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# *Acanthopanax senticosus* polysaccharide suppressing proliferation and metastasis of the human non-small cell lung cancer NCI-H520 cells is associated with Wnt/β-catenin signaling

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Lung cancer (LC) has the highest mortality of all tumors. Non-small cell lung cancer (NSCLC) accounts for about 80% of all LC. *Acanthopanax senticosus* polysaccharide (ASPS) is extracted from the root of *Acanthopanax senticosus* (AS). Herein, we examined the effect and molecular mechanism of ASPS on NSCLC. The proliferation, invasion and migration of NCI-H520 cells were detected by cell counting kit-8 (CCK-8), transwell assay and wound healing assay, respectively. The epithelial-mesenchymal transition (EMT) and Wnt/β-catenin pathway-related factors were evaluated using quantitative real-time PCR (qRT-PCR) and western blot assay. Our results showed that ASPS significantly decreased the proliferation of cells at 24 and 48 h. Moreover, ASPS markedly repressed the invasion and migration capacities of cells in a concentration-dependent manner. Besides, ASPS obviously downregulated the levels of matrix metalloproteinase-2 (MMP-2), MMP-9, fibronectin 1 (FN1), vimentin, wnt3a, phosphorylated-glycogen synthase kinase 3β (p-GSK3β) and cyclin D1, whereas E-cadherin level was upregulated. The level of GSK3β was not changed within the different groups. ASPS conspicuously inhibited the abilities of proliferation and metastasis in human non-small cell lung cancer cell line NCI-H520 possibly by suppressing Wnt/β-catenin pathway mediated-EMT.

*Key words: Acanthopanax senticosus polysaccharide, proliferation, metastasis, Wnt/β-catenin pathway*

Lung cancer (LC) has the highest mortality of all tumors and about 1.5 million people die of LC each year worldwide. According to the histologic characteristic, LC is divided into two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [1]. The proportion of NSCLC patients in newly diagnosed LC patients is up to 85% per year and the 5-year survival rate is less than 15% [2]. Although chest screening, low dose CT and other early screening methods are widely used in the early detection of NSCLC, the most patients are in middle and late stage when diagnosed, thereby losing the chance of surgical cure [3, 4]. At the present, treatment of NSCLC mainly includes chemotherapy, molecular targeted therapy and immunotherapy [4]. However, these treatments have some side effects and the patient's survival rate is still very low [5, 6]. Hence, we need to examine the specific pathogenesis of NSCLC and find a safe and effective treatment.

It is well known that the widespread use of Chinese herbal medicine in the treatment of various tumors is a hot spot of research [7–9]. Researchers have proved that the antitumor activity of Chinese herbal medicine is mainly derived from the plant extracts, including polysaccharides, saponins, steroids, etc. [10–12]. In recent years, the extensive biological activity of plant polysaccharides has been gradually recognized [13]. The antitumor research of polysaccharide has aroused wide attention of scholars both at home and abroad [14, 15].

*Acanthopanax senticosus* (AS) belongs to Chinese herbal medicine [16]. *Acanthopanax senticosus* polysaccharide (ASPS) is extracted from the root of AS and is one of the main active components of AS [17]. The bioactivities of ASPS include immunomodulatory, antitumor, antioxidant, anti-radiation and anti-inflammatory [18–21]. Meanwhile, it has the advantages of low toxicity, so it has a great potential in

clinical application [22, 23]. Nevertheless, the role of ASPS in NSCLC is not yet known.

The development of tumor is the evolutionary process of multifactor, multigene and multistage gradual accumulation. Cell signal transduction pathway plays a pivotal role in its development, invasion and metastasis [24, 25]. As we know, the Wnt/β-catenin signal transduction pathway plays an important regulatory role in cell differentiation, proliferation, survival and apoptosis, as well as cell carcinogenesis, tumor invasion and other pathological processes [26]. Wnt/β-catenin pathway is closely related to the tumors and the abnormal activation of the pathway is involved in the pathogenesis of a variety of human cancers, such as nasopharyngeal carcinoma, gastric cancer, liver cancer, breast cancer, prostate cancer, etc. [27–32].

