

Astragaloside IV increases MMP-2 mRNA and protein expression in human lung cancer A549 cells

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Abstract. Lung cancer is the leading cause of cancer-related death in Taiwan. In the clinical treatment of lung cancer patients in Asia, an *Astragalus*-based herbal mixture is commonly used in conjunction with chemotherapy. The principal component of *Astragalus* (also known as Huang-qi) is Astragaloside IV. The Astragaloside IV marker has been qualified for the phytochemicals of *Astragalus*. The recurrence and metastasis of lung cancer are positively correlated with matrix metalloproteinase-2 (MMP-2) mRNA and protein expression. This study examined the effects of Astragaloside IV on the mRNA and protein expression of MMP-2 in lung cancer A549 cells by RT-PCR and Western blotting. The cytotoxicity of Astragaloside IV in these cells was determined using CellTiter 96® Aqueous One Solution Reagent and the MTT assay. A549 cells were treated for different durations with Astragaloside IV concentrations of 10, 20, 50 and 100 ng/ml. The respective proliferation rates per amount (relative to the control) were as follows: 24 h, 96.85±1.12, 95.63±0.83, 93.92±0.84, 95.27±0.57%; 48 h, 98.86±1.56, 95.71±0.59, 94.09±0.68, 93.44±0.5%; 72 h, 99.48±0.16, 95.60±0.48, 95.23±0.67, 94.72±1.12%. MMP-2 mRNA expression as well as vascular endothelial growth factor mRNA expression were upregulated at concentrations of 10, 20 and 50 ng/ml. Additionally, protein expression of MMP-2 was increased at concentrations of 10, 20 and 30 µg/ml after 24 h of treatment. These results indicate that Astragaloside IV upregulates MMP-2 mRNA and protein expression in A549 cells, and therefore that it may increase recurrence and metastatic rates in lung cancer. This issue should be further examined in the clinical setting.

Introduction

Astragalus membranaceus (Leguminosae), also known as Huang-qi in Chinese, is an important 'Qi tonic' herb first recorded in the classical treatise Shen Nong Pen Tsao Ching (circa A.D. 100). It has been prescribed for centuries for general debility and to increase stamina and the overall vitality of the immune system, as well as for spleen deficiency symptoms such as a lack of appetite, fatigue and diarrhea. Additionally, *Astragalus* tonifies the lungs and is used in cases of frequent colds and shortness of breath (1). Currently, it is known to demonstrate a wide range of immunopotentiating effects (2-4) and to be efficacious as an adjunct cancer therapy in order to potentiate the activity and reduce the adverse toxicities of chemotherapeutic agents, inhibit recurrences and prolong survival time (5-7). *Astragalus* components include Astraisoflavan, Astragaloside I-VII, Astraperocarpan, Formononetin, Ononin, Soyasaponin-I, D-β-asparagine, calycosin and cycloastragenol (8). The Astragaloside IV marker has been qualified for the phytochemicals of *Astragalus* (9).

Cancer metastasis, which involves multiple processes and various cytophysiological changes, is one of the primary causes of cancer-related death. It has been reported that silibinin inhibits the invasion of human lung cancer cells by decreasing production of matrix metalloproteinase (MMP)-2 (10). Another study demonstrated that inhibition of MMP-2 and -9 activity by ONO-4817 suppressed lung metastasis (11). MMP-2 may play an important role in A549 cell migration with laminin-10/11 through an α3β1 integrin-dependent pathway (12). It has also been well documented that MMPs play an integral role in tumor spread and the metastatic cascade; human fibrosarcoma cells aggregate platelets via mechanisms that are mediated, in part, by MMP-2 (13).

According to a 2004 report from the 'People's Health Bureau of Taiwan', lung cancer is the leading cause of cancer-related death in the country. *Astragalus* root (*Radix Astragali*) has been clinically administered to many lung cancer patients post-operatively and/or following chemotherapy or radiotherapy. Though its functions are controversial, there is no available information addressing whether the herb affects the metastasis and recurrence of lung cancer. Hence, in the present study, we investigated the effects of Astragaloside IV on MMP-2 messenger ribonucleic acid (mRNA) and protein expression in human lung cancer A549 cells.

