International Immunopharmacology 23 (2014) 304–313

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Astragaloside IV inhibits migration and invasion in human lung cancer A549 cells via regulating PKC-α-ERK1/2-NF-κB pathway

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ARTICLE INFO

Article history:
Received 28 July 2014
Received in revised form 22 August 2014
Accepted 28 August 2014
Available online 16 September 2014

Keywords:
Astragaloside IV
Lung cancer
Invasion
Migration
Inflammatory response

ABSTRACT

The migration and invasion characteristics that are related to inflammatory response play important roles in the development of lung cancer. Astragaloside IV (AS-IV), an effective saponin component isolated from Astragali Radix, has been reported to inhibit metastasis of tumor cells. However, little is known about the underlying mechanism of AS-IV on inhibiting the migration and invasion characteristics of lung cancer cells. In the present study, cell proliferation was assessed by MTT colorimetric assay. Wound-healing assay and transwell chambers assay were used to detect the effects of AS-IV on the migration capacity and invasiveness of A549 cells. Metastasis-related bio-markers expressions were detected by Western blot analysis. Levels of inflammatory factors including transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in cell supernatant were tested by enzyme linked immunosorbent assay (ELISA). The expressions of PKC-α, ERK1/2 and NF-κB were analyzed by Western blot analysis. The results showed that the migration and invasion ability of A549 has been suppressed in presence of AS-IV. The levels of MMP-2, MMP-9 and integrin β1 were decreased significantly, whereas E-cadherin was increased by the treatment of different concentrations AS-IV. Furthermore, AS-IV also significantly decreased TGF-β1, TNF-α and IL-6 levels. Interestingly, PKC pathway inhibitor AEB071 (Sotrastaurin) (0.1 μM) or ERK inhibitor U0126 (1 μM) or NF-κB inhibitor PDTC (1 μM) could affect suppression of AS-IV on cell invasion, at least partially. Our results suggested that the migration and invasion of AS-IV in A549 cells might be related to the PKC-α-ERK1/2-NF-κB pathway. The result indicated that AS-IV could be used as a candidate for the inhibition of metastasis of human lung cancer.

1. Introduction

Lung cancer is one of the most aggressive human cancers. The late diagnosis of patients with lung cancer usually results in only 5-year survival rate of 10%–15%. The difficult situation has not been improved significantly over the last 30 years [1]. Lung cancer cells have metastasized into peripheral tissues before being diagnosed in many clinical cases [2]. Surgery, radiotherapy and chemotherapy can cause serious side effects which result in the low quality of life of patients [3]. Therefore, it is urgent for searching effective and safe drug to improve this disadvantage of the treatment of lung cancer.

Protein kinase C (PKC)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway plays important roles in cancer cell survival, proliferation, apoptosis, migration and invasion [4]. The PKCs over-expression, which has been regarded as one of the bio-markers for the diagnosis of cancers, can be activated by phorbol esters and also promote tumor development [5–7]. Previous studies have shown that inhibition of the expression of isoform PKC-α inhibits A549 cells invasion and migration [8]. PKCs mediated pathological signaling events in the migration and invasion of tumor cells through downstream signaling pathways such as ERK1/2 [9]. Accumulating literatures reported that the increase of ERK1/2 phosphorylation levels in tumor tissues was related to the clinical stage, invasion and migration of lung cancer, suggesting an integral role of ERK activation in regulating the signaling events [10,11]. The PKC-ERK1/2 pathway acts as important regulator contributes to the development of invasion and migration of lung cancer [12,13].

Additionally, tumor growth and metastasis are related to the microenvironment produced by inflammation [14]. There are a large number of inflammatory cells presented in tumor invasion front, especially macrophages [15]. Tumor-associated macrophages (TAMs) regulate inflammation and adaptive immunity by producing growth factors (such as TGF-β1) and cytokines (such as TNF-α and IL-6) enhances angiogenesis and promotes cell proliferation [16]. Macrophages affect the behavior and function of the tumor cells directly and are considered to be

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http://dx.doi.org/10.1016/j.intimp.2014.08.027
1567-5769/

essential factors in tumor cell migration and invasion [17]. Therefore, the inflammation plays important roles in tumor migration and invasion.

The traditional Chinese herb Astragali Radix is the root of *Astragalus membranaceus* (Fisch) Bge. or *Astragalus membranaceus* (Fisch) Bge.var. Mongholicus (Bge.) Hsiao., which has been used as folk herbal medicine in China for thousands of years. Accumulative data showed that Astragali Radix was beneficial for the treatment of lung cancer in clinical [3,18]. Astragaloside IV (AS-IV) is a purified saponin with a defined chemical structure (Fig. 1A) isolated from Astragali Radix and is recognized as the main effective compound in the extract [19]. It was reported that AS-IV inhibited tumor growth of lung cancer in model mice [20]. Additionally, the anti-migration and anti-invasion ability of AS-IV has been shown in other cells such as keratinocytes [21]. However, little is known about the underlying mechanism of AS-IV on inhibiting the migration and invasion characteristics of lung cancer cells.

Herein, we investigated the effects of AS-IV on the cell proliferation, adhesion, migration and invasion of lung cancer A549 cells and also explored its regulation effect on PKC-ERK1/2-NF-κB pathway. The effect of AS-IV on the macrophages-related growth factors TGF-β1, cytokines TNF-α and IL-6 was evaluated. The levels of integrin β1, E-cadherin, MMP-2 and MMP-9 related to metastasis affected by AS-IV were investigated.

2. Materials and methods

2.1. Reagents

AS-IV ($C_{41}H_{68}O_{14}$) (110781–201314) was purchased from the National Institutes for Food and Drug Control (Beijing, China). Human lung adenocarcinoma cell line A549 was obtained from ATCC (Manassas, VA, USA). Bovine serum albumin (BSA) was provided by Sigma (St. Louis, MO, USA). Basal DMEM medium and fetal bovine serum (FBS) were provided by Gibco (Grand Island, NY, USA). The 24-well transwell inserts (8.0 μm pores) were purchased from Corning (NY, USA). PKC inhibitor AEB071 (Sotrastaurin) was obtained from Sellek (Houston, TX, USA). ERK1/2 inhibitor U0126 and NF-κB inhibitor PDTC were purchased from Beyotime Institute of Biotechnology (Nantong, China). MMP-2, MMP-9, E-Cad, integrin β1 and PKC-α antibodies were from Boster Biological Engineering Co., Ltd. (Wuhan, China). TGF-β1 and TNF-α ELISA kits were purchased from Senbeijia Bio-tech. Co., Ltd. (Nanjing, China). Other reagents are analytical grade and from commercial sources.

2.2. Cell culture

A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 1% nonessential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. These cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Medium was changed every 2 days.

