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## Astragaloside IV Inhibits the Progression of Non-Small Cell Lung Cancer Through the Akt/GSK-3 $\beta$ / $\beta$ -Catenin Pathway

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Astragaloside IV (AS-IV) is an active ingredient in *Astragalus membranaceus* and is involved in various biological processes, such as regulating the immune system, and counteracting inflammation and malignancy. The aim of this study was to explore the effect of AS-IV on non-small cell lung cancer (NSCLC) cells. Cell counting kit (CCK)-8 assay and flow cytometry were performed to investigate cell survival and cell death, and Western blotting was performed to assess protein expression. We found that AS-IV inhibited the migration and proliferation of NSCLC cells and caused a noticeable increase in cell death. Furthermore, the expression of Bax, a marker of cell death, was increased, whereas the expression of Bcl-2, an antiapoptotic protein, was reduced. AS-IV also promoted cleavage of caspase-3, another indication of apoptosis. Finally, the Akt/GSK-3 $\beta$ / $\beta$ -catenin axis was suppressed in response to AS-IV. Taken together, these findings provide evidence that AS-IV inhibits NSCLC development via inhibition of the Akt/GSK-3 $\beta$ / $\beta$ -catenin signaling axis. We therefore propose that AS-IV represents a promising novel agent for the treatment of NSCLC.

**Key words:** Astragaloside IV (AS-IV); Non-small cell lung cancer (NSCLC); Akt; GSK-3 $\beta$ ;  $\beta$ -Catenin

### INTRODUCTION

Non-small cell lung cancer (NSCLC) is a major global public health problem and responsible for over 25% of cancer-related deaths annually<sup>1,2</sup>. Overall, NSCLC has a low 5-year survival rate compared to other malignancies, at 14% for men and 17% for women<sup>3,4</sup>. Current NSCLC therapies involve a combination of surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy<sup>5-7</sup>. Although significant advances have been made in the systemic treatment of NSCLC, one of the major limitations has been the development of cellular drug resistance<sup>8</sup>. Drug resistance can be either preexistent (intrinsic) or acquired after drug exposure. The majority of patients experience well-established toxicities from cytotoxic chemotherapy without a survival advantage<sup>9</sup>. Up to 50%–75% of patients who are initially responsive to the small molecule anti-EGFR inhibitor gefitinib become resistant to this therapy in 5–10 months, indicating tumor progression. At present, drug resistance to anti-EGFR inhibitors is the result of secondary EGFR gene mutations, EGFR/Met gene amplification, and/or activation of key signaling pathway<sup>10</sup>. As a result, it has become necessary

to develop innovative and effective strategies to prevent and/or overcome drug resistance<sup>11</sup>. A number of natural products have been shown to effectively regulate cancer progression and to also enhance the anticancer effects of various anticancer agents. For example, kanglaite is a Chinese herbal medicine that contains extracts from coix seeds. Various studies have shown that kanglaite has anticancer effects, particularly for gastric, lung, and liver cancer. This herbal medicine has undergone clinical trials and is now used in combination with conventional therapy to improve patient quality of life<sup>12-14</sup>.

Astragaloside IV (AS-IV) is isolated from a Chinese herb, *Astragalus mongholicus*. It is known to have a variety of immunomodulatory activities and has been widely used to regulate the immune system and provide neuroprotection<sup>15-18</sup>. It has previously been shown that AS-IV can inhibit the invasion and migration of malignant pulmonary cells via regulation of Treg cells and the PKC- $\alpha$ -ERK1/2-NF- $\kappa$ B axis<sup>19,20</sup>. The goal of the present study was to investigate the potential biological activities of AS-IV and to better understand the key signaling pathways involved in NSCLC.

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## MATERIALS AND METHODS

### Cell Culture and Reagents

HCC827, A549, and NCI-H1299 human NSCLC cell lines were obtained from ATCC (Manassas, VA, USA). AS-IV was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cells were cultured in DMEM containing 10% FBS, under 5% CO<sub>2</sub> conditions at 37°C for 24 h.

### Evaluation of Cell Growth and Proliferation

A CCK-8 assay was performed to evaluate cell growth and proliferation. Briefly, the culture media was supplemented with 100  $\mu$ l of CCK-8 liquid, and cells were incubated for 4 h at 37°C. Treatment was terminated at predetermined time points. A multiwell spectrophotometer (Bio-Rad; Hercules, CA, USA) at 490 nm was used to evaluate absorbance at 450 nm.