At the present study, we explored the effect of ASPS on the proliferation and metastasis of human non-small cell lung cancer line NCI-H520 and further studied whether the potential mechanism was the Wnt/β-catenin pathway involvement.

## Materials and methods

**Drug treatment.** *Acanthopanax senticosus* polysaccharide (ASPS) was extracted from the cortex of *Acanthopanax senticosus*. The powder of *Acanthopanax senticosus* polysaccharide was purchased from Shaanxi Ciyuan Biotechnology Co., Ltd. The color was brown and the purity was more than 80%. The powder was dissolved in serum-free Roswell Park Memorial Institute-1640 (RPMI-1640) (Biosun, Shanghai, China) and filtered through 0.22 μm membrane (Shenghua; Haining, Jiaxing, China). The solution of ASPS was diluted to 10, 20, 40, 80, 160 and 320 mg/ml in RPMI-1640.

**Cell culture.** Human non-small cell lung cancer cell line NCI-H520 was obtained from Shanghai enzyme linked Biotechnology Co., Ltd. Cells were incubated in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS; Lonsera, Shanghai, China) and penicilin-streptomycin solution (Leagene, Beijing, China) in 37 °C incubator (S-G80A-II; Sheyanyiqi, Shanghai, China) with 95% relative humidity and 5% $CO_2$.

**Table 1. Sequences of the primers.**

| Primer name | Sequence (5'-3') | Product size (bp) |
|---|---|---|
| MMP-2-Forward | CAGCCCTGCAAGTTTCCATT | 210 |
| MMP-2-Reverse | GTTGCCCAGGAAAGTGAAGG | |
| MMP-9-Forward | GAGACTCTACACCCAGGACG | 238 |
| MMP-9-Reverse | GAAAGTGAAGGGGAAGACGC | |
| FN1-Forward | TGGCACTGATGAAGAACCCT | 224 |
| FN1-Reverse | GGGAAACTGTGTAGGGGTCA | |
| Vimentin-Forward | AATAAGATCCTGCTGGCCGA | 225 |
| Vimentin-Reverse | GGTGTTTTCGGCTTCCTCTC | |
| E-cadherin-Forward | ACGCATTGCCACATACACTC | 217 |
| E-cadherin-Reverse | GGTGTTCACATCATCGTC | |
| β-actin-Forward | GGGAAATCGTGCGTGACATT | 219 |
| β-actin-Reverse | AGGTAGTTTCGTGGATGCCA | |

**Cell Counting Kit-8 (CCK-8) assay.** CCK-8 (Yeasen, Shanghai, China) was used to assess the cell proliferation as manufacturer's instructions recommend. Firstly, cells were inoculated into 96-well plates at the density of $1.5\times10^3$ cells/well and cultured in incubator for 24 h. Next day, cells were treated with PBS and different concentration of ASPS (0, 10, 20, 40, 80, 160 and 320 mg/ml) for 12, 24 and 48 h, respectively. Then, CCK-8 solution was dripped into each well and the plate was transferred into the incubator for 4 h. Finally, OD value at 450 nm was detected by HBS-1096B microplate reader (Detie, Nanjing, China).

**Transwell assay.** Transwell assay was carried out to evaluate cell invasion according to the manufacturer's instructions. Briefly, cells were inoculated into 6-well plates at the density of $2\times10^4$ cells/well and cultured in incubator for 24 h. Next day, cells were treated with PBS (control) and different concentration of ASPS: 40 (ASPS1), 80 (ASPS2) and 160 (ASPS3) mg/ml for 48 h, respectively. BD matrigel (Solarbio, Shanghai, China) was filled in the upper chamber of transwell at 37 °C for 30 min. Then, transwell was placed into the culture plate. RPMI-1640 was added into the upper chamber of transwell for 25 min and sucked away. 10% FBS was dropped into the lower chamber of the transwell. Subsequently, cell suspension was cultured in the upper chamber of transwell for 24 h. Cells were stained with 0.1% crystal violet (Zhongxin chemtech; Tianjin, China) for 20 min and washed with PBS for 3 times. Finally, cells were observed and photographed using inverted microscope (DYS-339; Dianying, Shanghai, China).