2.3. Cell viability assay

Cell viability was determined using MTT assay. Briefly, A549 cells in the logarithmic growth phase were digested by 0.25% trypsin, added to DMEM supplement with 10% fetal bovine serum and plated in 96-well plates at a final density of 0.6×10^4 cells/well. After incubation for 24 h, the cells were treated with various series diluted concentrations of AS-IV. Next, MTT (5 mg/mL, 10 μL) was added to each well, and the plates were incubated for 4 h at 37 °C. Subsequently, the supernatant was discarded, and dimethyl sulfoxide (DMSO, 100 μL) was added to each well. The optical density was read using Spectra Max 190 microplate reader (Molecular Devices, CA, United States) at 570 nm. The optical density of each well was compared with the control group treated with DMSO only. Cell viability was accounted to be the percentage of the optical density. The measurement was performed in triplicate.

2.4. Adhesion experiment

The 96-well plates covered with 50 μL matrigel (BD Biosciences, NJ, US) solutions (diluted with DMEM at concentration 1:8) and air dried overnight were used in this experiment. A549 cells (1×10^4 cells/well) were inoculated into the 96-well plates and treated with different concentrations of AS-IV (0, 5, 10 and 20 μM). After 4 h incubation, the medium was discarded, and wells were washed with 50 μL phosphate-buffered saline (PBS, pH 7.4) for 4 times to remove cells non-adherent cells. Subsequently, 10 μL MTT (Sigma; 5.0 mg/mL) was added into each well and the samples were incubated at 37 °C for 4 h. DMSO (100 μL) was added into each well and shaken for 10 min. The OD value at 570 nm was read using Spectra Max 190 microplate reader. Each group consisted of triplicates, and the cell adhesion inhibition rate was calculated.

2.5. Wound healing experiment

A wound healing assay was performed as described previously [22]. Briefly, A549 cells were seeded at a concentration of 1×10^5 cells/well into 12-well plates in DMEM supplement. Cells were incubated overnight yielding confluent monolayer for wounding. Wound tracks in the monolayer were scored in each well using a 200 μL pipette tip. Then the suspended cells were washed twice with DMEM, and the wounded cell monolayer was incubated in FBS-free medium with series AS-IV concentrations for 24 h. Photographs of the wound area were captured at the 0 and 24 h with IX73 microscope (Olympus, Tokyo, Japan). Data were quantified through analyzing the areas in the scratch not

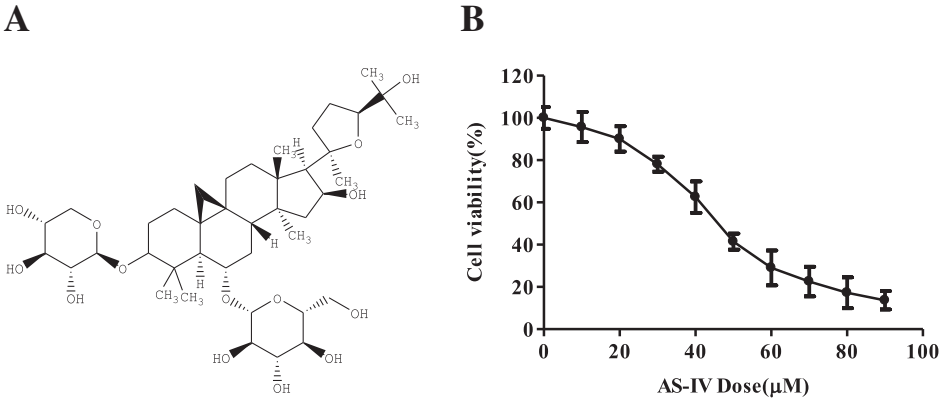

Fig. 1. Effect of AS-IV on cell viability of A549 cells. (A) The chemical structure of AS-IV. (B) A549 cells were treated with AS-IV with various concentration (0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 μM) for 24 h and measured cell viability with MTT assay. The results were shown as a percentage of control and represented as mean ± SD ($n = 6$).

covered by cells Image pro plus (IPP) software (Media Cybernetics, MD, USA). Closure rate was measured as percentage of the area in 0 h. The experiments were performed in triplicate.

2.6. Cell migration experiment

Transwell chambers (Corning, NY, USA) were used in the cell migration experiments as described previously [23]. Cells were placed on the upper layer of transwell chambers cell permeable membrane at a concentration of 1×10^4 cells/well treated with different concentrations of AS-IV (0, 5, 10 and 20 μM). The medium with 10% FBS was placed in lower chamber as chemotactic factor (600 μL/well). Following a 10 h incubation at 37 °C, cells that did not penetrate the polycarbonate membrane at the bottom of the upper chamber were erased with cotton swabs. The cells penetrated through membrane were fixed with methanol and 0.1% crystal violet for 10 min. Six visual fields of each chambers were selected under a IX73 microscope, and the number of cells was counted. The stained crystal violet was eluted by acetic acid solution. The optical density of the solution was detected with Spectra Max 190 microplate reader. Each experiment was repeated for 3 times. The migration activity was quantified by cell counter associated with the optical density.

2.7. Cell invasion experiment

Transwell chambers were used in evaluation cell invasion successfully as described previously [24]. The membrane at the bottom of each transwell chamber was coated with 50 μL matrigel and air-dried overnight. The chambers were blocked with 2% BSA, 50 μL/well and incubated at 37 °C for 2 h and were then rinsed with PBS buffer. Cells were inoculated into the upper layer of the transwell chambers at a concentration of 2×10^4 cells/well treated with different concentrations of AS-IV (0, 5, 10 and 20 μM). The 10% FBS for the experimental and control group was added into the lower layer of the transwell chamber (500 μL/well). The cells were cultured at 37 °C for 24 h. Cells that did not penetrate the polycarbonate membrane at the bottom of the chamber were erased with cotton swabs. The cells penetrated through membrane were fixed with methanol and stained with 0.1% crystal violet for 10 min. Six fields of vision of each chamber were chosen randomly under a IX73 microscope, and the number of cells was counted. The stained crystal violet was eluted by acetic acid solution. The optical density of the solution was read using Spectra Max 190 microplate reader at 590 nm. Each experiment was repeated for 3 times. The invasion activity was quantified by cell counter associated with the optical density.

2.8. ELISA for TGF-β1, TNF-α and IL-6 levels

A549 cells (6×10^5/well) were seeded in 6-well plates and treated with different concentration of AS-IV (0, 5, 10 and 20 μM) was cultured for 24 h. TGF-β1, TNF-α and IL-6 levels in the supernatant were measured using commercially available ELISA kits (SENBEIJIA, Nanjing, China). The detailed procedures were performed according to the manufacturer's protocols. After reaction, the optical density (OD) of samples was measured by a microplate reader at 450 nm. The contents of TGF-β1, TNF-α and IL-6 were calculated according to the standard curve.