### Flow Cytometry

Cells were washed with ice-cold PBS and then centrifuged at 1,000 rpm for 5 min. The supernatant was removed, and binding buffer was added to the cell suspension. Cells were stained with FITC-annexin V and propidium iodide (PI) for 10 min at room temperature. A FACS scan flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used to analyze the fluorescent signals, and FlowJo software version 7.6 was used to estimate the rate of cell death.

### Scratch Test

Cells were plated onto six-well plates (cell number:  $4 \times 10^5$  cells/well), and trypsin was used to digest the cells. Scratches were made in the cell monolayer using a sterile pipet tip (10  $\mu$ l). Cells were washed with antibiotic PBS to eliminate detached cells, and the medium was replaced with serum-free medium. Scratch distance was measured and documented immediately and after 24 h.

### Western Blot Analysis

Cell lysates were prepared using lysis buffer, and the Bradford assay (Bio-Rad) was performed to quantify the amount of cellular proteins, which were then subjected to SDS-PAGE using 8%–15% polyacrylamide gels (Bio-Rad). Proteins were subsequently transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% BSA and then incubated overnight at 4°C with specific primary antibodies diluted in TBST: anti-Bax (5023), anti-phospho-AKT (4060), anti-phospho-GSK (9323), anti-phospho- $\beta$ -catenin (4176), anti- $\beta$ -actin (3700), anti-Bcl2 (3498), anti-caspase 3 (9664), anti-AKT (4691), anti-GSK (12456), and anti- $\beta$ -catenin (8480) (Cell Signaling Technology, Beverly, MA, USA). The membranes were incubated with the appropriate secondary antibodies following washing. Bands were detected using the Pierce detection reagent (Pierce, Rockford, IL, USA) and analyzed by Chemiluminescence Imaging System (Ultra-Lum, Claremont, CA, USA).

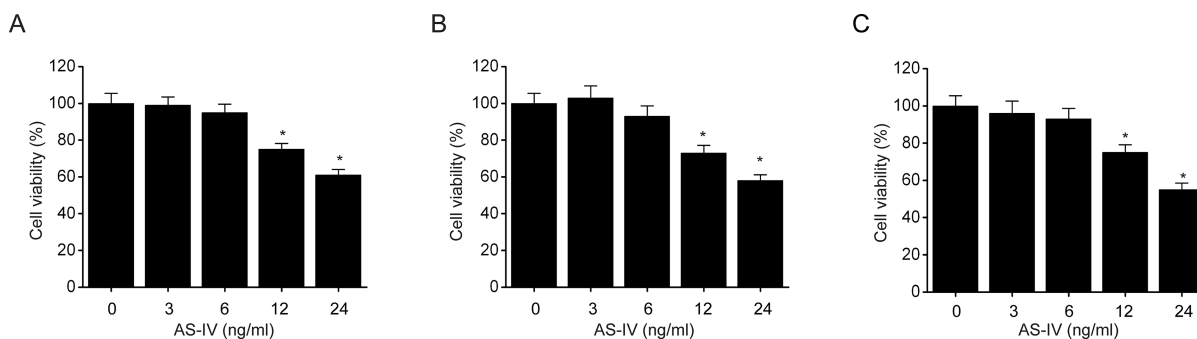
### Statistical Analysis

Results are presented as mean  $\pm$  SEM. Two-tailed Student's *t*-test or ANOVA before Tukey's post hoc analysis was performed to determine the differences between groups. Statistical significance was defined as a value of  $p < 0.05$ .

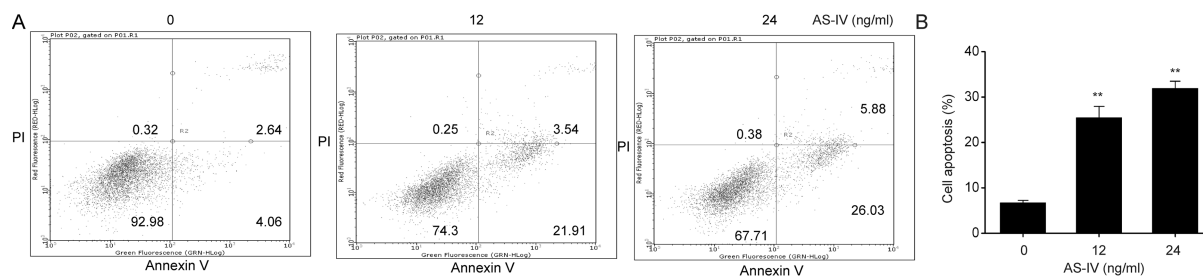
## RESULTS

### AS-IV Inhibits NSCLC Cell Proliferation

A CCK-8 assay was performed to investigate the cytotoxic effects of AS-IV on NSCLC cells. Three different human NSCLC cell lines, A549, HCC827, and NCI-H1299, were treated with AS-IV. We found that AS-IV (12 and 24 ng/ml) significantly inhibited the proliferation of A549 (Fig. 1A), NCIH1299 (Fig. 1B), and HCC827 (Fig. 1C) cells. These results show that AS-IV is able to prohibit NSCLC cell survival.



