

lncRNA AWPPH 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. AWPPH is a newly discovered long non-coding (lnc)RNA that serves an oncogenic role in the development of several types of cancer; however, its involvement in non-small cell lung cancer (NSCLC) is unknown. Therefore, the aim of the present study was to investigate the function of AWPPH in NSCLC. The results demonstrated that AWPPH expression levels were significantly upregulated in the lung tissues and serum samples of patients with NSCLC compared with in healthy controls. High expression levels of AWPPH effectively distinguished NSCLC patients from healthy controls. In addition, patients with high expression levels of AWPPH had significantly shorter survival time. AWPPH overexpression in NSCLC cells promoted proliferation and inhibited apoptosis, and activated the Wnt/ β -catenin signaling pathway, which is a classic signaling pathway involved in the development and progression of different types of cancers. Treatment with a Wnt/ β -catenin signaling pathway activator produced no significant effect on AWPPH expression. Therefore, it was concluded that lncRNA AWPPH could promote the growth of NSCLCs by activating the Wnt/ β -catenin signaling pathway.

Introduction

Lung cancer is one of the major causes of cancer-related mortality, causing ~154,050 cases of mortality and affecting over 234,000 new patients annually in the United States (1,2). In developing countries, including China, the incidence of lung cancer was predicted to further increase in the near future due to aggregated air pollution (3). Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, which accounts for over 85% of cases (4). Despite efforts made to improve treatment and prevention of NSCLC, prognosis for

patients with this disease remain poor because most patients are diagnosed with existing distant metastasis, which is not appropriate for radical surgical resection (5). At present, early diagnosis and treatment are critical for the survival of patients with NSCLC.

The human genome transcribes a large set of non-coding RNAs that are recognized as major players in both normal physiological processes and pathological changes (6). Long non-coding (lnc)RNAs are a subgroup of non-coding RNAs that are composed of over 200 nucleotides (7). It has been well established that nearly all critical aspects of the onset, development and progression of NSCLC require the involvement of different lncRNAs (8,9). lncRNA AWPPH has been demonstrated to serve as an oncogene in hepatocellular carcinoma and bladder cancer (10,11); however, to the best of our knowledge its involvement in NSCLC has not been reported. In addition, the Wnt/ β -catenin signaling pathway has pivotal roles in tumor growth, and the modulation of Wnt/ β -catenin signaling can in some cases be achieved through the interaction with different lncRNAs (12). In the present study, a systematic investigation of the functionality of AWPPH in NSCLC was carried out and the results revealed that AWPPH may promote tumor growth of NSCLC via activation of the Wnt/ β -catenin signaling pathway.

Materials and methods

Subjects. The present study recruited 88 patients with NSCLC who were diagnosed and treated at the Cangzhou Central Hospital from January, 2013 to January, 2018. These patients included 56 males and 32 females, aged 23-71 years (mean age, 46 \pm 8.9 years). Patients with other types of cancer, lung diseases and severe diseases (such as severe infections) were excluded from the study. Patients receiving treatment prior to admission, were also excluded. The control group comprised 88 healthy volunteers. The healthy volunteers included 58 males and 30 females, aged 26-70 years (mean age, 47 \pm 7.6 years). There were no significant differences in age and gender between the patient and control group.

The present study was approved by the Ethics Committee of Cangzhou Central Hospital (Cangzhou, China), and all participants provided written informed consent.

Specimen collection. Lung cancer tissue and healthy lung biopsies were collected from patients with NSCLC and

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healthy controls, respectively. Blood (~20 ml) was extracted from the elbow vein of both patients and healthy controls. The blood was kept at room temperature for 4 h, followed by centrifugation at 1,200 x g at room temperature for 15 min to collect the serum. All samples were stored in liquid nitrogen.

Cell lines and cell culture. The normal human lung tissue cell line WI-38, and two human NSCLC cell lines NCI-H23 and NCI-H522 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Eagle's minimal essential medium (EMEM; ATCC) containing 10% fetal bovine serum (FBS; ATCC) according to the manufacturer's protocol. The cells were collected during logarithmic growth phase for subsequent experiments.

For activation of Wnt signaling, 10 ng/ml Wnt agonist (catalog no. 853220-52-7; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added to the serum-free cell culture medium and cells were incubated for 6 h at 37°C.