2.9. Western blot assay

A549 cells (2×10^6/well) were seeded in 6-well plates overnight and treated with AS-IV (0, 5, 10 and 20 μM) for 36 h. The cells were harvested, washed twice with PBS and added the lysate at 4 °C with ice-cold RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris and 2 mM ethylenediaminetetraacetic acid [EDTA]) for 30 min. Equal amounts of proteins from each sample were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Protein bands were then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% (w/v) bovine serum albumin (BSA) for 2 h, washed in TBST thrice and incubated with primary antibodies MMP-2, MMP-9, PKC-α, p-ERK1/2, NF-κB (p65), integrin β1 and E-cadherin (1:1000) overnight at 4 °C. The membranes were washed and incubated with the secondary antibody conjugated with IgG-HRP for 1 h at room temperature and then washed in TBST thrice. Western blot chemiluminescence reagents were added for the visualization of the protein bands. The proteins detected were quantified by IPP software.

2.10. Statistical analysis

All data in this study were obtained from three independent experiments and then expressed as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) used for multiple comparisons and Student's *t* test to determine the difference between two groups. All the analysis performed on SPSS 17.0 software (SPSS, IL, USA). The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. AS-IV inhibited the viability of A549 cells

The effect of AS-IV on cell viability in A549 cells was evaluated by MTT assay. A549 cells were treated with series increasing doses (0–90 μM) AS-IV for 24 h. As shown in Fig. 1B, the result demonstrated that the viability of A549 cells was decreased significantly when treated with AS-IV at concentration more than 20 μM for 24 h. Meanwhile, the viability of A549 cells did not alter significantly when treated with the dosage below 20 μM. Therefore, the concentration of AS-IV lower than 20 μM was chosen for further experiments.

3.2. AS-IV suppressed A549 cell adhesion

The effect of AS-IV tumor cell adhesion was assessed by MTT assay. As shown in Fig. 2A, AS-IV could inhibit the adhesion of the A549 cells to matrigel. The cell adhesion ability of A549 cells was reduced by the treatment with AS-IV in a concentration-dependent manner. The cell adhesion rates of A549 cells exposed to AS-IV at concentration of 5, 10 and 20 μM reached 88.85 ± 5.12%, 79.98 ± 5.02% and 70.18 ± 7.79%, respectively, compared with control group. These data suggested that AS-IV could inhibit the adhesion of A549 cells.

3.3. AS-IV inhibited A549 cell migration

Wound-healing assay was performed to evaluate the suppression of AS-IV on A549 migration. The closure rate of A549 cells in each group was measured by IPP software. As shown in Fig. 2B and C, most of the wound area was cover with cell monolayer after 24 h incubation in control group. The closure rates of groups treated with AS-IV 5, 10 and 20 μM were 41.26 ± 4.03%, 34.33 ± 3.09% and 15.96 ± 1.48%, which were significantly smaller than the untreated group 69.20 ± 2.70% ($p < 0.01$).

After 10 h migration in transwell chambers, the number of migrating cells treated with AS-IV concentration of 5, 10 and 20 μM was significantly lower than control cells (Fig. 2D). The OD value of crystal violet indicated that migrating cells treated with AS-IV at 5, 10 and 20 μM were 75.09 ± 5.28%, 39.43 ± 7.05% and 22.65 ± 8.40% of control group cells ($p < 0.01$) (Fig. 2E).

3.4. AS-IV suppressed A549 cell invasion

Transwell invasion assay was performed to determine the suppression of AS-IV on the invasion of A549 cells. Cells penetrated through

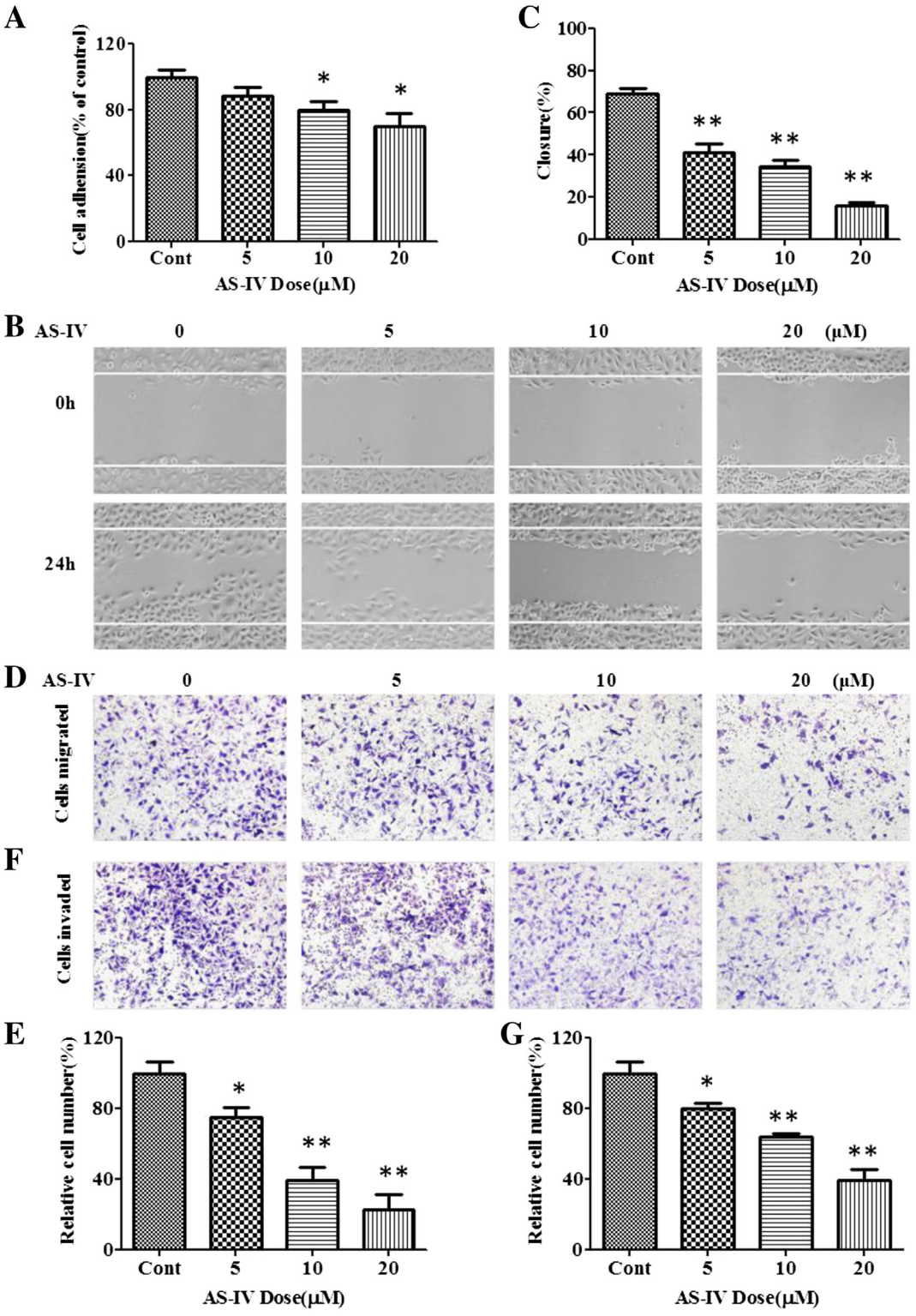

Fig. 2. Effect of AS-IV on adhesion, migration and invasion of A549 cells. (A) MTT assay for adherent ability of A549 cells. (B) Wound healing experiment for cells migration ability. (C) The area closed with migrating cells at time 0 and 24 h was measured with Image pro plus software. (D) A549 cells were inoculated in transwell chambers treated with AS-IV for 10 h to test cell migration. (E) The optical density of permeated cells. (F) A549 cells were inoculated in matrigel-coated transwell chambers treated with AS-IV for 24 h to test cell invasiveness. (G) Optical density for the permeated cells. The results were obtained from triplicate experiments and represented the means ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control group. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