**Wound healing assay.** Wound healing test was performed to analyze cell migration following a standardized method. Firstly, cells were inoculated into 6-well plates at the density of $2\times10^4$ cells/well and cultured in incubator for 24 h. Next day, cells were scratched about 500 μm width by 1 mL pipette tip and washed with RPMI-1640 for 2 times. Then, cells were treated with PBS (control) and different concentration of ASPS at 40 (ASPS1), 80 (ASPS2) and 160 (ASPS3) mg/ml for 0, 24 and 48 h, respectively. Finally, cells were observed and photographed using inverted microscope.

**Quantitative real-time PCR (qRT-PCR) assay.** Total RNA of was collected by TRIeasy™ Total RNA Extraction Reagent (Yeasen, Shanghai, China). cDNA was synthesized by ABScript II cDNA first strand synthesis kit (ABclonal, Beijing, China). The reaction reagents were as follows: 1 μg RNA, 2 μl $d(T)_{23}VN$, 10 μl ABScript II reaction mix, 2 μl ABScript II enzyme mix, 1 μl $dNTP_S$, nuclease-free $H_2O$ to a total volume of 20 μl. Reaction conditions were 80 °C for 5 min. Then, cDNA was amplified by SYBR Premix Taq™ II kit (Takara, Beijing, China). The reaction reagents were as follows: 25 μl SYBR Green Mix, 4 μl cDNA, 1 μl forward/reverse primer, nuclease-free $H_2O$ to a total volume of 50 μl. Reaction conditions were as followed: predegeneration: 95 °C

for 5 min, (denaturation: 95 °C for 15 s; annealing: 62 °C for 35 s) for 40 cycles, extending: 75 °C for 30 s. The primer sequence was listed in the Table 1. β-actin was regarded as internal control. The formula $2^{-\Delta\Delta CT}$ was used to compare the quantification.

**Western blot assay.** Total protein was lysed with high RIPA lysis buffer (Leagene, Beijing, China). Protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and bound to polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Shanghai, China). Then, membrane was sealed by 5% skim milk at room temperature for 1.5 h and incubated with anti-matrix metalloproteinase-2 (MMP-2) (R&D, IC903G-100UG, 1:1000), anti-MMP-9 (Abcam, EP1254, 1:500), anti-fibronectin 1 (FN1) (Abcam, ab32419, 1:800), anti-vimentin (R&D, AF2105, 1:700), anti-E-cadherin (R&D, MAB1838, 1:1000), anti-wnt3a (Abcam, ab28472, 1:800), anti-β-catenin (R&D, AF1329, 1:1000), anti-phosphorylated-glycogen synthase kinase 3β (p-GSK3β) (Abcam, ab75814, 1:1000), anti-GSK3β (Abcam, ab32391, 1:1000), anti-cyclin D1 (R&D, MAB4314, 1:900) and anti-β-actin (Abcam, ab13772, 1:800) at 4 °C refrigerator overnight. Next day, membrane was incubated with the secondary antibodies (Rabbit anti-mouse IgG, CST, #58802, 1:7000; Goat anti-rabbit IgG, Beyotime, A0423, 1:8000; Donkey anti-goat IgG, Beyotime, A0181, 1:8000) at 37 °C for 60 min. The protein was exposed using the ECL chromogenic solution (Biodragon, Beijing, China).

**Statistical analysis.** All experiments were carried out at least three times. Experimental data was presented as mean ± standard deviation (SD) using Microsoft Excel. One-way ANOVA was performed to evaluate the differences between groups. A $p<0.05$ was treated as a significant difference.