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Materials and methods

Chemicals and reagents. Astragaloside IV ($C_{41}H_{68}O_{14}$) was obtained from Fluka BioChemika (74777). The human lung cancer A549 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Tris-HCl was purchased from Sigma Chemical Co. (St. Louis, MO, USA), TE buffer from Merck Co. (Darmstadt, Germany) and penicillin-streptomycin, trypsin-EDTA, fetal bovine serum and glutamine from Gibco BRL (Grand Island, NY, USA). The CellTiter 96 AQueous One Solution Cell Proliferation Assay, TRIzol Reagent and the Reverse Transcription System were purchased from Promega Corp. (USA). Gene Taq DNA polymerase was purchased from Blossom Biotechnologies Inc. (Taiwan cat no. GP500), while MMP-7 and -2, along with vascular endothelial growth factor (VEGF) primers, were purchased from Mission Biotech (Taiwan). Minimum essential medium (MEM) was purchased from Hyclone (cat. no. SH30008.01).

The A549 human lung cancer cell line. Human lung cancer A549 cells were placed into 25-cm² tissue culture flasks and maintained in MEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell proliferation. A549 cells were plated in 96-well plates at a density of 2×10^4 cells/well and grown for 24 h. Various concentrations of Astragaloside IV were then added to the cells for final concentrations of 10, 20, 50 and 100 ng/ml, while MEM (solvent) alone was added for the control. Cells were then grown at 37°C in 5% CO₂ and 95% air for 24 h, 48 and 72 h. In order to determine cell proliferation, CellTiter 96 AQueous One Solution Reagent (20 µl/well) was added, and the plates were incubated for 2 h at 37°C in a humidified 5% CO₂ atmosphere. Absorbance was recorded at 490 nm using a 96-well plate reader. All cultures were assayed in triplicate.

Reverse transcriptase polymerase chain reaction (RT-PCR). A549 cells (5×10^6 /ml) were treated with or without various concentrations (0, 10, 20 and 50 ng/ml) of Astragaloside IV for 24 h. Total RNA was extracted from the cells using TRIzol Reagent, then 1 µg of the total RNA, 0.5 µg oligo(dT)₁₅ primer, 10 µM dNTP mixture and diethyl pyrocarbonate-treated water were combined in a microcentrifuge tube at a final volume of 20 µl. The entire mixture was heated at 70°C for 5 min and chilled on ice for at least 5 min. Reverse transcription was conducted using the Reverse Transcription System (Promega) according to the manufacturer's instructions. Reverse transcription products from the total RNA served as the template for PCR. For the amplification of target cDNA, the following components were combined in a 25 µl solution: 25 mM MgCl₂, 10 mM dNTP, 10 µM primer, Taq DNA polymerase, Reverse Transcription 10X buffer and first-strand cDNA reaction nuclease-free water. Primer sequences were: MMP-2 forward 5'-TGCGGTTTCTCGAATCCAT-3' and reverse 5'-GGAGTCCGTCCTTACCGTCAA 3'; MMP-7 forward 5'-GGTCACCTACAGGATCGTATCATAT-3' and reverse 5'-CATCACTGCATTAGGATCAGAGGAA-3'; VEGF