the membrane were stained and counted. As depicted in Fig. 2F, the number of penetrated cells was decreased significantly by the treatment of AS-IV at the concentration of 5, 10 and 20 μM in a concentration-dependent manner when compared with the control one ($p < 0.05$). The relative OD value of penetrated cells exposed to 5, 10 and 20 μM AS-IV were 79.73 ± 3.19%, 63.84 ± 1.96% and 39.49 ± 6.05%, respectively (Fig. 2G).

3.5. AS-IV suppressed the expressions of MMP-2, MMP-9, integrin β1 and increased E-cadherin

The increasing mobility and ECM degradation of tumor cells have been found to play crucial role in the invasion of tumor cells. Herein, the levels of matrix metalloproteinases and adhesion factors including MMP-2, MMP-9, E-cadherin and integrin β1 were examined by Western blot. Interestingly, after exposure of A549 cells to 5, 10 and 20 μM AS-IV for 24 h, the level of E-cadherin was increased remarkably to 1.37 ± 0.09, 1.87 ± 0.14 and 2.51 ± 0.16-fold of control group, whereas the level of integrin β1 was decreased markedly to 0.78 ± 0.05, 0.57 ± 0.03 and 0.47 ± 0.05-fold of control group (Fig. 3A).

The overexpression of MMP-2 and MMP-9 has been found to contribute to the adhesion and migration of tumor cells. After being treated with 5, 10 and 20 μM AS-IV, the protein relative contents of MMP-2 were 0.92 ± 0.04, 0.74 ± 0.05 and 0.68 ± 0.03-fold of the control one ($p < 0.05$) while MMP-9 were 0.66 ± 0.04, 0.47 ± 0.05 and 0.42 ± 0.03-fold ($p < 0.05$) (Fig. 3B). These results suggested that AS-IV inhibited the metastasis of A549 cells via reducing the expressions of integrin β1, MMP-2 and MMP-9 and increasing the expression of E-cadherin.

3.6. Effect of AS-IV on the levels of inflammation factors

Inflammation factors such as TGF-β1, TNF-α and IL-6 have been reported to play important roles in tumor migration, invasion and development. The inflammation factors were detected by ELISA kit. As shown in Fig. 3C, ELISA results showed that TGF-β1, TNF-α and IL-6 levels were decreased significantly after being treated with 5, 10 and 20 μM AS-IV, compared with the control group ($p < 0.05$).

3.7. Effect of AS-IV on the PKC-α, ERK1/2 and NF-κB levels of A549 cells

In order to reveal this underlying mechanism, the effect of AS-IV on PKC-α, ERK1/2 and NF-κB expression level was detected (Fig. 4A). Interestingly, a significant decrease of PKC-α was observed in membranes of A549 cells treated with 5, 10 and 20 μM AS-IV for 24 h while an increase in the cytoplasma than the control one ($p < 0.01$). The PKC-α levels in cell membrane were 0.71 ± 0.10, 0.51 ± 0.06 and 0.39 ± 0.05-fold of control group ($p < 0.01$), while cytoplasmic PKC-α levels were 3.01 ± 0.29, 3.70 ± 0.27 and 4.23 ± 0.43-fold of control group ($p < 0.01$).

The phosphorylation of ERK1/2 plays important roles in cancer invasion. The expressions of p-ERK1/2 exposed to 5, 10 and 20 μM AS-IV for 24 h were suppressed by 0.75 ± 0.08, 0.49 ± 0.08 and 0.44 ± 0.05-fold of control group markedly, while ERK1/2 expression changed insignificantly were 1.17 ± 0.03, 1.20 ± 0.05 and 1.22 ± 0.04 of control group.

The activation of NF-κB promotes cancer cells invasion results in cancer metastasis. After being treated with AS-IV at 5, 10 and 20 μM for 24 h, the expressions of NF-κB (p65) expressed in A549 cells were 0.88 ± 0.05, 0.60 ± 0.07 and 0.49 ± 0.04-fold of control group respectively ($p < 0.01$)(Fig. 4B).

3.8. AS-IV inhibited invasion and migration through suppressing PKC-α in A549 cells

To further verify the specific effect of AS-IV on PKC-α pathway, A549 cells were pretreated with PKC-α inhibitor AEB071 (0.1 μM) for 30 min and then incubated in the presence or absence of AS-IV (10 μM) for 24 h. As shown in Fig. 5A and B, the relative PKC-α level on cell membrane treated with AEB071 and AS-IV diminished