## Results

**ASPS decreased the proliferation of NCI-H520 cells.** The effect of ASPS on the proliferation of NCI-H520 cells was measured by CCK-8. The data showed that there was no obvious change in cell proliferation, when cells were administrated to ASPS at 10, 20, 40, 80, 160 and 320 mg/ml for

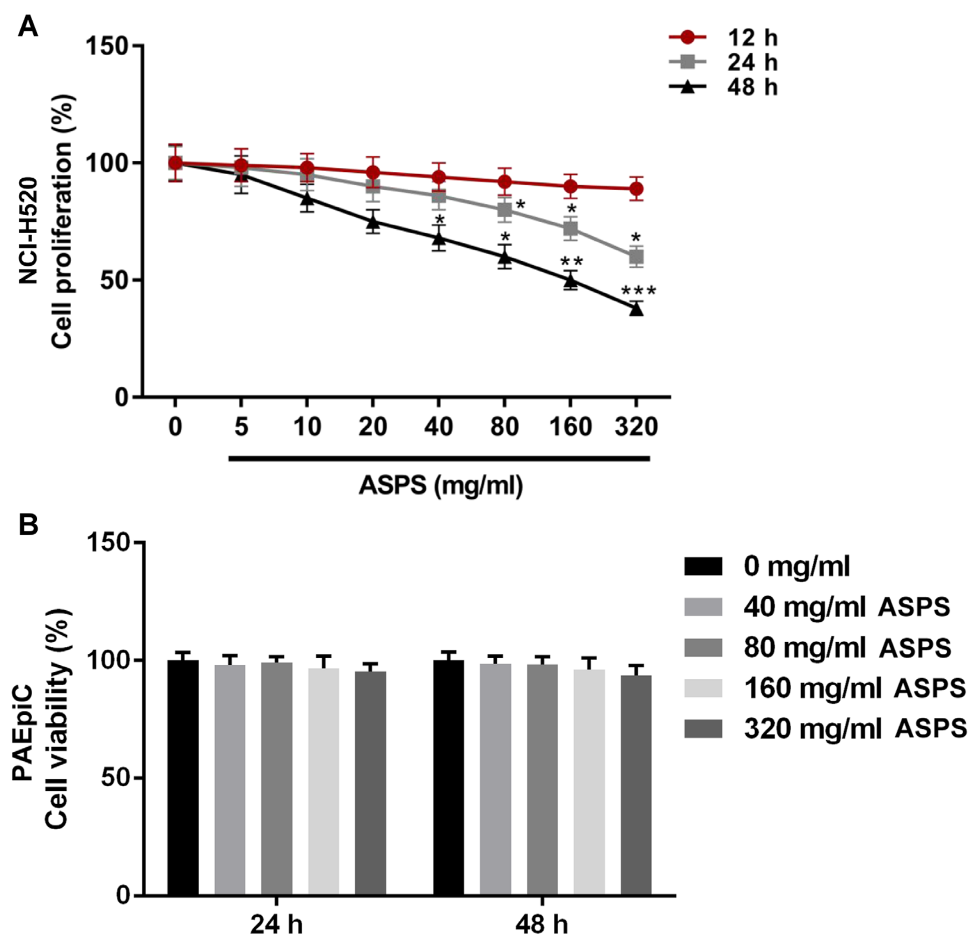


**Figure 1. The effect of ASPS on cell viabilities of NCI-H520 cell and human pulmonary alveolar epithelial cell (PAEpiC). (A) NCI-H520 cells were administrated to PBS (0) and different concentrations of ASPS: 0, 10, 20, 40, 80, 160 and 320 mg/ml for 12, 24 and 48 h, respectively. B) PAEpiC cells were administrated to PBS (0) and different concentrations of ASPS at 0, 40, 80, 160 and 320 mg/ml for 24 and 48 h, respectively. CCK-8 was used to evaluate the cell proliferation. *p<0.05, **p<0.01, ***p<0.001 vs. control (PBS).**

12 h. Besides, ASPS markedly inhibited cell proliferation for 24 and 48 h and 80 mg/ml ASPS significantly inhibited cell proliferation compared to control (0 mg/ml) at 24 h. The IC50 of ASPS was 160 mg/ml at 48 h. When the cells were treated with 320 mg/ml for 48 h, the rate of cell proliferation was less than 50% (Figure 1A). Moreover, study also demonstrated that 160 mg/ml ASPS treatment for 24 and 48 h had no significant inhibitory effect on human pulmonary alveolar epithelial cell viability (Figure 1B). Therefore, 40, 80 and 160 mg/ml ASPS treatment for 48 h were worth further investigating and were determined as the later experimental condition.