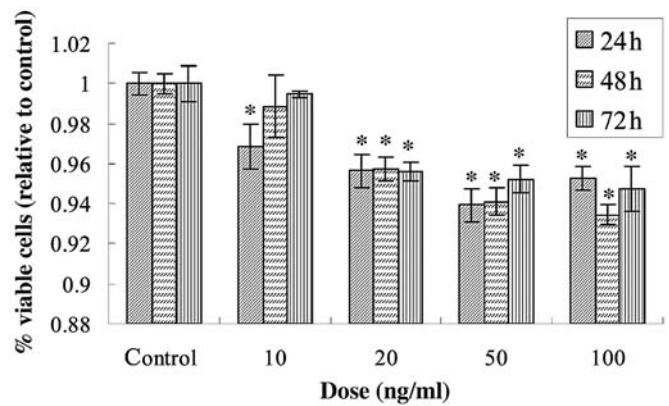


Figure 1. Cytotoxicity of Astragaloside IV on A549 cells. A549 cells were plated in 96-well plates at a density of 2×10^4 cells/well and grown for 24 h. Various concentrations of Astragaloside IV were then added to the cells for final concentrations 10, 20, 50 and 100 ng/ml, while MEM (solvent) alone was added for the control. Cells were then grown at 37°C in 5% CO₂ and 95% air for 24, 48 and 72 h. To determine cell proliferation, Cell Titer 96 AQueous One Solution Reagent was used as described in Materials and methods. Data represent the mean \pm SD. * $P < 0.05$, significant difference. All samples were assayed in triplicate.

forward 5'-GGGGGATCCGCTCCGAAACCATGAAC TT-3' and reverse 5'-CCCGAATTCTCCTGGTGAGAG ATCTGGTT-3'; and β -actin forward 5'-ACCGCTCGT TGCCAATAGTGATGA-3' and reverse 5'-CTGGCT GGCCGGCACCTGACA-3'.

Synthesis and analysis of RT-PCR products. RT-PCR products were synthesized using the Access RT-PCR System (Promega). Briefly, the RT-PCR conditions were 45 min at 45°C followed by 2 min at 94°C (RT), then 1 min at 94°C, 1 min at 58°C and 1 min at 72°C (PCR) for 35 cycles. The PCR products (8 µl) were analyzed using 3% agarose gel electrophoresis, and were readily visible by UV transillumination of an ethidium bromide-stained gel. The optical intensities of the PCR products were analysed using a Gel-Pro Analyzer image system. β -actin mRNA, which has been shown to be unaffected by stimulation, was measured in both normal and stimulated RNA samples at each point using the same cDNA. Integrated optical density (IOD) measurement of 10 separate β -actin samples did not vary >10% from the mean IOD, which is an expected variation resulting from experimental technique. For the target genes MMP-2, VEGF, MMP-7 and β -actin, the PCR product size was 605, 550, 373 and 294 bp, respectively.

Cell viability assay. Astragaloside IV concentrations in the A549 cells were increased to 2.5, 5, 20 and 30 µg/ml, and cytotoxicity was determined using the MTT assay. Briefly, 1×10^4 A549 cells in 200 µl media per well were plated onto a 96-well plate and incubated at 37°C in 5% CO₂ overnight to allow cells to attach to the wells. Various concentrations of Astragaloside IV dissolved in DMSO were added to each well followed by incubation at 37°C in 5% CO₂ for 24, 48 and 72 h to allow the drug to take effect. MTT solution was made at 5 mg/ml PBS, and 200 µl of the solution was added to each well. Incubation was carried out at 37°C in 5% CO₂ for 4 h to allow the MTT to be metabolized. The media was discarded and formazan (the MTT metabolic product) was resuspended