Construction of the AWPPH expression vector and transfection. Full-length AWPPH cDNA was inserted into the pIRES2-EGFP plasmid (Clontech Laboratories, Inc., Mountainview, CA, USA) to establish the AWPPH expression vector. The three cell lines were cultured overnight to reach 80-90% confluence, and 10 nM AWPPH vector was transfected into 4x10⁵ cells using Lipofectamine® 2000 (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). An empty vector was used as the negative control. Non-transfected cells were used as control cells. Cells were cultured in EMEM containing 10% FBS for 48 h at 37°C prior to subsequent experimentation.

Cell proliferation assay. The three cell lines were harvested during the logarithmic growth phase and were adjusted to a final cell density of 4x10⁴ cells/ml in cell culture medium. Then, 100 µl cell suspension (4x10³ cells) was transferred to each well of a 96-well plate. Cells were cultured at 37°C and 5% CO₂. A total of 10 µl Cell Counting Kit-8 solution (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to each well following 24, 48, 72 and 96 h of cell culture. Then, the cells were cultured for a further 4 h and optical density (OD) values at 450 nm were measured using a Fisherbrand™ accuSkan™ GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Inc.).

In cases where cells were treated with a Wnt inhibitor, 2.5 µM IWP-2 (Sigma-Aldrich; Merck KGaA) was added to cells at the beginning of culture.

Cell apoptosis assay. Cells were adjusted to a final cell density of 4x10⁴ cells/ml using medium containing 10 mM tetraethylammonium (Sigma-Aldrich; Merck KGaA) to induce cell apoptosis. Then, 10 ml cell suspension (4x10⁵ cells) was transferred to each well of a 6-well plate. Cells were cultured at 37°C for 24 h followed by digestion with 0.25% trypsin. Subsequently, cells were stained with Annexin V-fluorescein isothiocyanate (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and propidium iodide (Sigma-Aldrich; Merck KGaA), followed by the detection of apoptotic cells using flow cytometry. Cell apoptosis was normalized to the

control group using FCS Express 6 flow cytometry software (De Novo Software, Glendale, CA, USA).

In cases where cells were treated with a Wnt inhibitor, 2.5 µM IWP-2 (Sigma-Aldrich; Merck KGaA) was added to cells at the beginning of culture.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tumor tissues, healthy lung tissues and serum samples using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Tumor and healthy lung tissues were ground in liquid nitrogen prior to the addition of TRIzol® reagent. RNA quality, reflected by the A260/A280 ratio, was determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA samples with an A260/A280 ratio between 1.8-2.0 were subjected to RT to synthesize cDNA using SuperScript III Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.) using the following conditions: 55°C for 10 min and 75°C for 10 min. qPCR was performed using the Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Inc.). The following primers were used: AWPPH, forward, 5'-CTG GATGGTCGCTGCTTTT-3' and reverse, 5'-AGGGGG ATGAGTCGTGATTT-3'; β-actin, forward, 5'-GACCTCTAT GCCAACACAGT-3' and reverse, 5'-AGTACTTGCGCTCAG GAGGA-3'. The thermocycling conditions were as follows: 95°C for 45 sec, followed by 40 cycles of 95°C for 12 sec and 60°C for 40 sec. Relative expression levels of AWPPH were normalized to endogenous control β-actin using the 2^{-ΔΔC_q} method (13).

Western blotting. Total protein was extracted from cells using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) and quantified using the bicinchoninic acid assay method. Subsequently, 20 µg protein per lane was separated by SDS-PAGE using 10% gels. Proteins were transferred onto polyvinylidene difluoride membranes and blocked with 5% skimmed milk for 1 h at room temperature. The membranes were then incubated with rabbit anti-β-catenin (1:2,000; cat. no. ab32572; Abcam, Cambridge, UK) and mouse anti-GAPDH (1:1,000; cat. no. ab8245; Abcam) primary antibodies overnight at 4°C. The membranes were further incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase secondary antibody (1:1,000; MBS435036; MyBioSource, Inc., San Diego, CA, USA) at room temperature for 4 h. Finally, ECL™ Blotting Reagents GE Healthcare (Sigma-Aldrich; Merck KGaA) was added to visualize the proteins and membranes were scanned by a myECL™ imager (Thermo Fisher Scientific, Inc.). Relative protein expression levels of β-catenin were normalized to the endogenous control GAPDH using ImageJ software version 1.6 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Experiments were performed in triplicate. SPSS version 19.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. The χ² test was used to analyze countable data. Measurement data are presented as the mean ± standard deviation, and comparisons between two groups and multiple groups were performed using the unpaired Student's t-test and one-way analysis of variance followed

