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Astragaloside IV inhibits cell invasion and metastasis in vulvar squamous cell carcinoma through the TGF-β1/FAK/AKT signaling pathway

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ABSTRACT

Objectives: To investigate the mechanism of astragaloside IV (AS-IV) inhibiting the invasion and metastasis of vulvar squamous cell carcinoma (VSCC).

Material and methods: MTT and plate colony-formation assays were used to examine the cell proliferation of VSCC (SW962 cell line). Transwell and scratch wound-healing assays were used to analyse cell migration and invasion. Western blot was used to detect the expression of relevant proteins in terms of cell proliferation, invasion and metastasis, as well as the TGF-β1/FAK/AKT signaling pathway.

Results: The results showed that AS-IV inhibited the proliferation of SW962 cells in a concentration-dependent manner, as demonstrated by the upregulation of P53 and P21 expression and the downregulation of cyclin D1 expression. AS-IV decreased the ability of cell invasion and metastasis by the downregulation of MMP-2 and MMP-9 expression. When TGF-β1 was added to SW962 cells, the expression of the N-cadherin and Vimentin were upregulated and that of the E-cadherin was downregulated. Subsequently, fibroblast-like elongated spindle-shaped cells appeared, which suggests that TGF-β1 could induce EMT in SW962 cells. Furthermore, the expression of p-FAK, p-AKT, MMP-2 and MMP-9 were upregulated. The expression of these proteins exhibited the opposite effect after AS-IV intervention. Cell invasion and metastasis were suppressed.

Conclusions: AS-IV inhibits cell invasion and metastasis in VSCC through the TGF-β1/FAK/AKT signalling pathway.

Key words: astragaloside IV; vulvar squamous cell carcinoma; EMT; TGF-β1; FAK/AKT pathway; metastasis

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INTRODUCTION

As a gynecologic malignancy, vulvar cancer usually occurs in middle-aged and older women. Among vulvar cancer, vulvar squamous cell carcinoma (VSCC) nearly accounting for more than 90%. Currently, surgical resection is a standard treatment for VSCC patients. Post-surgical radiation and chemotherapy have proved to be effective in preventing recurrence and improving the outcome for VSCC patients. Nodal involvement and tumour recurrence are important clinical features of VSCC, which significantly decrease survival rates [1, 2]. Despite the presence of these adjuvant therapies, recurrence rates for VSCC remain as high as 40% [3], indicating the ineffective utilisation of adjuvant treatment options. Therefore, new therapeutic strategies and drugs are urgently needed. With the development of immunotherapy and targeted therapy, the anti-tumour Chinese herbs have also received much attention.

Astragalus membranaceus, one of the most used traditional Chinese herbs, has been used to nourish Qi and blood for thousands of years without toxicity [4]. Astragaloside IV (AS-IV) is the main active substance of *Astragalus membranaceus*. Previous studies have confirmed the beneficial effects of AS-IV in the therapy of many diseases, including fibrosis, inflammatory, autoimmune and cardiovascular diseases [5, 6]. Previous showed that AS-IV is valuable for cancer treatment due to its inhibitory effects on the proliferation, invasion and metastasis in various tumours, such as prostate cancer [7], breast cancer [8], gastric cancer [9], lung cancer [10] and glioma [11]. However, the effects of AS-IV on the invasion and metastasis in VSCC remain unclear.

Epithelial-to-mesenchymal transition (EMT) and invasion potential have been considered as essential factors in cancer metastasis, which is the major cause of cancer death. Transforming growth factor β1 (TGF-β1) is an essential in-

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ducer of EMT [12], and it is highly expressed in vulvar cancer [13]. Focal adhesion kinase (FAK) is a crucial participator in cancer metastasis [14]. It is unclear whether TGF-β1 and FAK cooperatively regulate the invasion and metastasis of VSCC. Cicchini et al. [15], reported that TGF-β1 could activate FAK to induce cell EMT. The phosphorylation of FAK enhances the activity of AKT; it then modulates the transactivation of various genes, including MMPs and EMT.

This study intends to investigate the mechanism of AS-IV inhibiting the invasion and metastasis of VSCC.