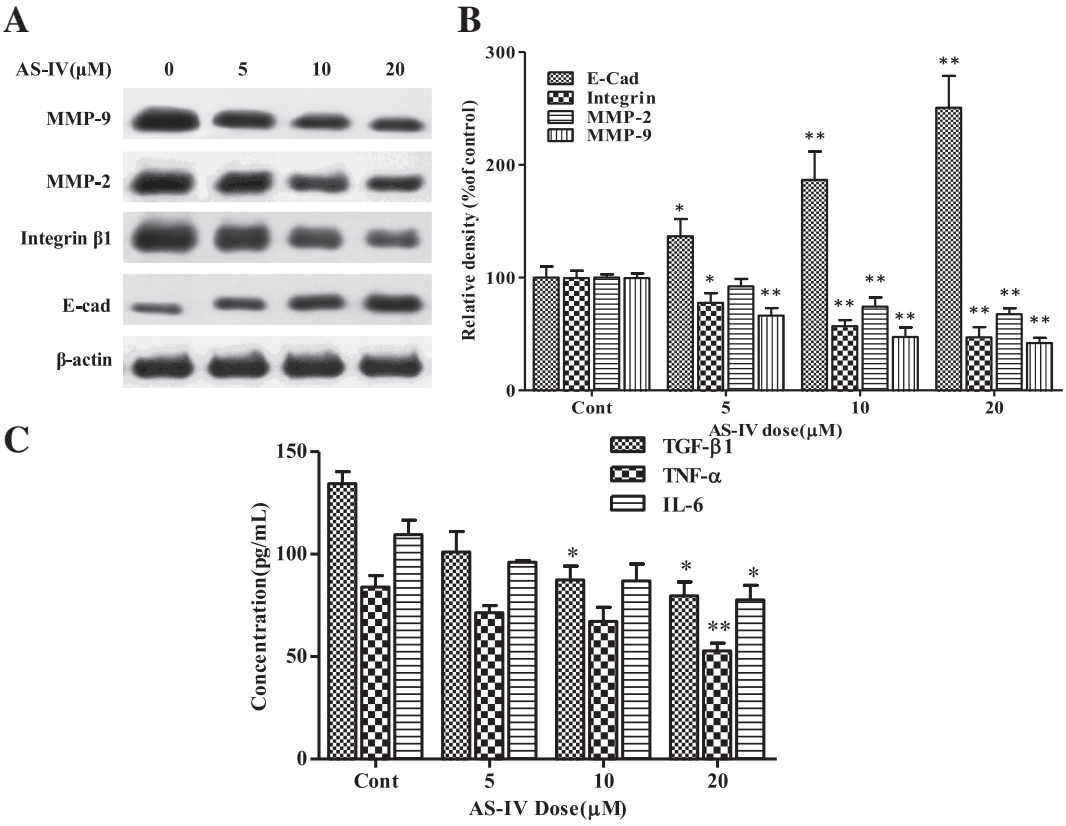

Fig. 3. AS-IV affected on the expression of cytokines with Western blot and ELISA assay. (A) Western blot analysis for the expressions of integrin β1, E-cadherin, MMP-2 and MMP-9. (B) The quantified results for Western blot. (C) ELISA for the TGF-β1, TNF-α and IL-6 levels in the supernatant. The results of AS-IV affected on the expression of cytokines represented as mean ± SD ($n = 3$). *$p < 0.05$ and **$p < 0.01$ versus the control group.

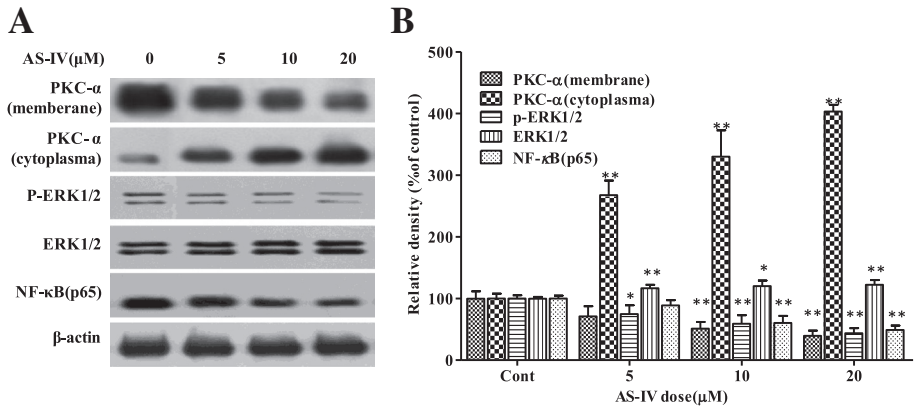

Fig. 4. The effect of AS-IV on the expression of PKC-α, p-ERK1/2, ERK1/2 and NF-κB with Western blot assay. (A) Western blot for the expressions of PKC-α, p-ERK1/2, ERK1/2 and NF-κB in A549 cells. (B) Quantification of the protein expressions. Values were represented the means ± SD ($n = 3$).*$p < 0.05$ and **$p < 0.01$ versus control group.

to 38.10 ± 7.34% significantly. It was lower than AS-IV (62.01 ± 4.79%) or AEB071 (54.22 ± 6.09%) alone. Additionally, both AEB071 and AS-IV could also reduce the expression levels of MMP-2, MMP-9 and integrin β1. The significant decreases of these protein expressions were observed between the AEB071 and AS-IV AEB071 and AS-IV co-treatment and AEB071 and AS-IV alone. However, a significant increase of E-cadherin level was observed between their co-treatment and AS-IV and AEB071 alone.

In the transwell invasion experiment, we observed that AS-IV or AEB071 alone reduced the relative permeated cell numbers to