**ASPS repressed the invasion of NCI-H520 cells.** The effect of ASPS on the invasion of NCI-H520 cells was detected by transwell assay. Our results showed that the number of cell invasion was drastically reduced, when cells were administrated to ASPS. As quantitative analysis of cell invasiveness showed, the rates of cell invasion were 65%, 45% and 30% in ASPS1, ASPS2 and ASPS3 groups, respectively. In comparison with control group, the invasiveness of cells was decreased by 35%, 55% and 70% in ASPS1, ASPS2 and ASPS3 groups, respectively (Figures 2A–B).

**ASPS suppressed the migration of NCI-H520 cells.** The effect of ASPS on the migration of NCI-H520 cells was analyzed by wound healing test. Our data showed that the number of cell migration was dramatically reduced, when the cells were administrated to ASPS. As quantitative analysis of cell migration showed, cell migration rates were 80%, 56% and 33% in ASPS1, ASPS2 and ASPS3 groups for 24 h, respectively. Cell migration rates were 60%, 50% and 25% in ASPS1, ASPS2 and ASPS3 groups for 48 h, respectively. The rates of cell migration were lessened by 20%, 44% and 67% in ASPS1, ASPS2 and ASPS3 groups for 24 h, respectively, compared with the control group. The rates of cell migration were reduced by 40%, 50% and 75% for 48 h, respectively, compared to the control group (Figures 3A–B).

**ASPS suppressed the metastasis of NCI-H520 cells by regulating EMT-related factors.** qRT-PCR and western blot assays were used to assess EMT-related factors. The qRT-PCR data revealed that the RNA levels of MMP-2, MMP-9, FN1 and vimentin were obviously declined and E-cadherin RNA level was enhanced in ASPS groups in dose-dependent manner, relative to the control group (Figure 4A). In addition, western blot data showed that the expression trend of protein was the same as that of RNA (Figure 4B).

**ASPS suppressed the proliferation and metastasis of NCI-H520 cells by downregulating Wnt/β-catenin pathway.** The molecular mechanism of ASPS on the proliferation and metastasis of NCI-H520 cells was examined by western blot assay. As western blot results showed that as compared with control group, ASPS significantly downreg-