MATERIAL AND METHODS

Cell culture

In this study, the human vulvar squamous cell carcinoma cell line SW962 came from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cells had been cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (FBS; GIBCO, MA, USA), penicillin (100 U/mL) and streptomycin (100 mg/mL; Sigma-Aldrich, St Louis, MO, USA) (5% CO_2, 37°C).

Chemicals, reagents and materials

Astragaloside IV (purity > 98%; Beijing Solarbio Science & Technology Co, Ltd, Beijing, China) had been dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St MO, USA) and stored at −20°C. The final concentration of DMSO in the culture media was less than 0.1%. Phosphate-buffered saline (PBS) and trypsin/ethylenediaminetetraacetic acid (EDTA) solution were purchased from Thermo Fisher Scientific Inc (MA, USA). Matrigel (0.5 mg/mL) and transwell chambers were purchased from BD Biosciences (NJ, USA). The bicinchoninic acid (BCA) protein assay kit was purchased from Nanjing KeyGen Biotechnology Co, Ltd. Recombinant human TGF-β1 (rhTGF-β1), crystal violet, acridine orange (AO) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) came from Beijing Solarbio Science & Technology (Beijing, China).

MTT assay

MTT assay was used to detect the cytotoxicity and viability. SW962 cells (3×10^3 cells/well) were seeded onto 96-well plates in a 200-μL culture medium for 24 h. After treated with AS-IV at concentrations of 0–800 μg/mL for 24 h, MTT (20 μL, 0.5 mg/mL) was added to each culture well. After 4 hours, the formazan crystals were dissolved by the addition of 100 μL DMSO. After complete solubilisation, the absorbance was assessed at 490 nm by microplate reader.

Plate colony formation assay

SW962 cells were treated with AS-IV at concentrations of 0, 100 and 200 μg/ml. After cultured for 14 d, the cells were washed by PBS and fixed with carbinol for 15 min. The colonies were stained by crystal violet solution for 15 minutes as well. Then, the colonies with more than 50 cells were counted under an inverted microscope (Olympus IX70, Japan).

Transwell assays

Cell invasion and migration assays were performed by transwell chambers which equipped with 8-μm pore size inserts. The filters used for invasion assays were coated with 60-μL pre-diluted Matrigel (0.5 mg/mL). SW962 cells (1×10^5 cell/well for migration assay, 2.5×10^5 cell/well for invasion assay) had been added to the upper chamber of each well and incubated with different doses of AS-IV for 24 h with and without TGF-β1 (10 ng/mL). The lower chamber was filled with DMEM containing 10% FBS. The migrated or invaded cells were stained with crystal violet staining solution (0.1%). Then inverted microscope was used to count the number of cells from five different visual fields.

Scratch wound-healing assay

SW962 cells (2.5×10^6 cells/well) were cultured in 6-well plates and incubated overnight to produce a confluent monolayer. A 10-μL pipette tip was used to make three linear wounds on the monolayer of the cells attached to the bottom of a Petri dish and washed with 0.1% FBS media. Then, AS-IV was added. Cells were incubated for 24 h to allow migration into the wound area. At the indicated time points, the monolayer was washed twice with PBS to remove the debris or suspended cells. The scratch width was photographed under an inverted microscope at 0 and 24 h after scratching.

Morphological observation of mesenchymal cell transformation

SW962 cells were seeded onto 24-well plates at a density of 1×10^3 cells/well and incubated overnight. After treated with TGF-β1 (10 ng/mL) alone or in combination with AS-IV (100 μg/mL) for 24 h, the cells were incubated with an AO staining solution (0.01%) at room temperature for five minutes. The morphological changes of epithelial-like SW962 cells, transformed into mesenchymal-like cells, were immediately observed and photographed under an inverted fluorescence microscope.