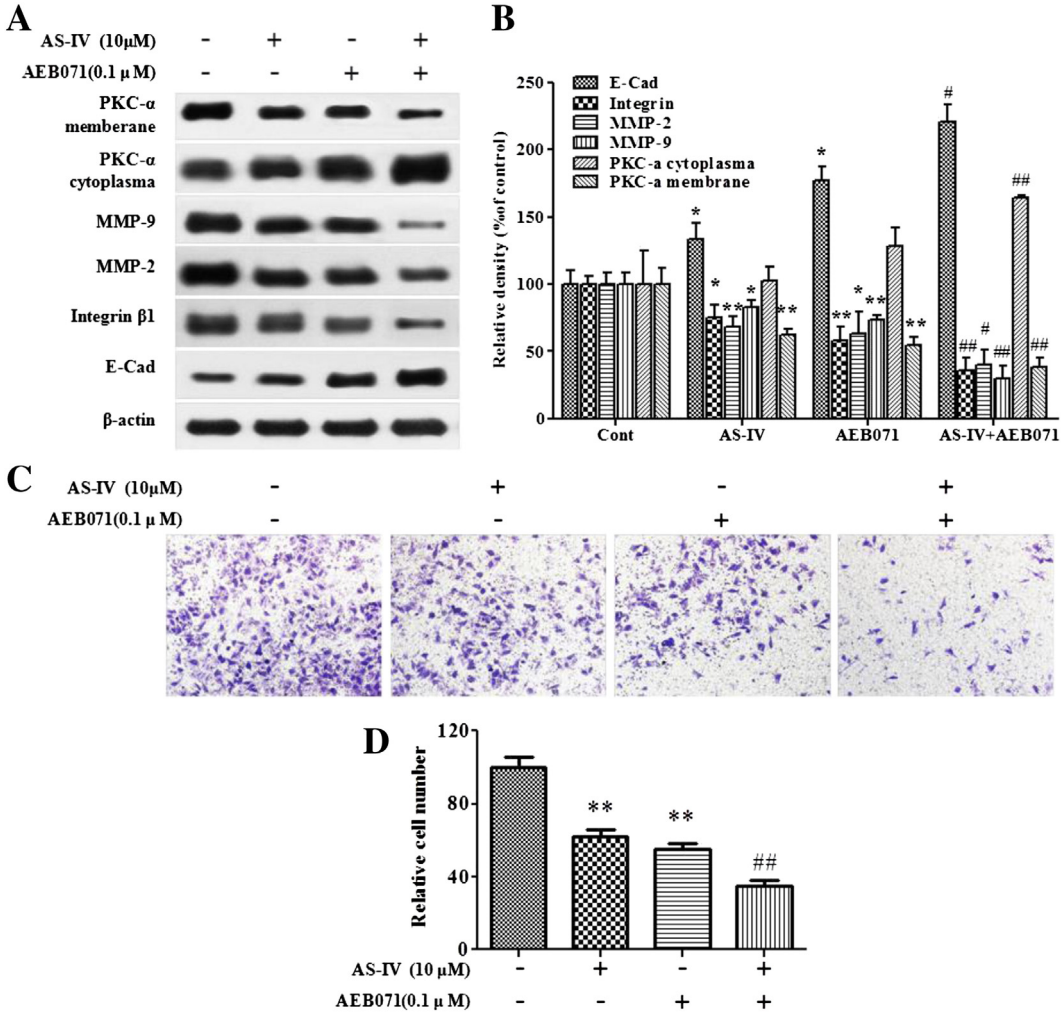

Fig. 5. AS-IV inhibited invasion by suppression of PKC-α. (A, B) The Western blot assay and quantitation results of proteins in A549 cells. (C) The invasion of A549 cells treated with AS-IV (10 μM), AEB071 (0.1 μM) and both were detected with transwell chambers assay. (D) The optical density of permeated cells was detected, and invasion rates were calculated. The results were represent as percentage of control group. The results were represented the means ± SD of triplicate independent experiments. *$p < 0.05$ and **$p < 0.01$ versus the control group, #$p < 0.05$ versus the group treated only AS-IV. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

61.96 ± 4.16% and 44.67 ± 6.52% while their co-treatment resulted in 34.95 ± 2.97%. There was a significant inhibition on the penetrated cells between the co-treatment and AS-IV and AEB071 alone ($p < 0.01$) (Fig. 5C and D). The results indicated that AS-IV inhibited the invasion by suppressing the activation of PKC-α on cell membrane in A549 cells.

3.9. AS-IV inhibited invasion and migration of A549 cells through refrain the ERK1/2 phosphorylation activation

In order to investigate whether AS-IV affect the down-regulation of ERK1/2 phosphorylation, ERK1/2 inhibitor U0126 (1 μM) was used in our experiment. Here, the protein express of ERK1/2 and p-ERK1/2 in A549 cells were detected with Western blot. The relative expression level of p-ERK1/2 was restrained to 26.19 ± 11.48% by the co-treatment of U0126 and AS-IV, which was significantly lower than 93.26 ± 6.58% and 60.64 ± 11.25% by AS-IV or U0126 alone. Meanwhile, the expressions of MMP-2, MMP-9 and integrin β1 were decreased remarkably by the co-treatment of U0126 and AS-IV. More importantly, these expression levels were much lower than AS-IV or U0126 alone. For the level of E-cadherin, a significant increase was observed in AS-IV and U0126 co-treatment group than AS-IV and U0126 treated alone. (Fig. 6A and B).

The invasion results indicated that relative permeated cell numbers were inhibited by AS-IV and U0126 co-treatment to 31.72 ± 3.23% significantly, compared with 57.85 ± 2.58% and 49.30 ± 4.88% by AS-IV or U0126 alone ($p < 0.01$) (Fig. 6C and D). These results demonstrated that the inhibition of AS-IV on A549 cell invasion might be associated with the suppression of ERK1/2 phosphorylation, at least in part.

3.10. AS-IV inhibited NF-κB activation in A549 cells

The expression of NF-κB p65 in A549 cells treated with AS-IV (10 μM) in the presence or absence of inhibitor PDTC (1 μM) was examined by Western blot. Meanwhile, the invasion ability affected with NF-κB inhibitor PDTC was evaluated by transwell invasion assay. As shown in Fig. 7A and B, the results demonstrated that treatment with PDTC (1 μM) and AS-IV (10 μM) diminished the relative expression level of NF-κB (p65) to 50.29 ± 5.27%, which was significantly lower than 82.20 ± 6.48% and 62.51 ± 7.24% by AS-IV (10 μM) or PDTC (1 μM) alone. Simultaneously, the expressions of MMP-2, MMP-9 and integrin β1 treated with PDTC and AS-IV together were lower than the AS-IV or PDTC alone. The level of E-cadherin increased more by co-treatment, compared with AS-IV and PDTC alone.

The relative permeated cell numbers were inhibited by AS-IV and PDTC co-action to 32.72 ± 3.83% significantly in the invasion experiment, compared with 65.74 ± 4.24% and 61.85 ± 3.58% by AS-IV or