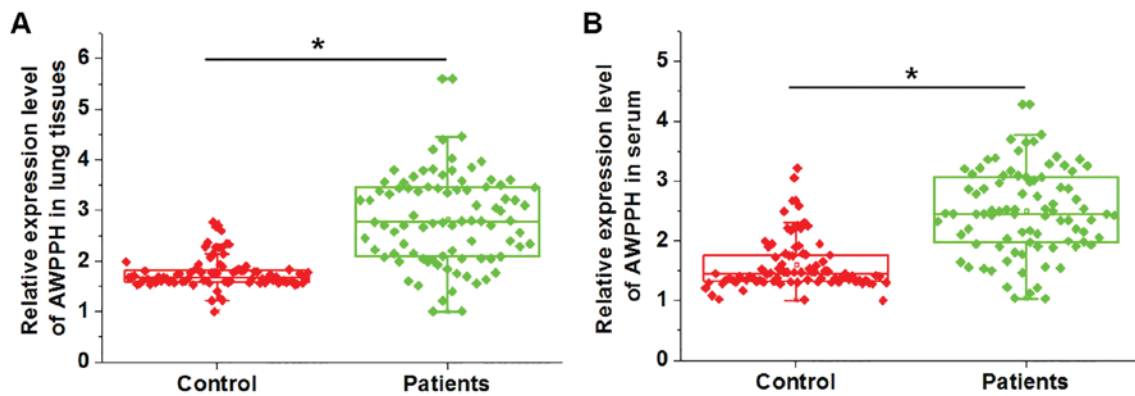


Figure 1. Expression of lncRNA AWPPH in patients with NSCLC and healthy controls. Expression levels of lncRNA AWPPH in (A) lung tissues and (B) serum collected from 88 patients with NSCLC and 88 healthy controls were detected via reverse transcription-quantitative polymerase chain reaction. Expression levels of lncRNA AWPPH in lung tissues and serum of patients with NSCLC were significantly higher compared with in healthy controls. * $P<0.05$. lnc, long non-coding; NSCLC, non-small cell lung cancer.

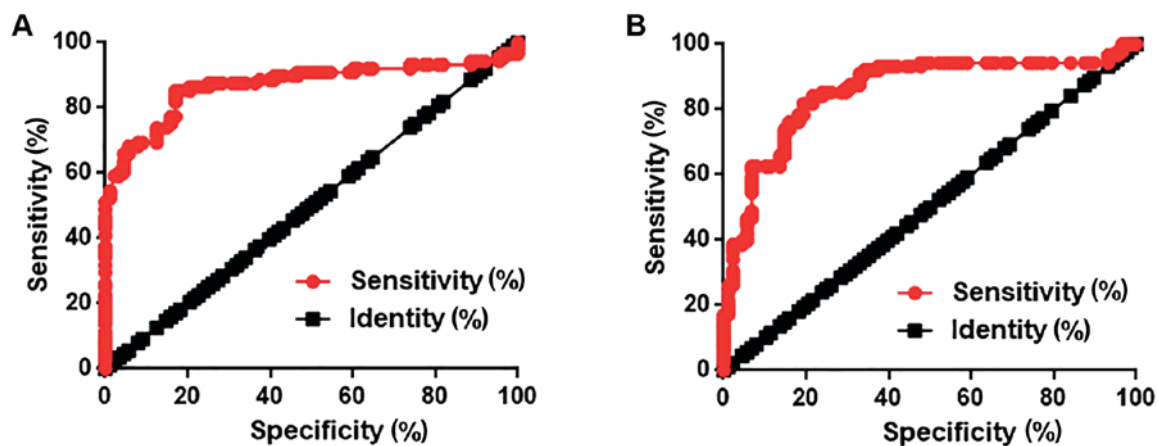


Figure 2. Diagnostic value of lncRNA AWPPH for NSCLC. Receiver operating characteristic curve analysis of lncRNA AWPPH expression in (A) lung tissues and (B) serum for the diagnosis of NSCLC. lncRNA AWPPH serves as a potential diagnostic marker for NSCLC. lnc, long non-coding; NSCLC, non-small cell lung cancer.