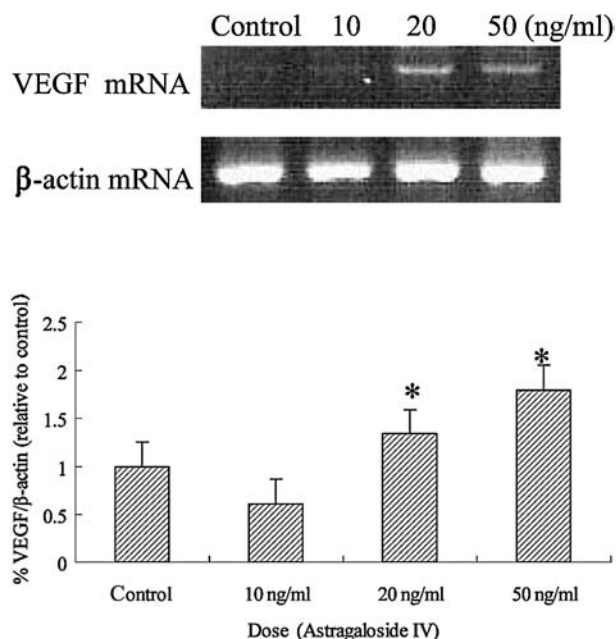


Figure 2. Representative RT-PCR showing changes in the levels of VEGF mRNA expression in A549 cells after exposure to Astragaloside IV. A549 cells (5×10^6 /ml) were treated with or without Astragaloside IV concentrations of 0, 10, 20 and 50 ng/ml for 24 h. Total RNA was extracted from the cells using the protocol specific to TRIzol Reagent. RT-PCR was conducted and determined as described in Materials and methods. Data represent the mean \pm SD. * $P < 0.05$, significant difference. All samples were assayed in triplicate.

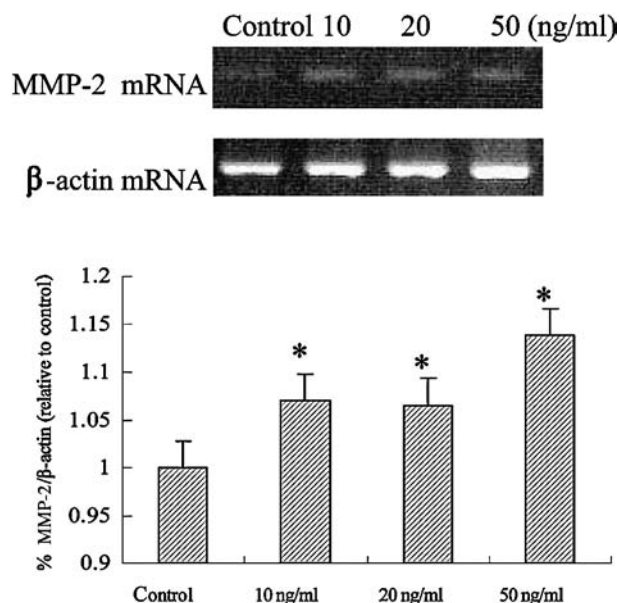


Figure 3. Representative RT-PCR showing changes in the levels of MMP-2 mRNA expression in A549 cells after exposure to Astragaloside IV. A549 cells (5×10^6 /ml) were treated with or without Astragaloside IV concentrations of 0, 10, 20 and 50 ng/ml for 24 h. Total RNA was extracted from the cells using the protocol specific to TRIzol Reagent. RT-PCR was conducted and determined as described in Materials and methods. Data represent the mean \pm SD. * $P < 0.05$, significant difference. All samples were assayed in triplicate.

in 200 μ l DMSO then placed on a shaking table at 150 rpm for 5 min to thoroughly combine it with the solvent. Optical density was read at 560 nm, and background noise was subtracted at 590 nm.

Cell cycle analysis. The percentages of cells in the sub-G1, G0/G1, S and G2/M phases were determined by flow cytometry as previously described (14). Briefly, 1×10^6 cultured A549 cells in 10-cm dishes were treated with 10, 20 and 30 μ g/ml of Astragaloside IV for 48 h. The harvested cells were washed twice with PBS, gently fixed with ice-cold 70% ethanol overnight and then resuspended in PBS containing 40 μ g/ml propidium iodide, 0.1 mg/ml RNase (Sigma) and 0.1% Triton X-100. The cells were maintained at 37°C for 30 min, and the cell cycle was analyzed by a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser at 488 nm.

Protein preparation. For protein extraction, 2×10^6 A549 cells were seeded onto 10-cm cultured dishes and treated with 10, 20 and 30 μ g/ml of Astragaloside IV for 24 h before being harvested by centrifugation. Proteins were extracted as previously described (15). Briefly, cell pellets were resuspended in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet p-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin and 5 μ g/ml antipain) for 30 min at 4°C. Lysates were immediately centrifuged at $13,000 \times g$ for 20 min at 4°C, and the supernatant was collected, aliquoted (50 μ g/tube) and

stored at -80°C until used for assay. The extracted protein concentrations were determined using the Bradford method (16).