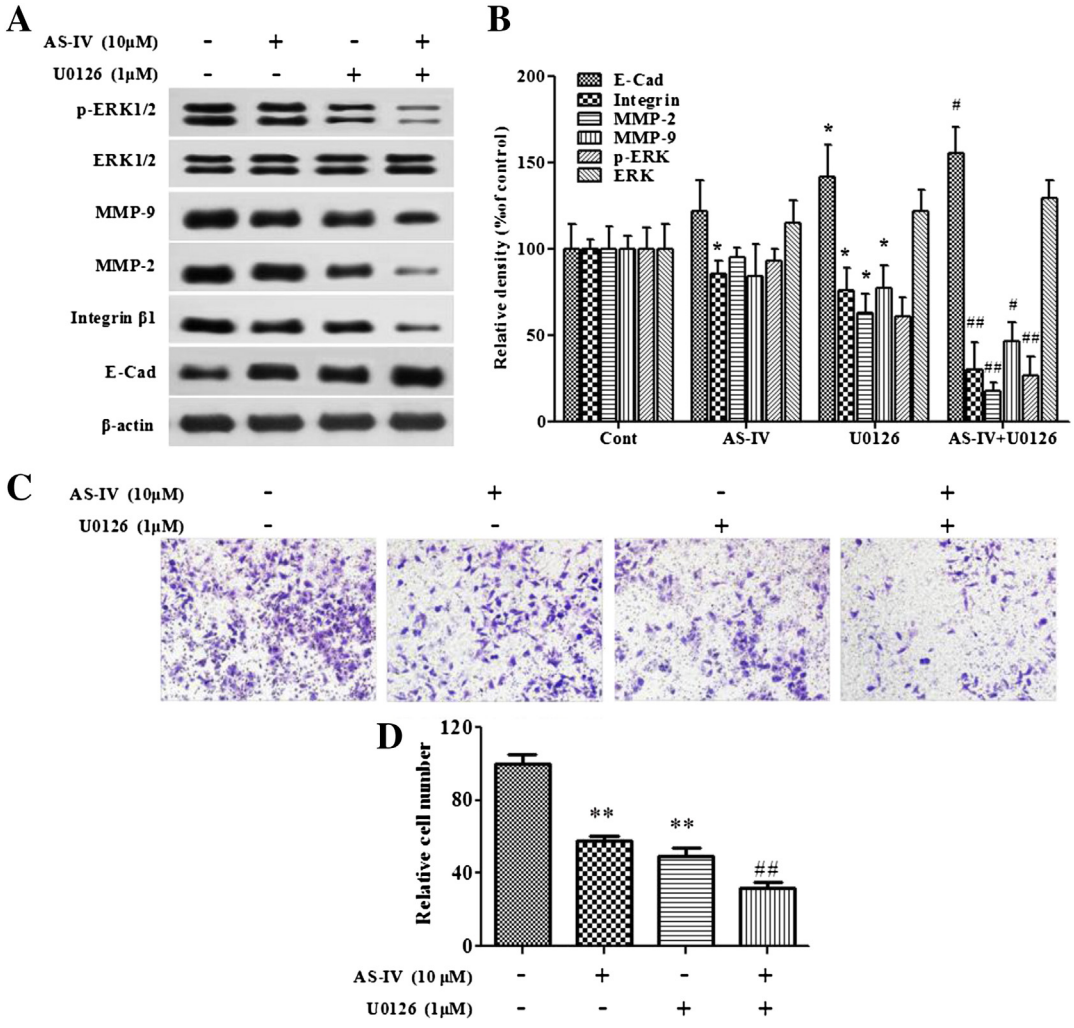

Fig. 6. The effect of ERK1/2 inhibitor U0126 on the invasion of A549 cells inhibited AS-IV. (A, B) The protein expressions were detected by Western blot and calculated. (C) Transwell chambers assay for cell invasion. A549 cells were inoculated treated with AS-IV (10 μM) without or with U0126 (1 μM) for 24 h. (D) The results of cell percent invasion rate were detected and calculated by optical density. Values were represented the means ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group, $^{\#}p < 0.05$ versus the group treated only AS-IV. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

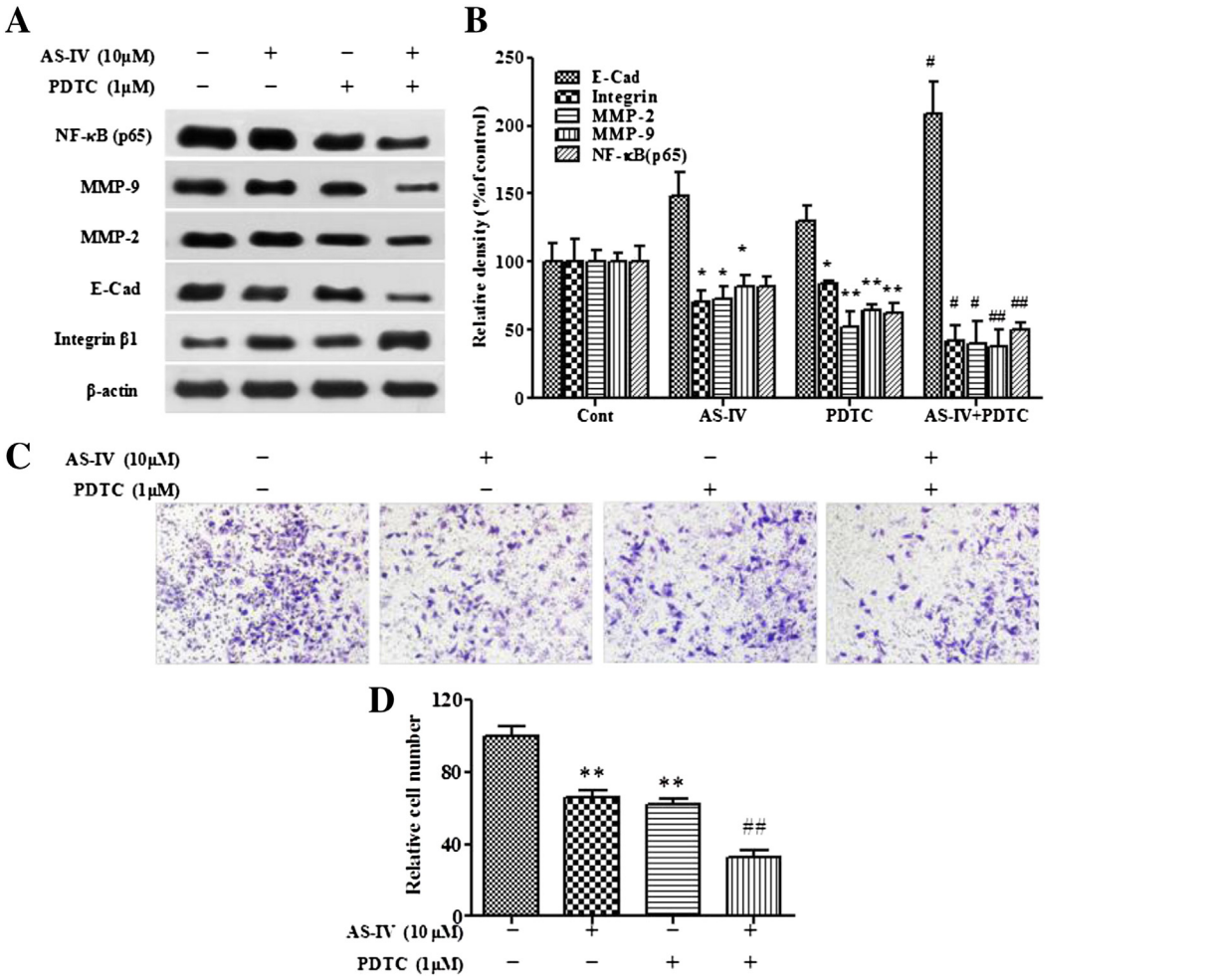

Fig. 7. PDTC was associated with the invasion inhibition by AS-IV. (A, B) The proteins of A549 cells in presence of AS-IV (10 μM), PDTC (1 μM) and co-treatment for 24 h were detected with Western blot. (C) Transwell chambers assay of A549 cells treated with AS-IV (10 μM) without or with PDTC was used to detect cell invasion. (D) The inhibition of cell invasion was detected and calculated with optical density. The results performed with three dependent experiments were represented the means ± SD ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the control group; $^{\#}p < 0.05$ versus the group treated only AS-IV. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

PDTC alone ($p < 0.01$) (Fig. 7C and D). These results indicated that the suppression of NF-κB activation played an important role in the inhibition of AS-IV on the invasion of A549 cells.

4. Discussion

The migration and invasion capabilities of cancer cells have been proved to play important roles in metastasis and recurrence of cancers. Existing studies have shown that the inhibition of invasion and metastasis can effectively reduce the development and recurrence of cancers [25]. AS-IV has been reported to inhibit cell proliferation and migration by activating JAK2/STAT3 and ERK1/2 signaling pathways [26]. However, the anti-metastatic effect and underlying mechanism of AS-IV on A549 cells remains unclear. Experiments described in present study indicate that AS-IV could inhibit the migration and invasion of A549 cells through inhibiting the PKC-α-ERK1/2-NF-κB pathway to regulate migration and invasion-related protein expression such as the E-cadherin, integrin β1 and MMPs.