by least significant difference post hoc test, respectively. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic value of lncRNA AWPPH expression. Survival curves were plotted using the Kaplan-Meier method and compared by log rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of lncRNA AWPPH in patients with NSCLC and healthy controls. Expression levels of lncRNA AWPPH in the lung tissues and serum samples of patients with NSCLC and healthy controls were detected by RT-qPCR. As shown in Fig. 1, the expression levels of lncRNA AWPPH in the lung tissues (Fig. 1A) and serum samples (Fig. 1B) of patients with NSCLC were significantly higher compared with those in healthy controls. These data suggested that upregulation of AWPPH may be involved in the pathogenesis of NSCLC.

Diagnostic value of lncRNA AWPPH for NSCLC. ROC curve analysis was performed to evaluate the diagnostic value of

lncRNA AWPPH expression in lung tissues and serum for NSCLC. As shown in Fig. 2A, AWPPH expression levels in lung tissues could distinguish patients with NSCLC from healthy controls with an area under the curve (AUC) of 0.8686 and 95% confidence interval (CI) of 0.8102-0.9271 ($P<0.0001$). AWPPH expression levels in serum could distinguish patients with NSCLC from healthy control with an AUC of 0.8569 and 95% CI of 0.7983-0.9156 ($P<0.0001$; Fig. 2B). These data suggested that lncRNA AWPPH serves as a potential diagnostic marker for NSCLC.

Prognostic value of lncRNA AWPPH for NSCLC. All the patients were followed-up for 5 years to record their survival information. Patients were divided into high ($n=40$) and low ($n=40$) expression groups, according to the median expression level of AWPPH in lung tissue and serum. As shown in Fig. 3, the overall survival rate of patients with high expression levels of AWPPH in both lung tissues (Fig. 3A) and serum (Fig. 3B) was significantly lower ($P<0.001$) compared with patients with low expression levels of AWPPH. These data suggested that lung tissue and serum AWPPH may serve as a biomarker for the prognosis of NSCLC.

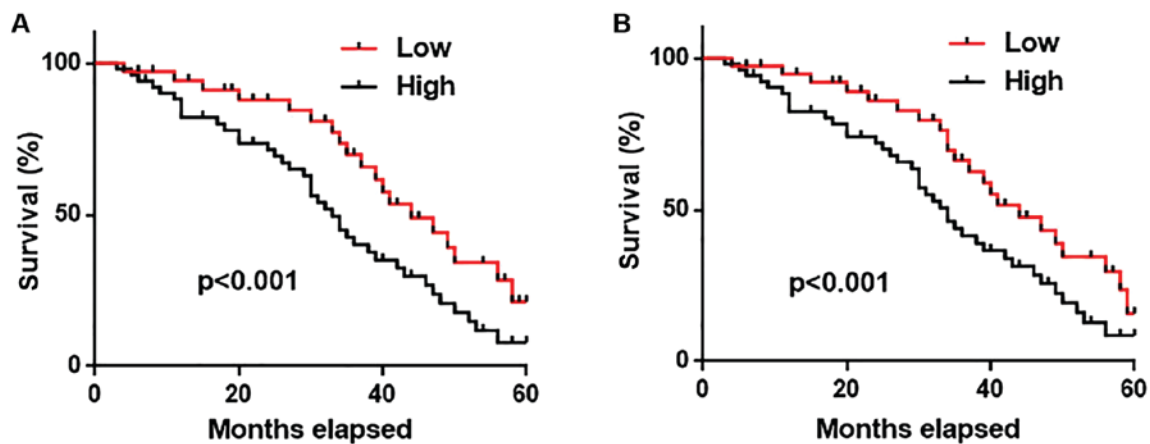


Figure 3. Prognostic value of lncRNA AWPPH for NSCLC. Survival analysis of patients with high and low expression levels of lncRNA AWPPH in (A) lung tissues and (B) serum. lncRNA AWPPH may serve as a potential prognostic marker for NSCLC.