Western blotting. All protein samples were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Life Science Products, Hercules, CA, USA) as previously described (15). The SDS-separated proteins were then equilibrated in transfer buffer (50 mM Tris, pH 9.0, 40 mM glycine, 0.375% SDS and 20% methanol) and transferred onto an Immobilon-P membrane (Millipore Corp., Bedford, MA, USA). The membranes were incubated with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h, then washed and incubated with appropriate dilutions of specific antibodies such as VEGF, NF- κ Bp65, MMP-2 and β -actin (Upstate Biotechnology, Lake Placid, NY, USA) at 4°C overnight. After incubation with anti-mouse peroxidase-conjugated antibody (Santa Cruz, CA, USA), the immunoreactive bands were visualized with an enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech). β -actin was used as an internal control.

Statistical analysis. The significance of the differences between results was assessed using the Student's t-test. P-values < 0.05 were considered significant. All samples were assayed in triplicate.

Results

Cytotoxic effects of Astragaloside IV on A549 cells. The results of cell proliferation indicate that concentrations of

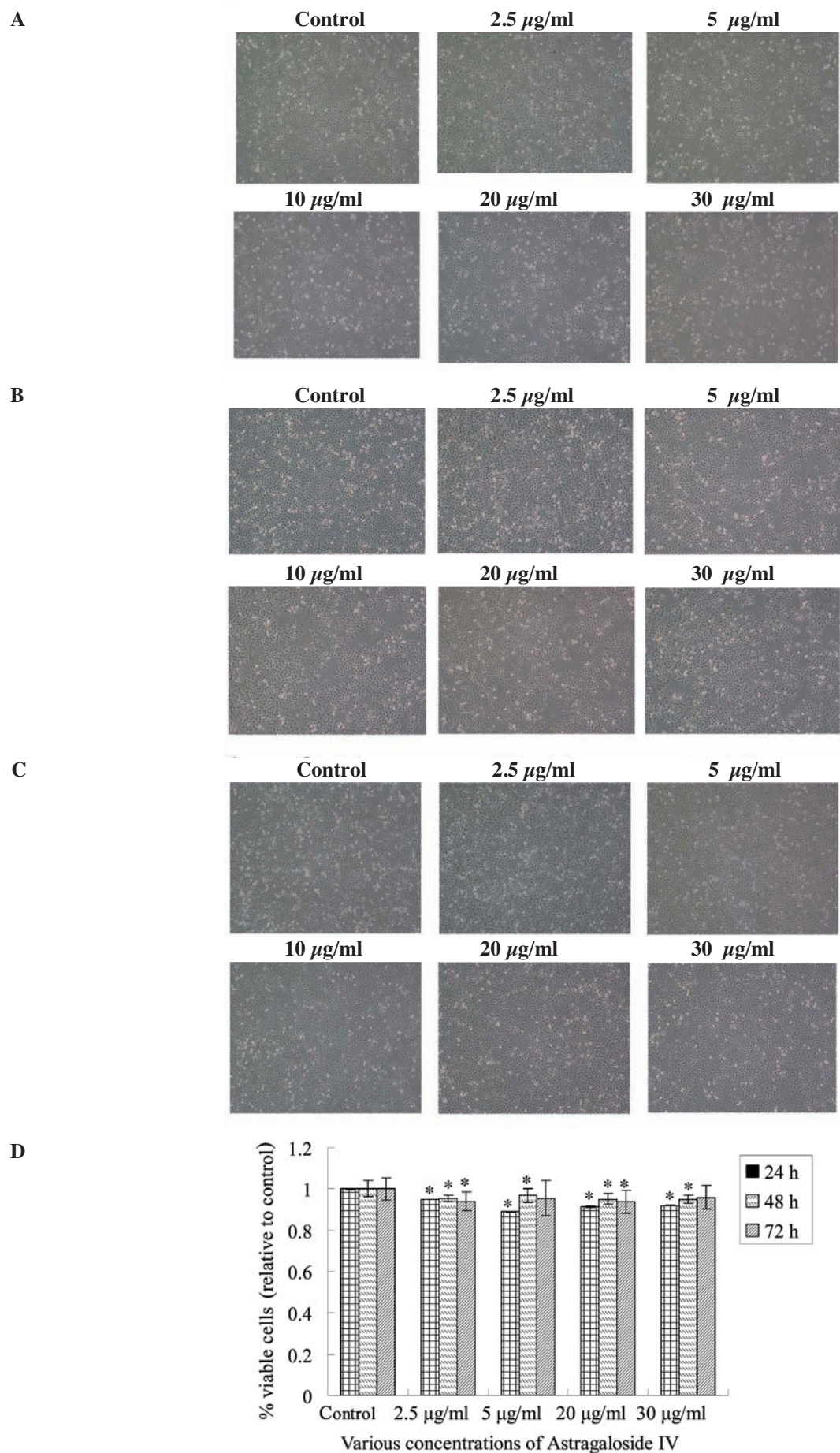


Figure 4. A549 cells were treated with increased Astragaloside IV concentrations of 0, 2.5, 5, 20 and 30 $\mu\text{g/ml}$ for (A) 24 h, (B) 48 h and (C) 72 h. The results were viewed under a contrast-phase microscope (x100) (D) The cytotoxicity of Astragaloside IV in A549 cells was determined using the MTT assay as described in Materials and methods. Astragaloside IV significantly inhibited A549 cell growth. Each point is the mean \pm SD of three experiments. * $P < 0.05$.

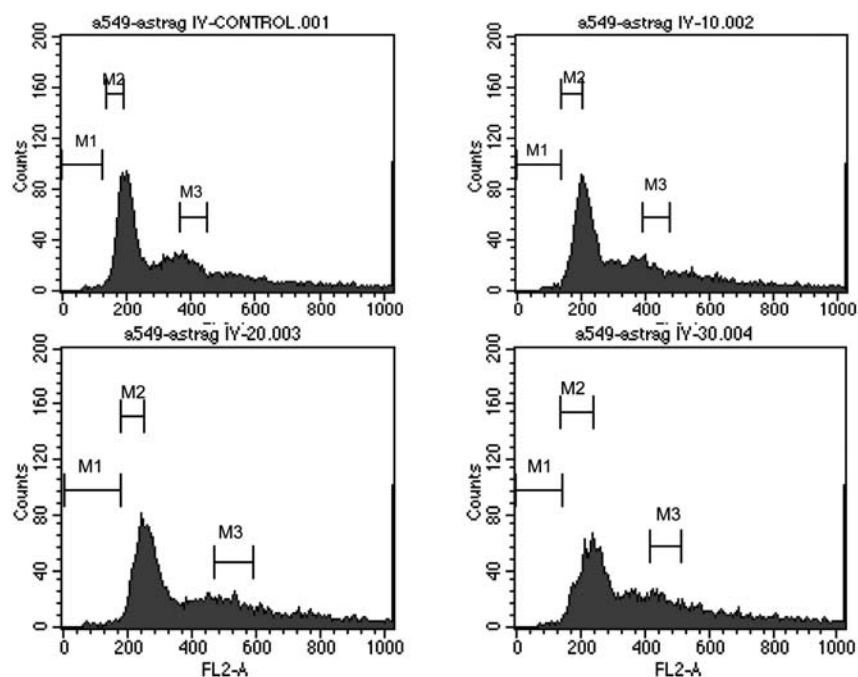


Figure 5. A549 cells were exposed to Astragaloside IV concentrations of 10, 20 and 30 $\mu\text{g/ml}$ for 48 h. The cells were harvested and cell cycle analysis was performed by flow cytometry as described in Materials and methods. The percentages of cells in the sub-G1 phase exhibited a significant increase after treatment with 10, 20 and 30 $\mu\text{g/ml}$ of Astragaloside IV for 48 h.