The migration process of tumor cells requires the coordinated regulation of not only E-cadherin-mediated cell–cell adhesions but also integrin-mediated cell-ECM adhesions [27]. The down-regulation or lack of E-cadherin expression can reduce the connection between tumor cells and also increase the invasion of cancer cells to surrounding tissue through the basement membrane, resulting in the development of tumor [28]. The expression of integrin β1 is involved in NSCLC adhesion to lymphatic endothelium to contribute to the invasion and metastasis of lung cancer [29]. In the present study, AS-IV significantly inhibited integrin β1 and increased E-cadherin protein expression, resulting in the suppression of A549 cells migration and invasion. The results indicated that the inhibition of AS-IV on the migration and invasion of A549 cells might be associated with the regulation of integrin β1 and E-cadherin proteins.

ECM degradation by proteolytic enzymes MMPs, especially MMP-2 and MMP-9, has been regarded as an important mechanism in cancer metastasis because of the easier metastasis of cancer cells to other tissues and organs via the vascular or lymphatic system. The invasion and metastasis of tumor cells could be archived by the blockade of MMP-2 and MMP-9 proteins [30,31]. Herein, we found that AS-IV inhibited the expressions of MMP-2 and MMP-9, as well as cell invasion. These results indicated that the anti-metastatic effect of AS-IV on A549 cells was relevant to its inhibition on MMP-2 and MMP-9 expression.

Additionally, chronic inflammation of tumor microenvironment triggers cellular events, which promote malignant development of cancer cells [32]. Cancer metastasis could be promoted by inflammatory cytokines [33,34]. The expression of TNF-α in the tumor microenvironment is a common feature of many malignant tumors. Accumulative studies have pointed out that TNF-α plays a cancer promoting role in inflammation driven carcinogenesis and promotes many tumor cells metastasis and invasion [35]. Evidences support a fact that the stimulation of TNF-α could increase MMP-9 activity and expression in dose-dependent

manner in cancer cells [36]. Furthermore, the inflammatory cytokines IL-6 is another important inflammatory cytokines linked between inflammation and cancer. IL-6 can activate TGF-β1/MMP-9 signaling and then trigger the increased invasion of cancer cells [37]. Growth factor TGF-β1 produced by macrophages plays an important role in promoting tumor through the promotion of tumor growth, survival, motility and invasion. TGF-β1 can enhance the ability of MMP2/9 and result in the increase the invasion and metastasis ability of cancer cells [38]. In this study, inflammation factors TGF-β1, TNF-α and IL-6 levels in cell supernatant were inhibited by AS-IV in a concentration-dependent manner. The results indicated that the attenuation of AS-IV on the inflammatory responses could prevent the invasion of A549 cells.

The proteinase activation of E-cadherin, integrin β1, MMP-2 and MMP-9 was regulated by multiple signaling pathways including PKC-α signal pathway. The overexpression of PKC-α induced by phorbol ester was recognized to promote tumor cell motility by activating MMPs expression and inhibiting E-cadherin expression [39,40]. The cytosol-to-membrane translocation of PKC-α in A549 cells could be induced by IL-1β, resulting in MAPK activation and migration promotion [41]. The suppression of PKC-α translocation to membrane could decrease proliferation and invasion of cancer cells [42]. The suppression of PKC-α inhibitors has been found to reduce the motility and invasion of A549 cells as well as MMPs expression [43,44]. In the present study, the treatment of AS-IV significantly inhibited PKC-α, integrin β1, MMP-2 and MMP-9 expression levels in A549 cells membrane compared with control cells. When pretreated with PKC-α inhibitor AEB071, these decreases were also observed. More importantly, significant decreases of PKC-α, integrin β1, MMP-2 and MMP-9 expression levels in A549 cells were observed in the co-treatment group than AS-IV or AEB071 treated alone. The results suggested that the inhibition of AS-IV on the invasion of A549 cells was archived by down-regulation of the PKC-α pathway.

The PKC-α expression affects the migration and invasion of A549 cells by triggering downstream signaling pathway such as ERK1/2, followed with NF-κB-dependent MMP-2 and MMP-9 activations [45]. ERK1/2 has been believed to be one of the key molecules in the migration and invasion of tumor cells [46]. In various tumor cells, the activation of ERK1/2 into p-ERK1/2 has a positive correlation to the expression and activity of downstream invasion-related protein MMP-2 [47,48]. In this study, the phosphorylation of ERK1/2 could be suppressed by AS-IV in a dose-dependent manner. Additionally, the suppression of ERK1/2 phosphorylation could also be inhibited by an inhibitor U0126. Of note, there was a stronger inhibition on ERK1/2 phosphorylation in their co-treatment than AS-IV and U0126 alone. The results suggested that AS-IV inhibited the invasion ability of A549 cells by suppression of the ERK1/2 pathway.

NF-κB can be activated by many signals such as ERK1/2 and then result in regulating the expressions of multiple genes involved in cell invasion [49–51]. Activated NF-κB by ERK1/2 promoted the migration and invasion of carcinoma cells by repressing E-cadherin expression [52]. In this study, we demonstrated that the levels of NF-κB (p65) were inhibited by AS-IV or NF-κB special inhibitor PDTC. Moreover, the levels of NF-κB (p65) were decreased significantly by the co-treatment of AS-IV and PDTC compared with AS-IV and PDTC alone. The result indicated that the regulation of AS-IV on NF-κB (p65) expression participated in the inhibition of the invasion of A549 cells.

Results of this study demonstrated that AS-IV could inhibit the metastasis and development of lung cancer cells, which is coincident with previous research [3,14,25]. Interestingly, a research reported that AS-IV up-regulated MMP-2 protein expression in A549 cells, which might result in promoting the development of lung cancer [53]. The cause of the contrast results might be related to the regulation of AS-IV on ERK1/2 activation. Accumulating literature suggests that the suppression of ERK1/2 activation may result in the inhibition of MMP-2 expression and cell invasion. However, it was reported that the activation of ERK1/2 could also reduce the MMP-2 expression and inhibit the metastasis of cancer cells [54]. The expression of ERK1/2 inhibited by AS-IV might play different roles in the proliferation and invasion of A549 lung cancer cells. The speculation needs to be confirmed in further experiments.

In conclusion, we demonstrated that AS-IV could inhibit the invasion and migration of A549 cells by suppressing PKC-α-ERK1/2-NF-κB signaling pathway. The results may provide a new insight into the molecular mechanisms of AS-IV on treating lung adenocarcinoma. Therefore, we suggest that AS-IV may be a useful compound for the inhibition of metastasis of lung adenocarcinoma cancer.

Conflict of interest

None of the authors had a conflict of interest regarding the study or have anything to disclose.

Acknowledgments

This work is supported by the Suzhou Science and Technology program (ZXY2012009) and the College Graduate Research and Innovation Project of Jiangsu Province (CXLX13_599).

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