Western blot analysis

The cells were washed with PBS and lysed with RIPA (radio Immunoprecipitation Assay) buffer to extract the total protein. Equal amounts of protein (60 μg) were separated via SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) on a 10% gel, then transferred onto polyvinylidene difluoride membranes (Billerica, USA). After blocked with 5% nonfat dry milk for 2 h, the membranes

were incubated with primary antibodies. Anti-FAK (1:1000), anti-p-FAK (tyr397, 1:500), anti-AKT (1:1000) and anti-p-AKT (ser473, 1:500) came from Affinity Biosciences (OH, USA); anti-P53 (1:1000), anti-P21 (1:1000) and cyclin D1 (1:500) came from Abcam (Cambridge, UK); anti-MMP-2 (1:500), anti-MMP-9 (1:1000), anti-E-cadherin (1:1000), anti-N-cadherin (1:1000), anti-vimentin (1:500) and anti-GAPDH (1:2000) came from Santa Cruz Biotechnology (CA, USA). The blots were visualised with enhanced chemiluminescence (ECL, Pierce).

Statistical analysis

Prism 5.0 software (Graphpad Software, Inc, CA, USA) was used for data analysis. The data had been described as mean ± standard deviation (SD), and Student's t-test was used for comparison between groups. A $p < 0.05$ indicate a statistically significant difference.

RESULTS

Cytotoxicity of AS-IV was related to its concentration

The cytotoxicity of AS-IV was evaluated in order to ascertain its effect on the invasion and metastasis of VSCC cells at nontoxic concentrations. The MTT assay showed that AS-IV inhibited the viability of SW962 cells in a dose-dependent manner, and the significant inhibitory effect appeared at the high concentration goup of 200~800 μg/mL, not at the low concentration group of 0~100 μg/mL (Fig. 1A). In addition, when SW962 cells were treated with high concentration of AS-IV (200 μg/mL) for 24 hours, the cell morphology was altered to an abnormally shrinkable appearance, and the number and area of colonies were significantly diminished. However, the low concentration group (100 μg/mL) had no significant effects on the cell morphology and colony-formation ability (Fig. 1B). Furthermore, the effect of AS-IV on the expression of proliferation-related proteins showed that AS-IV downregulated the expression of cyclin D1, whereas upregulated the expression of P21 and P53 (Fig. 1C and Fig. 1D).

AS-IV inhibits the migration and invasion of SW962 cells. When SW962 cells were treated with AS-IV (0–100 μg/mL) for 24 h, transwell assay showed that the number of cells that had passed through the chamber membrane remarkedly decreased in a dose-dependent manner. In addition, compared with the control group, the migratory and invasive ability of the cells treated with 100 μg/mL AS-IV declined by 60% and 70%, respectively. In the wound-healing assay, compared with the scratch width at 0 h with that at 24 h after scratching, the closure rates of SW962 cells treated with 0, 25, 50 and 100 μg/mL AS-IV were 100%, 85%, 62% and 30%, respectively, suggesting that AS-IV at the concentration of 100 μg/mL exhibited the optimum inhibition effects on SW962 cell migration and invasion (Fig. 2A and Fig. 2B). Furthermore, Western blot analysis discovered that the expression levels of MMP-2 and MMP-9 were markedly downregulated upon AS-IV (100 μg/mL) treatment (Fig. 2C and Fig. 2D).

After exposed to TGF-β1 (10 ng/mL) for 24 h, we observed that the cells changed from a cobblestone-like morphology and cluster formation — the typical features of epithelial cells — into a fibroblast-like spindle shape and the cell–cell contact reduced. With the addition of AS-IV treatment, the fibroblast-like spindle-shaped cells were significantly reduced (Fig. 3A). Furthermore, Western blot analysis revealed that TGF-β1 stimulation downregulated the expression of E-cadherin and markedly upregulated the expression of N-cadherin and Vimentin in SW962 cells. When combined with AS-IV, the above proteins' expressions under TGF-β1 stimulation exhibited the opposite effect (Fig. 3B and Fig. 3C).

The data are expressed as mean ± SD (n = 3); *$p < 0.05$; **$p < 0.01$ vs NC; NC, the negative control; AO — acridine orange; AS-IV, astragaloside IV; TGF-β1 — transforming growth factor-β1.