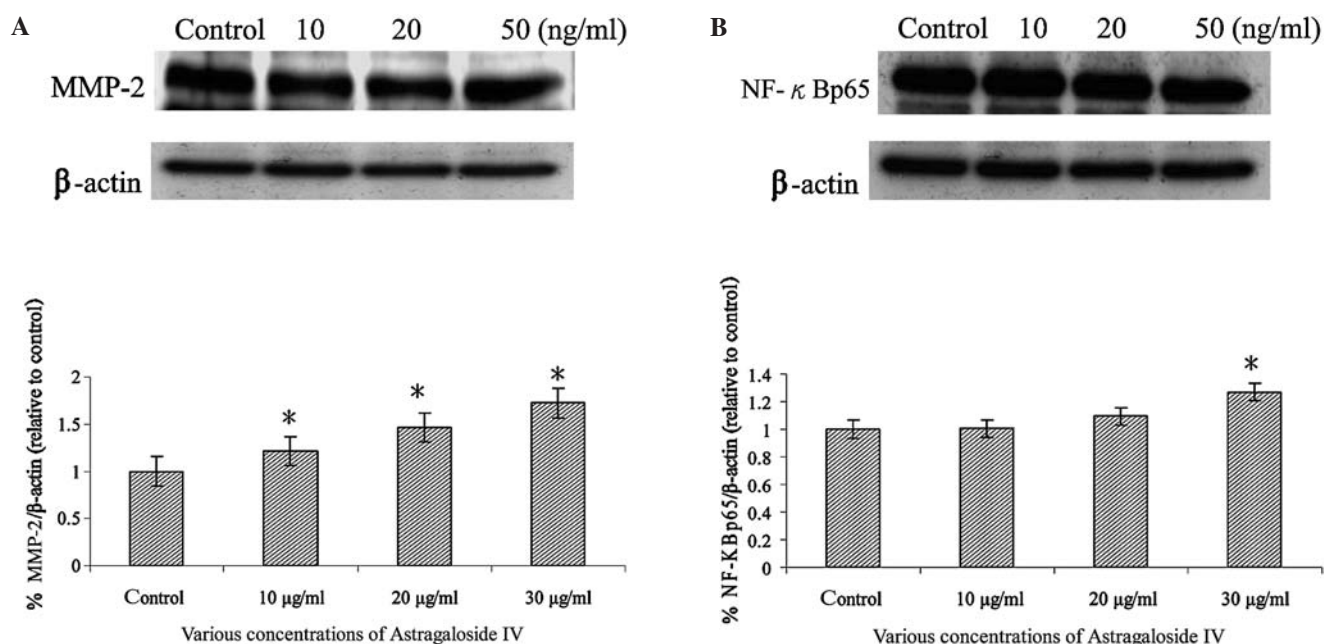


Figure 6. Protein expression of MMP-2 and NF- κ Bp65 in A549 cells after exposure to Astragaloside IV. A549 cells ($2 \times 10^6/\text{ml}$) were treated with different Astragaloside IV concentrations of 10, 20 and 30 $\mu\text{g/ml}$ for 24 h before being harvested by centrifugation. Total protein was prepared and determined as described in Materials and methods. Levels of (A) MMP-2 and (B) NF- κ Bp65 expression were estimated by Western blotting as described in Materials and methods. Protein expression of (A) MMP-2 was significantly increased after treatment with Astragaloside IV at concentrations of 10, 20 and 30 $\mu\text{g/ml}$ for 24 h. The protein expression of (B) NF- κ Bp65 was significantly increased after treatment with Astragaloside IV at a concentration of 30 $\mu\text{g/ml}$ for 24 h. Each point is the mean \pm SD of three experiments. * $P < 0.05$.

Astragaloside IV of 10, 20, 50 and 100 ng/ml in the A549 cells respectively resulted in proliferation rates (relative to the control) of 96.85 ± 1.12 , 95.63 ± 0.83 , 93.92 ± 0.84 , $95.27 \pm 0.57\%$ after 24 h; 98.86 ± 1.56 , 95.71 ± 0.59 , 94.09 ± 0.68 , $93.44 \pm 0.5\%$ after 48 h; and 99.48 ± 0.16 , 95.60 ± 0.48 , 95.23 ± 0.67 , $94.72 \pm 1.12\%$ after 72 h (Fig. 1).