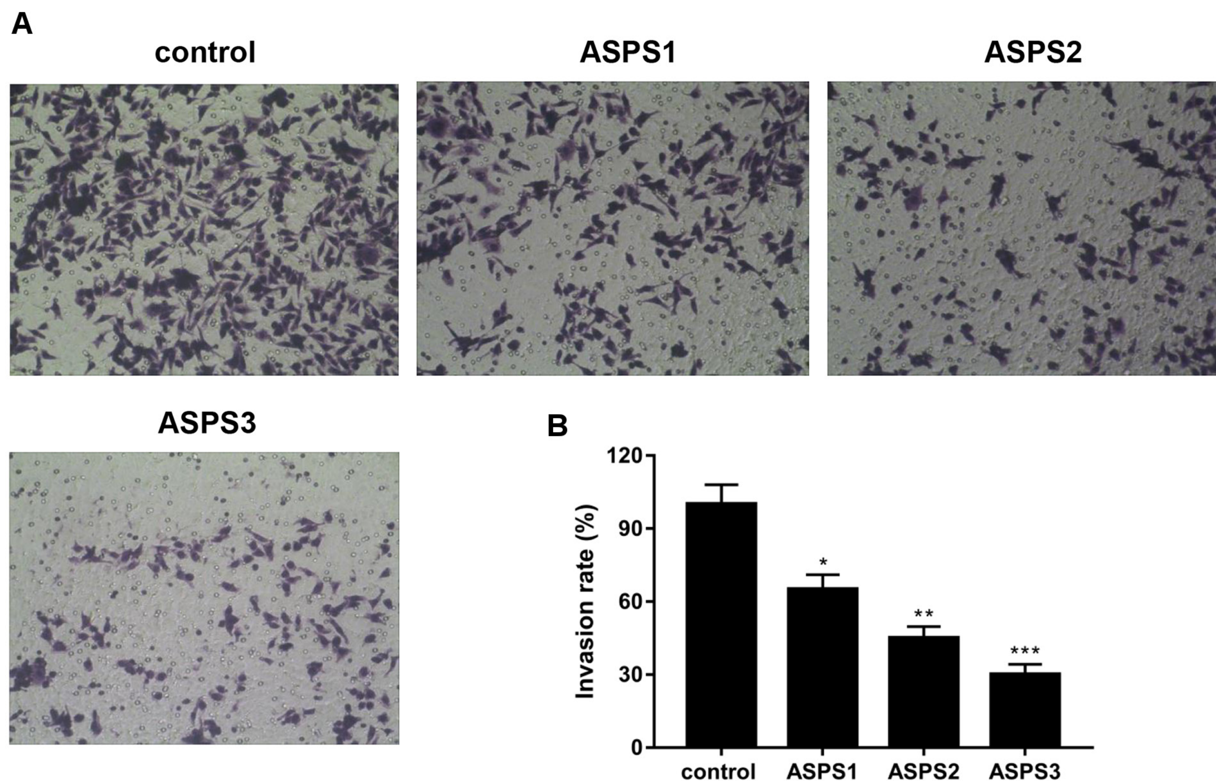


**Figure 2. ASPS repressed the invasion of NCI-H520 cells. A) Cells were treated with PBS (control) and different concentrations of ASPS: 40 (ASPS1), 80 (ASPS2) and 160 (ASPS3) mg/ml for 48 h, respectively. Transwell assay was performed to detect cell invasion. B) Quantitative analysis of cell invasiveness was carried out with GraphPad prism 7. *p<0.05, **p<0.01, ***p<0.001 vs. control.**