AS-IV suppresses SW962 cell migration and invasion by blocking the TGF-β1/FAK/AKT signalling. Western blot analysis results showed that TGF-β1 (10 ng/mL) significantly increased the expression of p-FAK and p-AKT in SW962 cells. At the same time, the protein levels of MMP-2 and MMP-9 were correspondingly upregulated. When combined with AS-IV (100 μg/mL) treatment, the expression of p-FAK, p-AKT, MMP-2 and MMP-9 mediated by TGF-β1 were de-

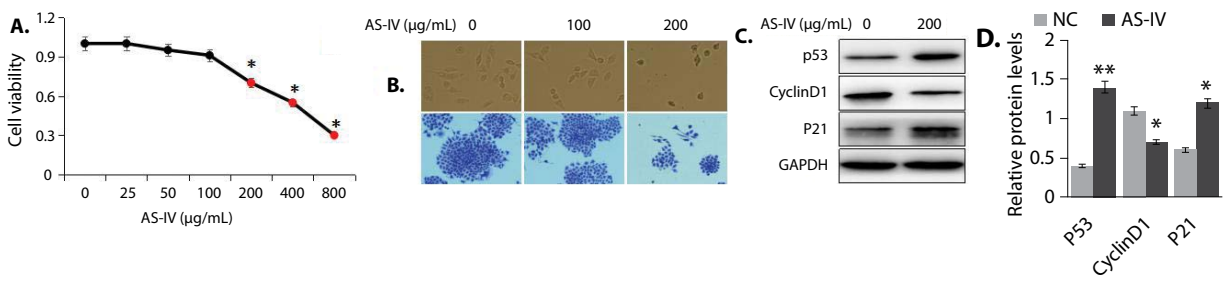

Figure 1. AS-IV inhibits the proliferation of SW962 cells; **A.** SW962 cells were incubated with increasing doses of AS-IV (0–800 μg/mL) for 24 h, MTT was used to detect the cell viability; **B.** After treated with 0, 100 and 200 μg/mL AS-IV for 24 h, the cell morphology was directly observed and photographed under a microscope (magnification, × 200), and the long-term growth ability was detected using a colony-formation assay (magnification, × 100); **C.** Protein expression levels of cyclin D1, P53 and P21; **D.** The intensity was quantified

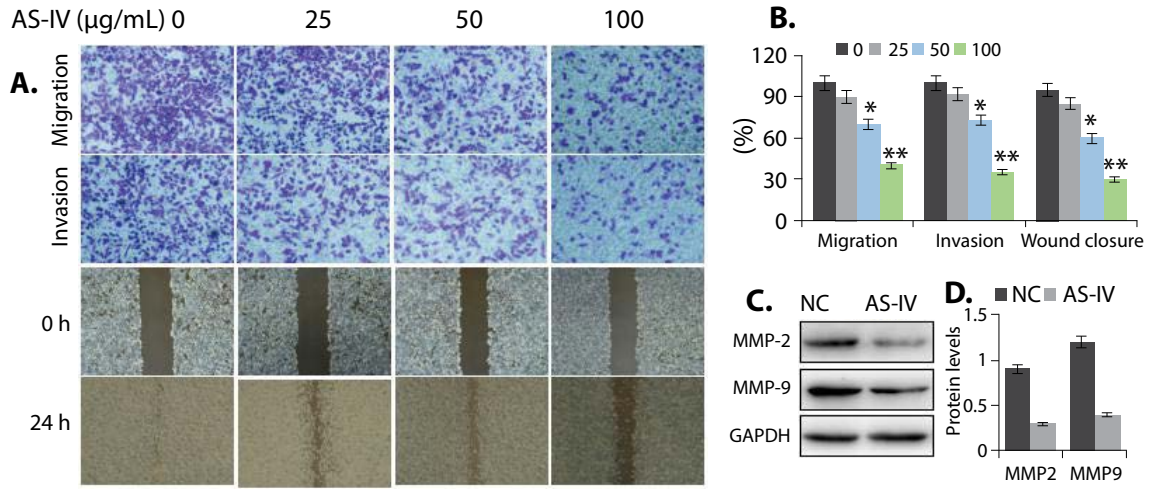

Figure 2. AS-IV inhibits the migration and invasion of SW962 cells (n = 3); **A.** (top) Transwell (magnification, × 100); (bottom) scratch wound-healing assays (magnification, × 40) were performed to detect the effects of AS-IV on the cell migration and invasion; **B.** The results were quantified, respectively; **C.** Western blot analysis of MMP-2 and MMP-9 expression levels; **D.** The intensity was quantified; *p < 0.05; **p < 0.01