Effects of Astragaloside IV on VEGF and MMP-2 mRNA expression in A549 cells. RT-PCR revealed VEGF mRNA expression to be upregulated at Astragaloside IV concentrations of 20 and 50 ng/ml (Fig. 2). MMP-2 mRNA expression was upregulated at concentrations of 10, 20 and 50 ng/ml (Fig. 3).

Cytotoxicity of Astragaloside IV in human lung cancer A549 cells. The results of the cell viability assay, visualized using a contrast-phase microscope at a magnification of $\times 100$ (Fig. 4A, B and C), indicate that when A549 cells were exposed to increased Astragaloside IV concentrations of 0, 2.5, 5, 20 and 30 $\mu\text{g/ml}$, the numbers of viable cells were 94.93 ± 0.15 , 88.88 ± 0.1 , 91.28 ± 0.23 and $91.86 \pm 0.15\%$ at 24 h; 95.48 ± 1.53 , 96.79 ± 3.4 , 95.07 ± 1.97 and $95.08 \pm 1.81\%$ at 48 h; and 93.94 ± 4.61 , 95.47 ± 8.55 , 93.73 ± 5.63 and $95.95 \pm 5.27\%$ at 72 h, respectively. This indicates that Astragaloside IV did not significantly inhibit the growth of A549 cells (Fig. 4D).

Effects of Astragaloside IV on the cell cycle. In order to determine the effects of Astragaloside IV on cell growth and apoptosis in A549 cells, cell cycle distribution was analyzed by flow cytometry. The results indicate that the percentage of cells in the sub-G1 phase did not increase after 48 h of treatment with Astragaloside IV concentrations of 10, 20 and 30 $\mu\text{g/ml}$ (Fig. 5).

Metastasis-associated protein expression. Astragaloside IV-mediated cell metastasis was investigated by determining the expression of metastasis regulator proteins using Western blotting. The results indicate that protein expression of MMP-2 was significantly increased after 24 h of treatment with Astragaloside IV at concentrations of 10, 20 and 30 $\mu\text{g/ml}$ (Fig. 6A). The protein expression of NF- κB p65 was significantly increased after 24 h of treatment with Astragaloside IV at a concentration of 30 $\mu\text{g/ml}$ (Fig. 6B).

Discussion

Astragalus membranaceus (family *Leguminosae*) is a genus widely distributed throughout the temperate regions of the world, principally across Europe, North America and Asia. Approximately 2000 species have been described (17). It has been reported that adjunctive therapy with an *Astragalus*-containing herbal remedy (Fu-Zheng) protects adrenal cortical function, reduces gastrointestinal toxicity and ameliorates bone marrow suppression (18,19), and *Astragalus*-containing herbal mixtures are commonly used in conjunction with chemotherapy to treat cancer patients (20).

Our results indicate that VEGF mRNA expression was upregulated by Astragaloside IV in human lung cancer A549 cells. VEGF is essential to vascularization at the very early stages of tumor formation and is a key promoter of metastasis (21). In many studies, VEGF expression in the primary tumor has been correlated with a greater risk of recurrence and a poor prognosis in non-small cell lung cancer (22).

Our results also demonstrate that Astragaloside IV increased MMP-2 mRNA and protein expression in the A549 cells. It is well documented that the MMPs play a central role in tumor invasion and angiogenesis (11), and the expression of MMP-2 is thought to be associated with the growth, invasion and metastasis of human lung adenocarcinoma cell lines (12,23,24).

This is the first report to suggest that Astragaloside IV upregulates VEGF mRNA and MMP-2 mRNA and protein expression in human lung cancer A549 cells *in vitro*. This implies that Astragaloside IV may in fact increase the recur-

rence and metastatic rates of lung cancer A549 cells. This issue requires further clinical study.

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