Associations between expression levels of lncRNA AWPPH in lung tissue and serum, and clinicopathological data. According to the median expression level of lncRNA AWPPH in lung tissue and serum, patients were divided into high and low expression groups. Associations between expression levels of lncRNA AWPPH in lung tissue and serum, and clinicopathological data of patients were analyzed by χ^2 test. As shown in Tables I and II, there were no significant associations between the expression levels of lncRNA AWPPH in lung tissue and serum with the patients' sex, age, drinking habit as well as distant tumor metastasis. However, the expression levels of lncRNA AWPPH in lung tissue and serum showed significant association with tumor size and smoking habit. Therefore, altered expression of lncRNA AWPPH may be induced by lung cancer and smoking.

Interaction between AWPPH and the Wnt/ β -catenin signaling pathway. The results in the present study indicated that AWPPH may be involved in lung tumor growth. It is known that the Wnt/ β -catenin signaling pathway has a pivotal role in tumor growth (12). Therefore, normal human lung tissue cell line WI-38, and two human NSCLC cell lines NCI-H23 and NCI-H522 were employed to explore potential interactions between AWPPH and the Wnt/ β -catenin signaling pathway *in vitro*. RT-qPCR revealed that AWPPH expression levels were significantly higher in NCI-H23 and NCI-H522 cells compared with in WI-38 cells ($P < 0.05$; Fig. 4A). Subsequently, the cells were transfected with the AWPPH expression vector. As shown in Fig. 4B, AWPPH overexpression was successfully achieved in all three cell lines. AWPPH overexpression significantly upregulated the protein expression levels of β -catenin in NCI-H23 and NCI-H522 cells ($P < 0.05$), but not in WI-38 cells ($P > 0.05$; Fig. 4C). A Wnt agonist was used to investigate the effects of the activation of Wnt/ β -catenin signaling pathway on AWPPH. However, treatment of cells with the Wnt agonist demonstrated no significant effect on AWPPH expression in all three cell lines ($P > 0.05$; Fig. 4D). Therefore, AWPPH is likely an upstream activator of Wnt/ β -catenin signaling pathway.

Effect of AWPPH overexpression and Wnt inhibition on cell proliferation and apoptosis. AWPPH expression vectors

were transfected into WI-38, NCI-H23 and NCI-H522 cells. As shown in Fig. 5, AWPPH overexpression promoted proliferation (Fig. 5A), but significantly inhibited apoptosis (Fig. 5B) of NCI-H23 and NCI-H522 cells. Overexpression of AWPPH in WI-38 cells did not affect cell proliferation or apoptosis. In addition, treatment with the Wnt inhibitor IWP-2 reduced the effects caused by AWPPH overexpression on cell proliferation and apoptosis. These data suggested that AWPPH may promote proliferation and inhibit apoptosis of NSCLC cells by activating the Wnt/ β -catenin signaling pathway.

Discussion

lncRNA AWPPH has been demonstrated to be involved in several types of cancer (10,11). Zhao *et al* previously reported that AWPPH is highly expressed in hepatocellular carcinoma tissue, and high expression levels of AWPPH are closely correlated with advanced TNM stage, microvascular invasion, encapsulation incomplete and Barcelona Clinic Liver Cancer stage (10). In another study, AWPPH was reported to be overexpressed in bladder cancer, indicating its role as an oncogene in the disease (12). To the best of our knowledge, the expression patterns of AWPPH in other diseases have not been explored. In the present study, the expression levels of lncRNA AWPPH in lung tissues and serum of patients with NSCLC and healthy controls were detected. The results showed that lncRNA AWPPH expression was upregulated in patients with NSCLC compared with in healthy controls in both lung tissues and serum. These data suggested that AWPPH may have an oncogenic role in NSCLC.

Early diagnosis and accurate prognosis is critical for the survival of patients with NSCLC. Early diagnosis and prediction of prognosis of diseases usually requires highly sensitive markers. Development of human disease is usually accompanied by changes in blood biomarkers. Therefore, monitoring the levels of these markers in blood may provide guidance for the treatment of diseases (14). In the present study, ROC curve analysis revealed that expression levels of AWPPH in lung tissues and serum could be used to effectively distinguish NSCLC patients from healthy controls. In

Table I. Association between expression levels of AWPPH in lung cancer tissue and clinicopathological data of patients with non-small cell lung cancer.