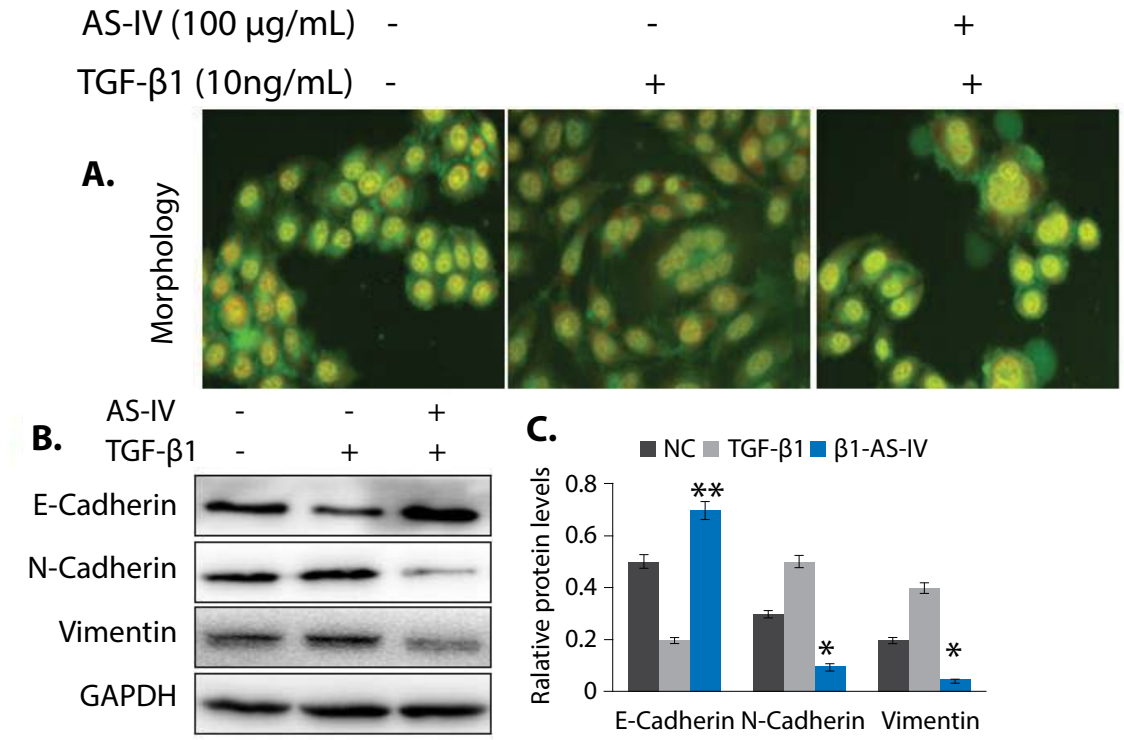

Figure 3. AS-IV reverses the TGF-β1-induced EMT in SW962 cells; **A.** The cells were stained with AO solution and the fibroblast-like morphological transformation were observed under an inverted fluorescence microscope (magnification, × 200); **B.** and **C.** The expression levels of E-cadherin, N-cadherin and Vimentin were measured via Western blot analysis

creased. However, the total FAK and AKT expression levels were not changed by TGF-β1 or by combination with AS-IV (Fig. 4A and Fig. 4B). Furthermore, after TGF-β1 stimulation, SW962 cell migration and invasion were markedly enhanced. With the addition of AS-IV treatment, the migration and invasion mediated by TGF-β1 were strongly inhibited (Fig. 4C and Fig. 4D).

The data are expressed as mean ± SD (n = 3); *p < 0.05; **p < 0.01 as indicated; p-FAK — phosphorylated FAK tyr397; p-AKT — phosphorylated AKT ser473.