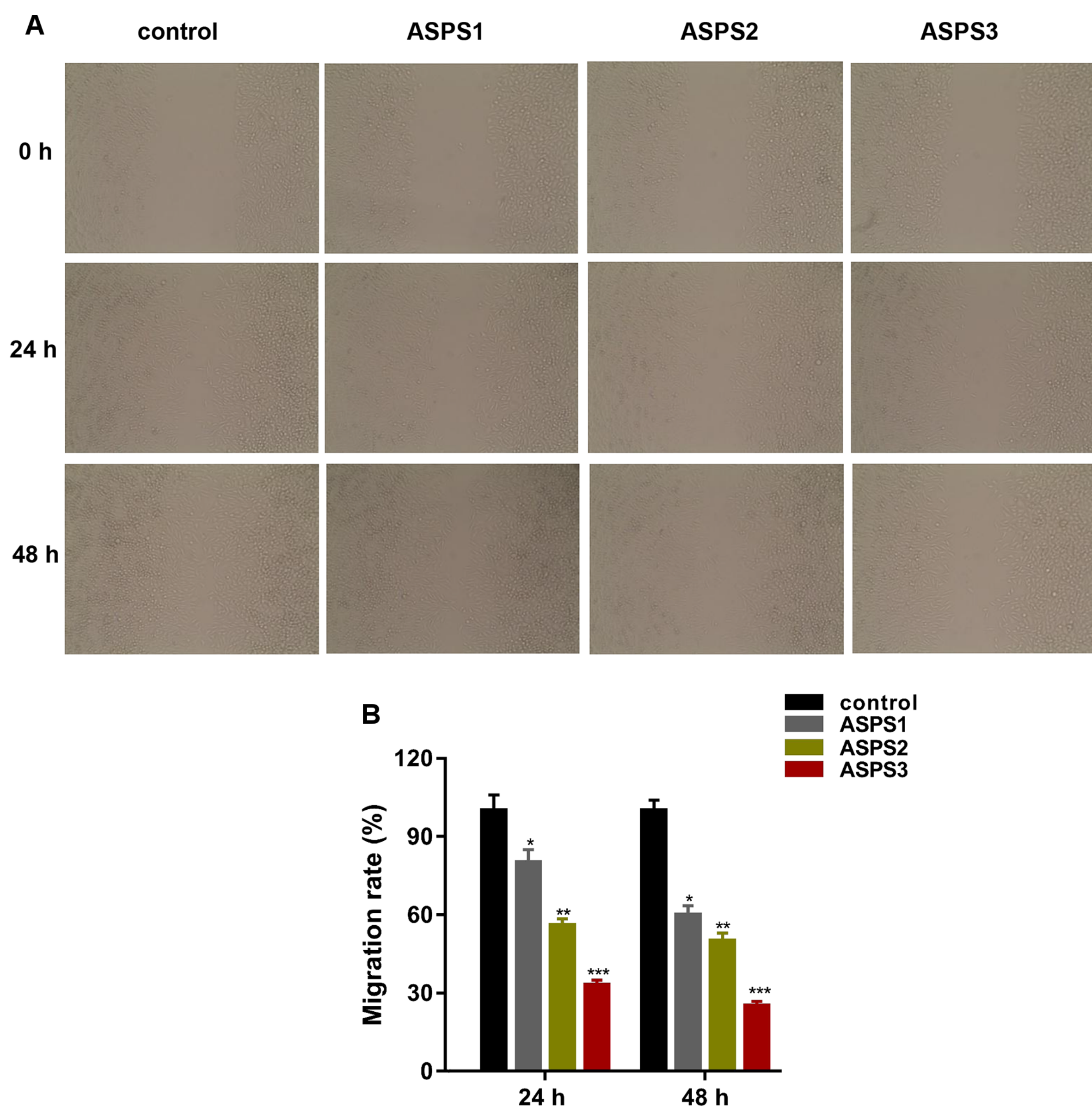


**Figure 3. ASPS suppressed the migration of NCI-H520 cells. A) Cells were treated with PBS (control) and different concentrations of ASPS: 40 (ASPS1), 80 (ASPS2) and 160 (ASPS3) mg/ml for 0, 24 and 48 h, respectively. Wound healing assay was used to explore cell migration. B) Quantitative analysis of cell migration rate was carried out with GraphPad prism 7. *p<0.05, **p<0.01, ***p<0.001 vs. control.**

ulated the protein levels of wnt3a, β-catenin, cyclin D1 in a concentration-dependent manner. Meanwhile, the phosphorylation level of GSK3β was attenuated in ASPS groups in dose-dependent manner. Moreover, there was no significant change in the expression of GSK3β protein between different groups (Figure 5).

## Discussion

For decades, accumulating evidence testifies that plant polysaccharides have obvious anticancer activity [14, 15, 33]. Han et al. have found that *Sargassum henslowianum* polysaccharide can treat gastric cancer by improving the immunomodulatory function [14]. Ayeka PA *et al.* have confirmed that licorice polysaccharide inhibits the growth of tumor, thereby improves the health of mice [33]. Therefore, we inferred that ASPS had the effect of anti-NSCLC. As expected, ASPS distinctly repressed the proliferation of NCI-H520 cells in concentration-dependent manner at 24 and 48 h.

Cancer metastasis is the most dangerous stage in the process of tumorigenesis and evolution and is the most important cause of cancer death, as well a key factor

**Figure 4. ASPS suppressed the metastasis of NCI-H520 cells by regulating EMT-related factors. A) RNA levels of MMP-2, MMP-9, FN1, vimentin and E-cadherin were analyzed by qRT-PCR assay. B) The protein levels of MMP-2, MMP-9, FN1, vimentin and E-cadherin were measured by western blot assay and normalized to β-actin expression. Gray value was detected and counted by quality one. *p<0.05, **p<0.01, ***p<0.001 vs. control.**

