and complex. With the gradual application of molecular biological techniques in diseases, the search for appropriate and effective molecular markers and therapeutic targets is a new strategy for the treatment of NSCLC, currently.

LncRNAs play important regulatory roles in cellular activities. MALAT-1, one of the lung cancer-related lncRNAs, positively regulates the proliferation and metastasis of lung cancer cells mainly by interacting with corresponding miRNAs and proteins[14,15]. Moreover, CCAT-1 has been proved to be highly expressed in lung

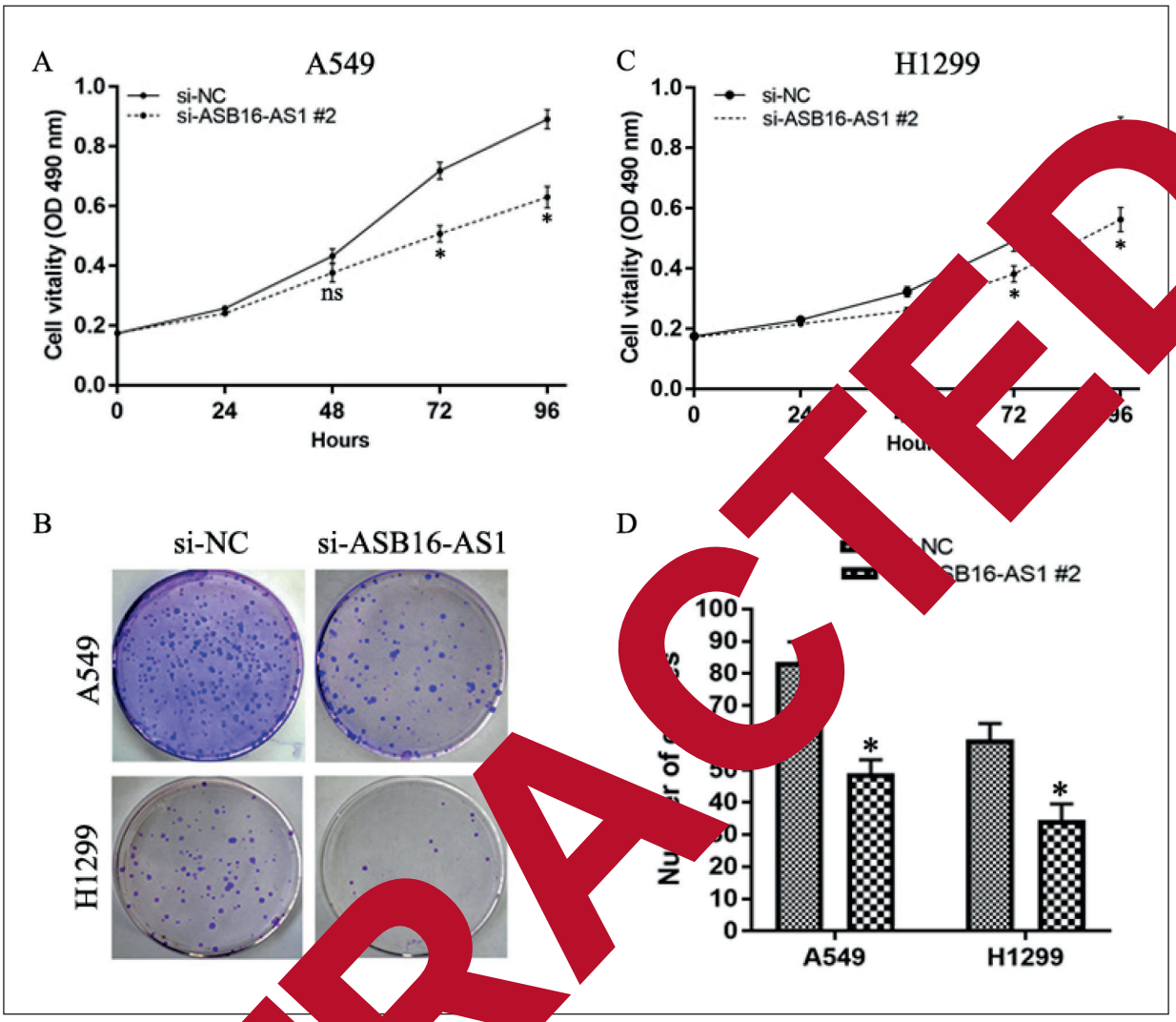

Figure 2. Effect of ln[illegible]ASB16-[illegible] proliferation of NSCLC cells. **A, C,** The results of CCK-8 assay reveal that the proliferation ability [illegible]LC cells is w[illegible]d after the interference in lncRNA ASB16-AS1 expression. **B, D,** The results of the colony for[illegible] reveal that [illegible]oliferation ability of the NSCLC cells declines in the experimental group compared with t[illegible]ontrol [illegible] (**p<0.01,*p 0.05) (magnification ×40).

ade[illegible]rcinom[illegible]nd colorectal cancer, which can [illegible]ed[illegible]an im[illegible]rtant molecular marker for tum[illegible]gnosis[illegible]. Our findings for the first [illegible]den[illegible]rat[illegible]hat lncRNA ASB16-AS1 wa[illegible]pregu[illegible]d NSCLC tissues and cells. Th[illegible]nockdow[illegible] of lncRNA ASB16-AS1 could i[illegible]ration and promote apoptosis of [illegible]SCLC cells.