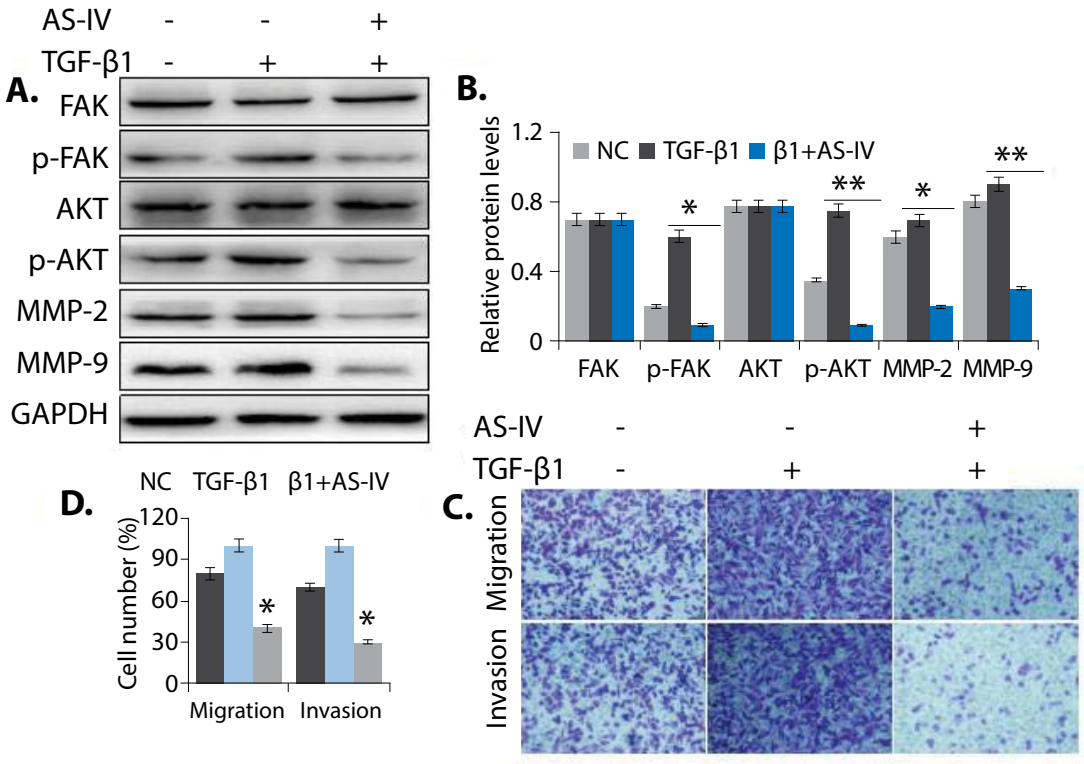

Figure 4. AS-IV supresses SW962 cell migration and invasion through bloking the TGF-β1/FAK/AKT signaling; **A.** and **B.** Western blot analysis detected the protein levels of p-FAK, p-AKT, FAK, AKT, MMP-2 and MMP-9; **C.** and **D.** Transwell assays were performed to examine the cell migration and invasion (magnification, × 100)

DISCUSSION

Abnormal proliferation and metastasis of tumour cells are crucial pathological processes with malignant tumor progression. Therefore, it is important to find an effective means to inhibit the proliferation, invasion and metastasis of cells to improve the prognosis of patients with vulvar cancer. AS-IV is one of the active compounds extracted from the Chinese medicinal plant Astragalus membranaceus. It has been widely used as an active ingredient in the clinical treatment of fibrosis and autoimmune diseases, and so on. In recent years, the anti-tumor effect of AS-IV have been paid great attention. AS-IV can inhibit cell proliferation, invasion and metastasis in some tumors, such as colon cancer.