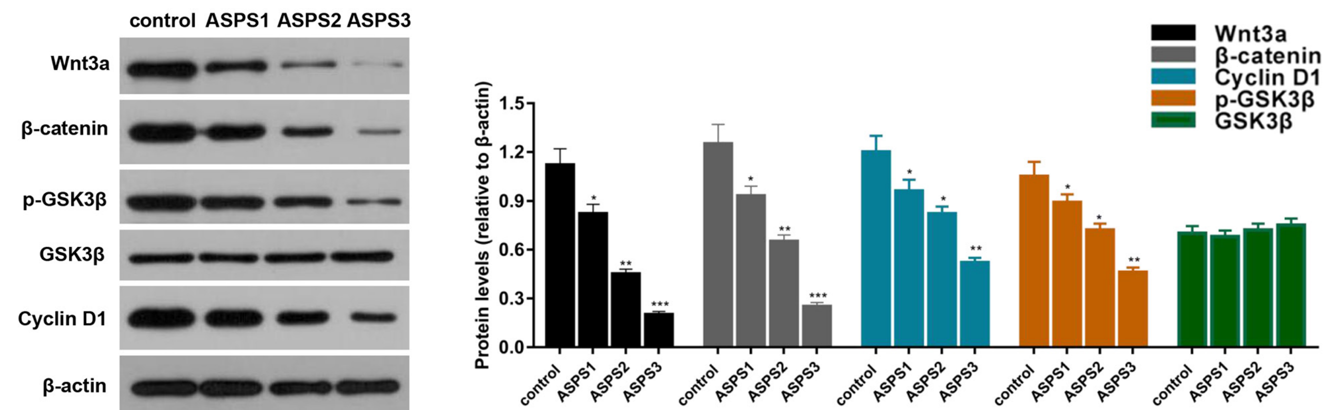


**Figure 5. ASPS suppressed the proliferation and metastasis of NCI-H520 cells by downregulating Wnt/β-catenin pathway. The protein levels of Wnt3a, β-catenin, Cyclin D1, p-GSK3β and GSK3β were evaluated by western blot assay. β-actin was regarded as internal control. *p<0.05, **p<0.01, ***p<0.001 vs. control.**

affecting the effect of tumor treatment [34]. Recent research has demonstrated that *Hizikia fusiformis* polysaccharide represses the invasion ability of human fibrosarcoma cells [35]. Furthermore, it has been found that *Acorus calamus* L. polysaccharide reduces the metastasis of lung cancer cells [36]. Similar to previous studies, our data showed that ASPS markedly reduced the invasion and migration capacities of NCI-H520 cells in a dose-dependent manner.

Metastasis of tumor cells involves the regulation of epithelial-mesenchymal transition (EMT) process-related molecules. EMT-related factors include MMPs, FN, vimentin, E-cadherin, and so on [37–39]. A study has proved that FBXO11 facilitates the capacities of proliferation and metastasis in gastric cancer cells through inducing EMT process [37]. Another research confirms that SPARC reduces the growth, invasion and migration of T-cell non-Hodgkin's lymphoma cells by inhibiting EMT process [40]. In addition, accumulating evidence substantiates that when the metastasis of the tumor cells is suppressed, the EMT process is blocked, accompanied by a decrease in the expression levels of MMP-2, MMP-9, vimentin and FN and an increase in E-cadherin expression [38, 39]. In this study, we observed that ASPS repressed the proliferation and metastasis of NCI-H520 cells by blocking EMT process, which downregulated levels of MMP-2, MMP-9, vimentin and FN1 and upregulated E-cadherin levels.

A large number of studies have shown that genes and drugs inhibit the growth and metastasis of various cancer cells by regulating the Wnt/β-catenin pathway [27–29, 31]. Moreover, Wnt/β-catenin pathway can induce EMT transformation by inhibiting GSK3β-mediated phosphorylation and the degradation of β-catenin and further represses downstream target genes, such as cyclin D1 [31, 41]. A previous report indicated that transmembrane 4 L6 family proteins silencing depressed the metastasis of NSCLC via downregulating Wnt/β-catenin and EMT signaling [42]. As expected, our results illustrated that ASPS significantly lessened metastasis of NCI-H520 cells via Wnt/β-catenin signaling pathway mediated-EMT. Though, validation experiments were also required, it was the limitation of the current study and we are planning to use antagonists or agonists to validate the possible signaling pathway.

All in all, our research testified that ASPS significantly repressed the proliferation, invasion and migration abilities of human non-small cell lung cancer NCI-H520 cells through suppressing Wnt/β-catenin pathway mediated-EMT. Based on the results of this study, ASPS may be developed as a drug for the treatment of NSCLC in the future.

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