**Figure 1.** Astragaloside IV (AS-IV) inhibits non-small cell lung cancer (NSCLC) cell proliferation. NSCLC cell lines A549 (A), NCI-H1299 (B), and HCC827 (C) were incubated for 48 h with different amounts of AS-IV (3, 6, 12, and 24 ng/ml). Cell survival was measured using a CCK-8 assay. Results are presented as mean  $\pm$  SEM of three independent experiments. \* $p < 0.05$ , compared to the control group.



**Figure 2.** AS-IV enhances NSCLC cell death. A549 cells were incubated with AS-IV (12, and 24 ng/ml) for 48 h. (A, B) Flow cytometric analysis of apoptosis. Results are presented as mean±SEM of three independent experiments. \*\**p*<0.01, compared to the control group.

*AS-IV Enhances NSCLC Cell Death*

To determine the role of AS-IV in NSCLC cell death, flow cytometry was performed to evaluate annexin V-FITC/PI double staining in the three NSCLC cell lines. We found that AS-IV (12 and 24 ng/ml) significantly induced apoptosis in A549 cell lines (Fig. 2)

*AS-IV Inhibits NSCLC Cell Migration*

We next determined the effect of AS-IV on NSCLC cell migration using the well-established scratch test. Migration was noticeably reduced in A549 cells supplemented with AS-IV compared to controls (Fig. 3). These findings show that AS-IV suppresses the migration of NSCLC cells.

*AS-IV Regulates the Expression of Bcl-2 and Bax in NSCLC Cells*

Western blotting was performed to determine the effect of AS-IV on Bcl-2 and Bax expression in NSCLC cells. We found that AS-IV treatment significantly reduced Bcl-2 expression but promoted Bax expression in NSCLC cells, compared to the control group (Fig. 4A–C). Moreover, AS-IV treatment caused a marked increase

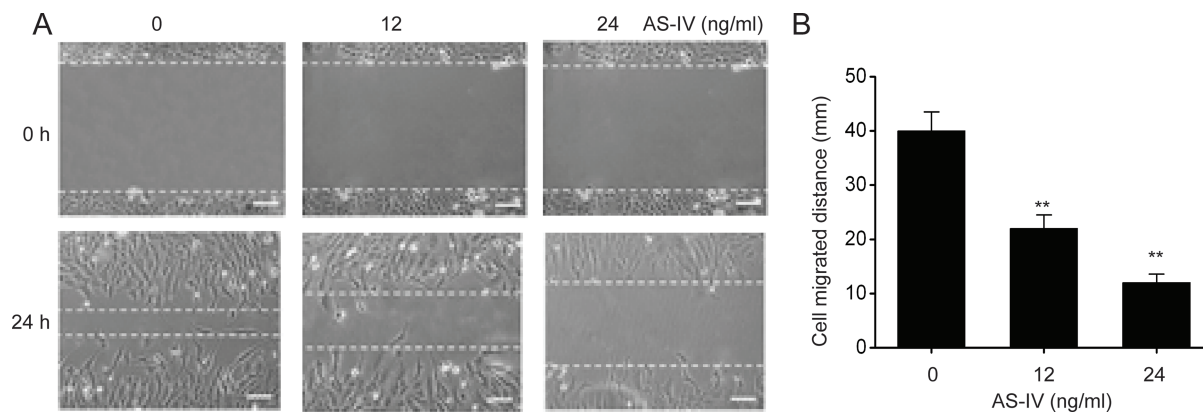
in the expression of cleaved caspase 3 compared to the control group (Fig. 4D). These results show that AS-IV enhances the expression of proteins that are related to NSCLC cell death.