Variables	No. of patients	High-expression	Low-expression	χ^2	P-value
Sex				0.79	0.382
Male	56	26	30		
Female	32	18	14		
Age				0.73	0.391
≥45 years	42	19	23		
<45 years	46	25	21		
Primary tumor diameter				19.45	<0.001
>7 cm	30	22	8		
3-7 cm	32	18	14		
<3 cm	26	4	22		
Distant tumor metastasis				0.73	0.393
Yes	40	18	22		
No	48	26	22		
Smoking				5.86	0.024
Yes	55	33	22		
No	33	11	22		
Drinking				0.84	0.363
Yes	60	32	28		
No	28	12	16		

Table II. Association between serum levels of AWPPH and clinicopathological data of patients with non-small cell lung cancer.

Variables	No. of patients	High-expression	Low-expression	χ^2	P-value
Sex				0.2	0.663
Male	56	27	29		
Female	32	17	15		
Age				0.73	0.390
≥45 years	42	19	23		
<45 years	46	25	21		
Primary tumor diameter				12.45	0.098
>7 cm	30	21	9		
3-7 cm	32	17	15		
<3 cm	26	6	20		
Distant tumor metastasis				0.73	0.392
Yes	40	18	22		
No	48	26	22		
Smoking				8.19	0.004
Yes	55	34	21		
No	33	10	23		
Drinking				0.84	0.362
Yes	60	32	28		
No	28	12	16		

addition, high expression levels of AWPPH in lung tissues and serum were closely associated with poor survival after discharge. The results suggested that AWPPH may serve as

a sensitive diagnostic and prognostic biomarker for NSCLC. Compared with an invasive lung biopsy, a blood test is a non-invasive method that would be preferred in the clinic.