The Wnt/β-catenin signaling pathway is a [illegible]y conserved signal transduction pathway, wh[illegible]n is closely related to the incidence of various human diseases. It is abnormally activated in liver cancer and lung cancer. The activation of the Wnt/β-catenin signaling pathway can promote the occurrence and development of tumors by modulating proliferation, migration, and apoptosis of tumor cells[18]. In the presence of a Wnt signal, the complex can be formed through a series of processes. Once it is activated, the nuclear βcatenin binds to the TCF/LEF protein and activates target genes, such as c-myc and CyclinD1[19]. Wnt/β-catenin signaling pathway is reported[20,21] to be regulated by many lncRNAs and further participates in tumor progression. This study disclosed that lncRNA ASB16-AS1 could promote proliferation and inhibit apoptosis of NSCLC cells by regulating the Wnt/β-catenin signaling pathway.

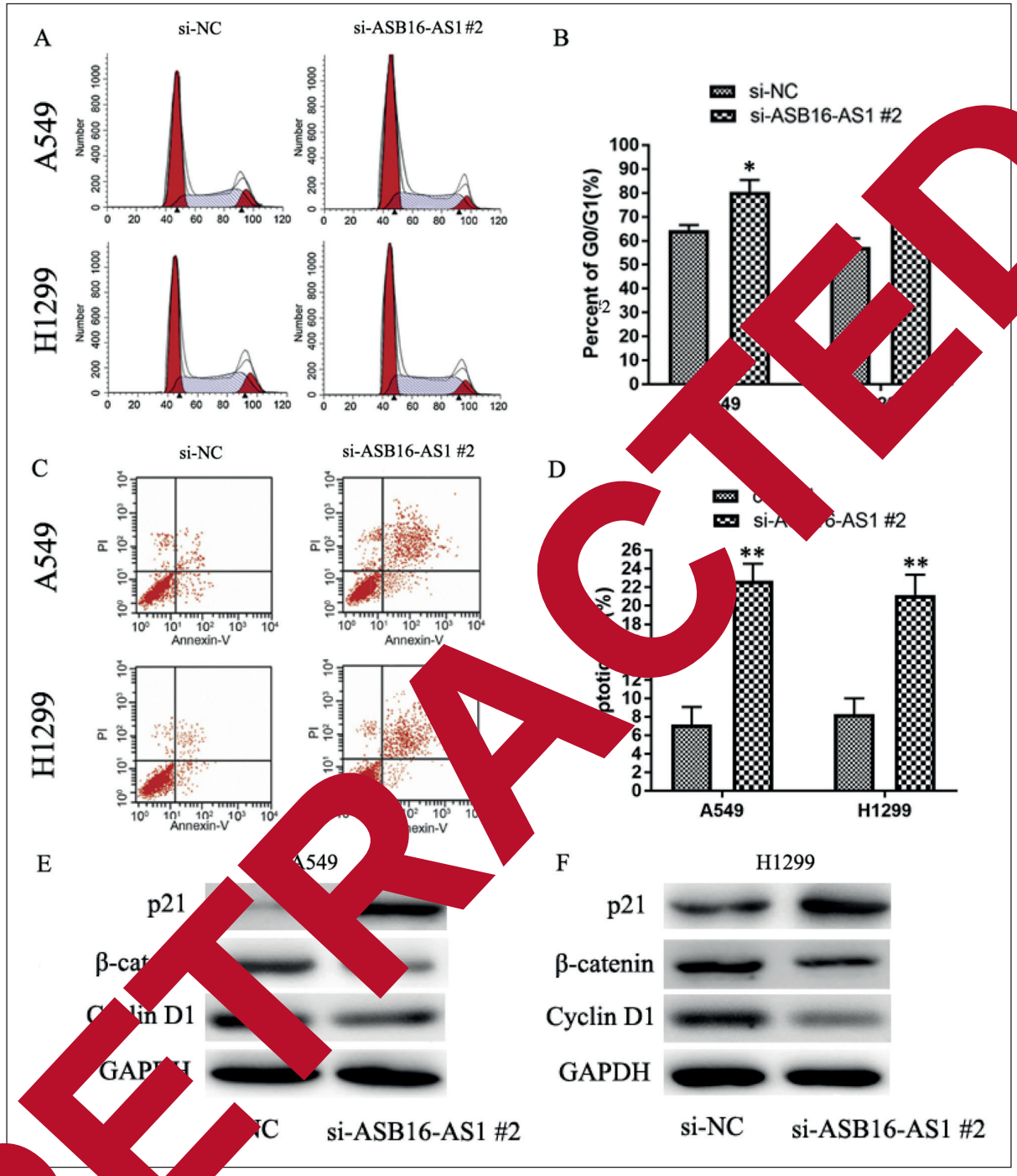

Fig. 3. Effe cRNA ASB16-AS1 on cell cycle and apoptosis of NSCLC cells. **A, B,** The results of flow cytometry sho that the ce ycle is arrested in G0/1 phase after interference in lncRNA ASB16-AS1 expression in NSCLC cells. **C, D,** T cytometry showed that the apoptosis rate is increased after interference in lncRNA ASB16-AS1 expression SCL in both groups (**$p<0.01$, *$p<0.05$). **E, F,** Western blotting results showed the expression changes in the olecular markers in the Wnt/β-catenin signaling pathway after interference in lncRNA ASB16-AS1 expression.

Conclusions

In summary, lncRNA ASB16-AS1 is upregulated in NSCLC tissues and cells, and the highly expressed lncRNA ASB16-AS1 promotes proliferation and inhibits apoptosis of NSCLC cells through the Wnt/β-catenin signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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