*AS-IV Inhibits Stimulation of the Akt/GSK-3β/β-Catenin Axis in NSCLC Cells*

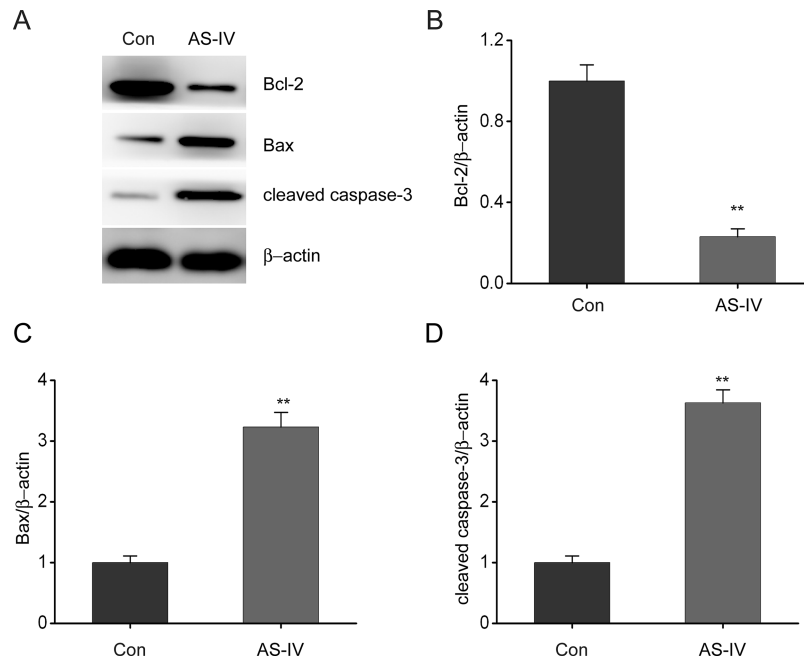
Previous studies have shown that the Akt/GSK-3β/β-catenin axis is involved in cell growth and proliferation in a wide range of human cancers<sup>21</sup>. In order to assess the underlying mechanisms by which AS-IV was exerting its cytotoxic effects, we performed Western blot analysis to examine the expression of components of the Akt/GSK-3β/β-catenin axis. AS-IV treatment resulted in significant inhibition of the phosphorylation of β-catenin, GSK-3β, and Akt (Fig. 5). These findings suggest that AS-IV inhibits NSCLC progression by suppressing stimulation of the Akt/GSK-3β/β-catenin axis.

**DISCUSSION**

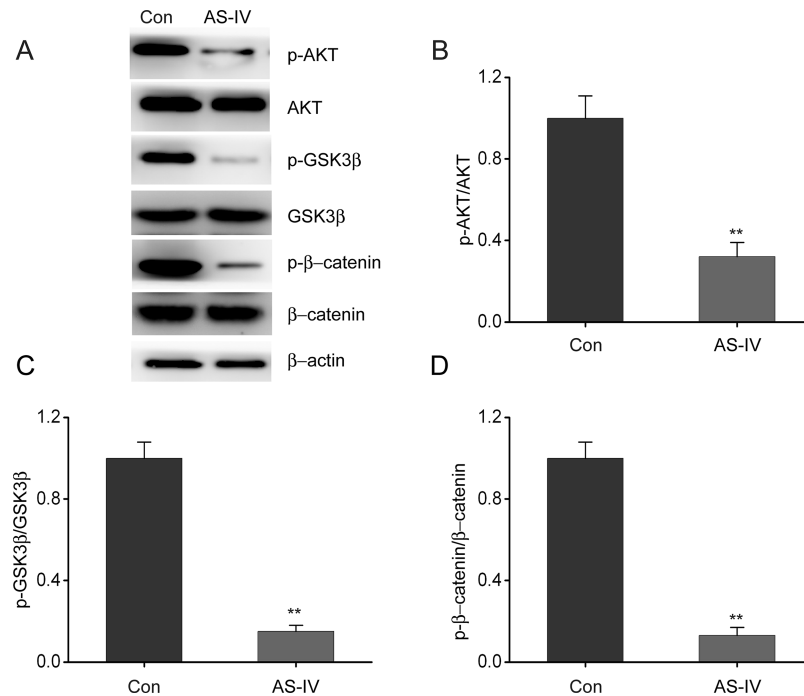
The major finding of the present study is that AS-IV inhibits NSCLC growth and proliferation via inhibition of the Akt/GSK-3β/β-catenin axis. AS-IV prevents



**Figure 3.** AS-IV suppresses migration of NSCLC cells. A549 cells were treated with AS-IV (12 and 4 ng/ml) for 24 h. (A) Scratch test was performed to assess cell migration. (B) Cell migration distance. Results are presented as mean±SEM of three independent experiments. \*\**p*<0.01, compared to the control group.