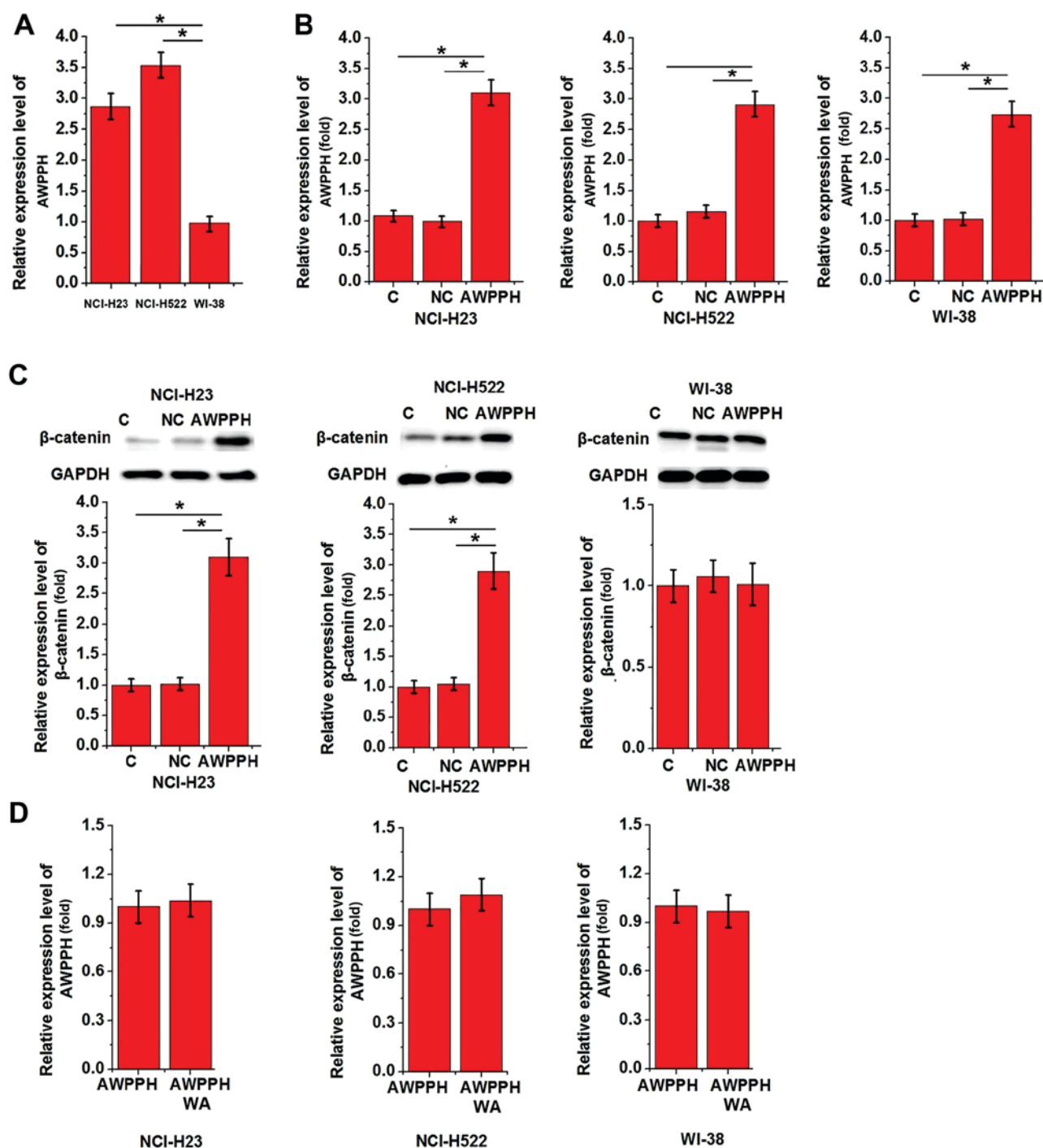


Figure 4. Interaction between AWPPH and the Wnt/β-catenin signaling pathway. (A) Expression levels of lncRNA AWPPH in two human NSCLC cell lines NCI-H23 and NCI-H522, as well as a normal human lung tissue cell line WI-38 were determined by RT-qPCR. (B) AWPPH overexpression was successfully achieved in all cell lines following transfection with the AWPPH expression vector. (C) Western blot analysis of β-catenin protein expression levels following AWPPH overexpression. (D) Expression levels of AWPPH in cells overexpressing AWPPH in the presence and absence of a WA were measured using RT-qPCR. AWPPH overexpression significantly upregulated the expression levels of β-catenin in NSCLC cell lines NCI-H23 and NCI-H522, but not the normal human lung tissue cell line WI-38. Treatment with the WA produced no significant effect on AWPPH expression in all three cell lines. * $P < 0.05$. AWPPH, cells transfected with the AWPPH expression vector; C, non-transfected cells; NC, negative control cells transfected with an empty vector; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WA, Wnt agonist.

It is known that the expression of certain lncRNAs can be affected by factors, including aging (15), smoking (16) and alcohol consumption (17). In the present study, no significant associations were found between AWPPH expression levels and the patients' sex, age and drinking habit. However, AWPPH expression levels in lung tissues and serum were

significantly associated with the patients' smoking habit. Therefore, smoking should be considered alongside other biomarkers in the diagnosis and prognosis of NSCLC using AWPPH.

The present study also demonstrated that expression levels of AWPPH in lung tissues and serum were significantly