This study showed that AS-IV administration inhibited cell viability in a concentration-dependent manner. Furthermore, the high concentration of AS-IV greatly shrunk SW962 cell morphology and decreased the number of cell colonies, whereas the low concentration had no those effects. Previous studies confirmed that AS-IV administration could inhibit the cell growth and proliferation of colorectal cancer cells [16, 17]. We also found that cyclin D1 expression was decreased and P21 and P53 proteins expression was increased. Although AS-IV has cytotoxicity, the low concentrations (0~100 μg/mL) of AS-IV have little effect on the cell growth.

EMT was a reversible process in which epithelial cells differentiate into cells with mesenchymal characteristics. Cadherin is a switch from E-cadherin to N-cadherin among EMT [18]. Paredes et al. [19], reported that E-cadherin expression increases invasiveness of tumor cells and metastasis. TGF-β1 is a potent EMT inducer during tumor progression [20]. In the present study, TGF-β1 upregulated N-cadherin and downregulated E-cadherin in SW962 cells. To further confirmed whether TGF-β1 induces EMT, we detected Vimentin protein expression and observed the ratio of spindle-shaped cells in SW962 epithelial cells under a fluorescence microscope. The results showed Vimentin expression upregulated, and the fibroblast-like spindle-shaped cells increased, indicating that TGF-β1 can induce SW962 cell EMT. After the treatment of SW962 cells with AS-IV, the above results were reversed, showing that N-cadherin and Vimentin expression was downregulated and E-cadherin expression was upregulated, the fibroblast-like spindle-shaped cells decreased, as a consequence, AS-IV may prevent TGF-β1-induced EMT in SW962 cells.

TGF-β1 is a kind of EMT inducer in tumor progression [21]. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase which participates in many important cellular processes such as cell invasion and migration [22]. It plays

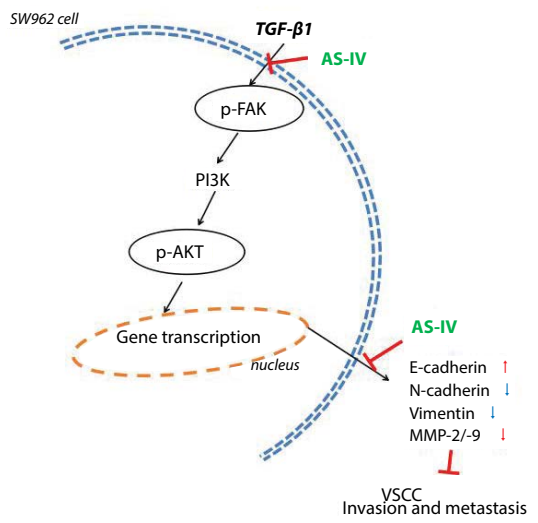

Figure 5. Graphical abstract of the present study. In VSCC cell line SW962, TGF-β1 promotes the activation of FAK/AKT signaling, which induces EMT initiation and MMP-2/MMP-9 upregulation, leading to the enhanced cell migration and invasion ability, while AS-IV blocks the TGF-β1/FAK/AKT signaling axis to inhibit VSCC cell migration and invasion

a crucial role in tumour metastasis through the activation of AKT pathway [23]. However, the role of FAK in VSCC still unknown. Our study found that treatment of SW962 cells with TGF-β1 enhanced cell migration and invasion, activated p-FAK and P-AKT, increased MMP-2 and MMP-9 expression, indicating that TGF-β1 enhanced SW962 cell migration and invasion through activation of the FAK/AKT pathway.

Cicchini et al. [15], reported that TGF-β1 can activate FAK to induce cell EMT. Furthermore, phosphorylation of FAK at tyr397 creates a binding site for the SH2 domains of PI3K, which in turn enhanced the activity of its downstream target AKT through phosphorylation at ser473, and then to modulate the transactivation of various genes including MMPs and EMT. After the treatment of AS-IV, cell invasion and metastasis induced by TGF-β1 were suppressed, p-FAK and p-AKT expression decreased, the same as MMP-2 and MMP-9 expression reduced (Fig. 5). Taken together, these results suggest that AS-IV mediated downregulation of p-FAK, p-AKT, MMP-2, MMP-9 and cell-invasive ability through suppressing the TGF-β1/FAK/AKT signaling cascade in SW962 cells.

Conflicts of interest

The authors declare no conflict of interests.

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