**Figure 4.** AS-IV regulates expression of Bax, Bcl2, and caspase 3 in NSCLC cells. A549 cells were treated with AS-IV (24 ng/ml) for 24 h. (A–D) Representative immunoblots (A), Bcl-2 (B), Bax (C), and cleaved caspase 3 expression (D) in A549 cells. Results are presented as mean  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$ , compared to the control group.



**Figure 5.** AS-IV inhibits stimulation of the Akt/GSK-3 $\beta$ / $\beta$ -catenin axis in NSCLC cells. A549 cells were treated with AS-IV (24 ng/ml) for 24 h. (A–D) Representative immunoblots (A), p-AKT (B), p-GSK-3 $\beta$  (C), and p- $\beta$ -catenin (D) expression in A549 cells. Results are presented as mean  $\pm$  SEM of three independent experiments. \*\* $p < 0.01$ , compared to the control group.

stimulation of the Akt/GSK-3 $\beta$ / $\beta$ -catenin axis, which promotes Bax expression and suppresses Bcl2 expression. AS-IV also enhances caspase 3 stimulation, ultimately leading to cell death. Moreover, with increased concentrations, AS-IV shows significant effect on the suppression of cell migration.

Cytotoxic chemotherapy has been the main systemic approach used to treat advanced, metastatic NSCLC. Nevertheless, drug resistance, caused by extensive use, has made chemotherapy difficult and often unsuccessful<sup>22-24</sup>. Traditional Chinese medicine has been widely utilized due to its promising ability to treat inflammatory disorders<sup>25</sup>, allergic responses<sup>26</sup>, and malignancies<sup>27</sup>. Previous studies have shown that AS-IV is effective at relieving inflammatory reactions, as well as treating cardiovascular illnesses and promoting sensitivity to drugs with respect to malignant cells, via modulation of intracellular signaling pathways<sup>16,28</sup>. In the present study, we showed that AS-IV inhibits cell growth, proliferation, and migration, and is able to induce NSCLC cell death. Moreover, Bcl-2 proteins participate in the regulation of cell death, which is dependent on mitochondria. Upstream of mitochondria, Bax promotes cell death while Bcl-2 suppresses cell death. The comparative ratio of the two proteins (Bax/Bcl2) is crucial to the stimulation of cell death<sup>29</sup>. Consequently, we found that AS-IV suppressed Bcl-2 expression while promoting Bax expression, which then results in caspase 3 cleavage leading to activation of cell death.

The class I PI3K/Akt signaling axis mediates several key cellular processes, including cell viability, migration, and cell cycle progression<sup>30</sup>. PI3K/Akt malfunction enhances the development of malignancies, as well as vascularization of numerous tumors<sup>31,32</sup>. Previous studies have shown that AKT stimulates GSK3 $\beta$  phosphorylation, which triggers the nuclear aggregation of  $\beta$ -catenin, which is related to TCF4. This then stimulates the expression of various transcription factors linked to EMT, such as Twist1, ZEB1, and SNAI1<sup>33</sup>. GSK-3 $\beta$  also participates in preventing malignancy development by cytoplasmic phosphorylation as well as degeneration of  $\beta$ -catenin via the Akt pathway<sup>34</sup>. There is increasing evidence to suggest that  $\beta$ -catenin is upregulated in NSCLC tumor cells when compared to normal cells. It has been shown that activation of the  $\beta$ -catenin pathway stimulates proliferation and leads to the generation and development of NSCLC<sup>35,36</sup>. In the present study, we showed that AS-IV inhibited the Akt phosphorylation and subsequent GSK-3 $\beta$  function, which in turn stimulated the ubiquitination of  $\beta$ -catenin. These findings demonstrate that AS-IV can promote  $\beta$ -catenin phosphorylation modulated via GSK-3 $\beta$ . The phosphorylation of  $\beta$ -catenin causes it to become unstable and consequently reduces its nuclear aggregation.  $\beta$ -Catenin-regulated transcription is crucial for cell proliferation, as well as the progression of NSCLC.

In summary, our research shows that the biological effects of AS-IV in NSCLC are mediated via inhibition of the Akt/GSK-3 $\beta$ / $\beta$ -catenin signaling pathway. Although further in vivo animal studies are required to confirm its in vitro preclinical cytotoxicity, our findings suggest that AS-IV is a promising novel agent for the treatment of NSCLC.

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