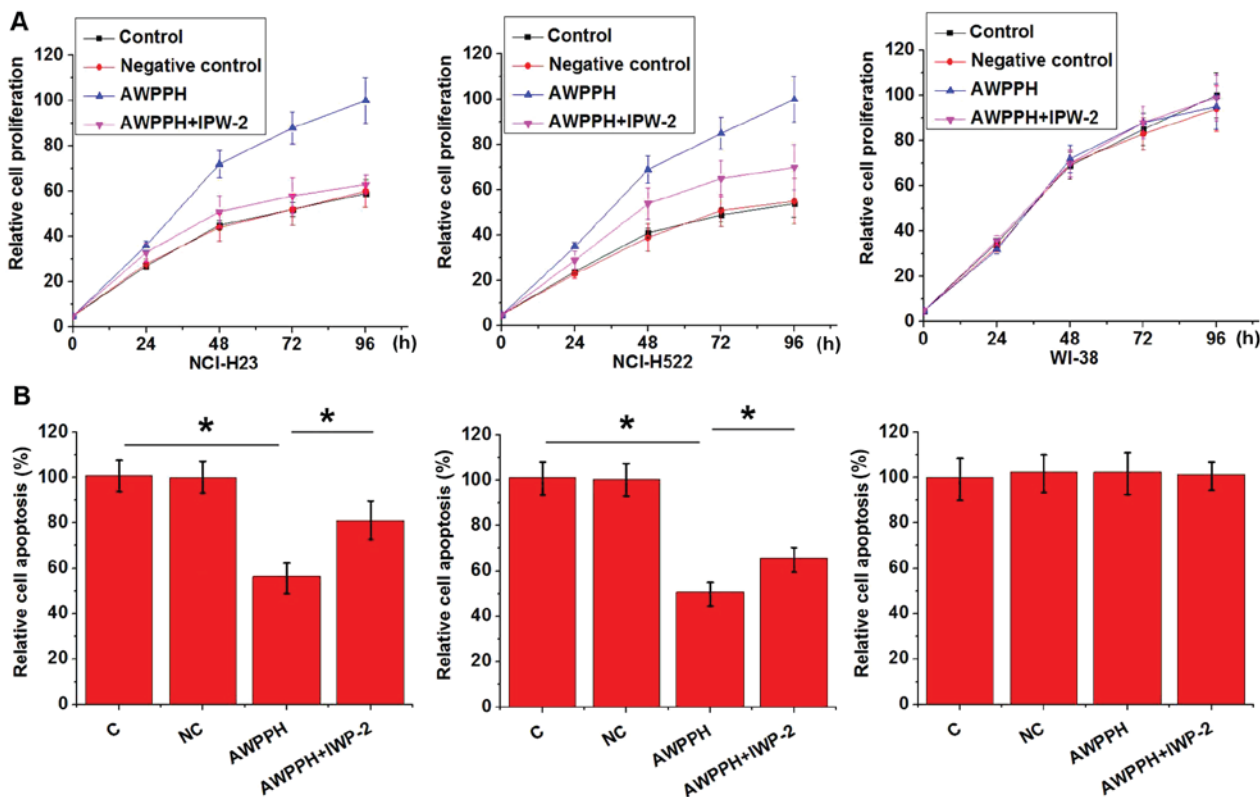


Figure 5. Effect of AWPPH overexpression and Wnt inhibition on cell proliferation and apoptosis. (A) Cell proliferation and (B) cell apoptosis were measured in different cell lines following AWPPH overexpression in the presence or absence of a Wnt inhibitor, using Cell Counting Kit-8 and MTT assays, respectively. * $P < 0.05$. AWPPH, cells transfected with the AWPPH expression vector; C, nontransfected cells; IWP-2, Wnt inhibitor; NC, negative control cells transfected with an empty vector.

associated with tumor size, but not tumor metastasis, indicating the involvement of AWPPH in tumor growth. The Wnt/ β -catenin signaling pathway serves a pivotal role in the growth of different types of cancer, including NSCLC (10,18), and inhibition of the Wnt/ β -catenin signaling pathway is considered to be a therapeutic target for the inhibition of tumor growth (19). It is known that the Wnt/ β -catenin signaling pathway can interact with lncRNAs to carry out its biological function (20,21). In the present study, transfection with the AWPPH expression vector significantly upregulated the expression of β -catenin in two NSCLC cell lines, while treatment with the Wnt agonist (or the activation of Wnt signaling) did not produce a significant effect on AWPPH expression. In addition, the Wnt inhibitor significantly reversed the enhancing effects of AWPPH overexpression on cell proliferation and its inhibitory effects on cell apoptosis. These data suggested that AWPPH may promote the growth of NSCLC by serving as an upstream activator of the Wnt/ β -catenin signaling pathway. Notably, AWPPH overexpression, and treatment with the Wnt inhibitor and activator did not significantly effect the proliferation and apoptosis of the normal human lung tissue cell line WI-38. Therefore, AWPPH may be a potential target for the treatment of NSCLC.

In conclusion, AWPPH was overexpressed in patients with NSCLC. AWPPH expression may have diagnostic and prognostic value for NSCLC. AWPPH may participate in the progression of NSCLC by promoting proliferation and inhibiting apoptosis of NSCLC cells through activation of the Wnt/ β -catenin signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZS and BS designed the experiments. ZS, JD and LZ performed experiments. BS drafted the manuscript. ZS, JD and LZ received and reviewed the manuscript. All authors approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Cangzhou Central Hospital (Cangzhou, China), and all participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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