Article

Discovery Medicine 2025; 37(193): 394–405
https://doi.org/10.24976/Discov.Med.202537193.32

Angiotensin II and AGTR1 in ESCC Proliferation and Invasion: Insights from the JAK/STAT3 Pathway

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Published: 20 February 2025

Background: Angiotensin II (Ang II) and its receptor, Angiotensin II receptor type 1 (AGTR1), have been implicated in the proliferation of cancer cells across various tumor types. This study aims to examine the impact of Ang II and AGTR1 on esophageal squamous cell carcinoma (ESCC) cells.

Methods: The clonogenicity and proliferation of tumor cells were evaluated through Clone Formation and Cell Counting Kit-8 (CCK-8) assays. Cell migration and invasion were determined utilizing Transwell assays. Flow cytometry was employed to analyze the cell cycle. Additionally, to investigate the expressions of genes associated with cell growth, migration, infiltration, and Janus kinase-signal transducer and activator of transcription 3 (JAK/STAT3) signaling pathways, quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting were utilized.

Results: In the current study, it was observed that increasing the concentration of Ang II significantly augmented the proliferation of ESCC cells. However, with the addition of its inhibitor losartan, the proliferative activity of ESCC cells was significantly reduced with the increase of losartan concentration ($p < 0.05$). The inhibition of AGTR1 also markedly reduced the proliferative activity of ESCC cells, counteracting the effect induced by Ang II treatment ($p < 0.05$). Additionally, Ang II was found to stimulate the migration and invasion of ESCC cells, facilitate the transition of these cells from the first gap (G1) to the synthesis (S) phase, and impede apoptosis ($p < 0.05$). However, treatment with losartan and AGTR1 inhibition significantly diminished the number of migratory and invasive cells, inhibited the transition from G1 to S phase, and promoted apoptosis in ESCC cells ($p < 0.05$). Regarding the mechanism, our research team found that Ang II and AGTR1 can enhance the proliferation and invasion of ESCC cells and inhibit their apoptosis via the JAK/STAT3 signaling pathway. Nevertheless, the AGTR1 blocker, losartan, effectively obstructed this process.

Conclusion: The activation of the JAK/STAT3 signaling pathway by Ang II and AGTR1 promotes the advancement of ESCC tumors. Consequently, targeting Ang II and AGTR1 could potentially emerge as a promising strategy for ESCC treatment.

Keywords: Angiotensin II; Angiotensin II receptor type 1; esophagus squamous cell carcinoma; mechanism; invasion; migration

Introduction

Esophageal cancer holds the eighth position in global prevalence among malignant tumors, with the sixth highest fatality rate across all tumor types, exhibiting geographical variability in its occurrence [1,2]. It is primarily classified into two types: esophageal squamous cell carcinoma (ESCC) and adenocarcinoma [3,4]. Notably, in China, ESCC constitutes more than 90% of esophageal cancer cases [5]. Despite extensive research and ongoing advancements in treatment methods, the mortality rate associated with ESCC remains largely unchanged. Early symptoms of esophageal cancer often go unnoticed, leading to the ease of lymphatic metastasis and tissue invasion, resulting in a grim prognosis [6]. The formation of microvessels in the tumor microenvironment is crucial for inducing tumor invasion and metastasis. Preliminary investigations conducted by our team have revealed a correlation between tumor size, tumor differentiation level, TNM stage in ESCC patients, and microvessel density as well as lymphatic vessel density in ESCC tissues [7].

Losartan, a medication designed to block Angiotensin II receptors, is commonly prescribed for managing high blood pressure and heart failure. However, recent studies have suggested that losartan might possess anti-tumor properties against certain types of tumors [8]. Investigations have revealed that losartan can impede tumor growth and metastasis through various mechanisms [9]. Firstly, it can inhibit angiogenesis, thus interrupting the blood supply to the tumor and depriving tumor cells of essential nutrients and oxygen. Furthermore, losartan demonstrates the capability to suppress the proliferation and replication of cancer cells, resulting in a reduction in both tumor size and quantity. Moreover, it can also stimulate tumor cell apoptosis,

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or self-destruction, further curbing tumor progression [10]. Nonetheless, it's imperative to acknowledge that current research on the impact of losartan on tumors is somewhat limited. The majority of investigations have been conducted on cellular and animal models, underscoring the necessity for additional clinical investigations to ascertain its precise effects and efficacy against tumors [11]. Additionally, being a pharmaceutical agent, losartan comes with certain side effects and safety concerns that warrant careful consideration in clinical application. In conclusion, while losartan may exhibit potential antitumor effects, particularly against esophageal squamous cell carcinoma, further research is indispensable to validate its efficacy and safety.

The renin-angiotensin system (RAS) functions as a vital circulating endocrine system, crucial for maintaining blood pressure, electrolyte balance, and fluid levels [12]. Its traditional components include angiotensinogen (AGT), derived from the liver; renin, secreted by paraglomerular cells; angiotensin-converting enzyme (ACE); and Angiotensin II (Ang II). Ang II, a pivotal bioactive peptide within the RAS, plays a critical role in blood pressure regulation and facilitates cellular processes such as proliferation, differentiation, and programmed cell death. Research indicates that Ang II contributes to the progression of various chronic diseases by stimulating the infiltration and activation of fibroblasts [13,14] and macrophages [15,16]. These cell types are integral stromal components within the tumor microenvironment. Activation of fibroblasts leads to the formation of tumor-associated fibroblasts, while tumor-associated macrophages significantly contribute to the establishment of an immunosuppressive milieu within tumors [17]. The Ang II receptors, particularly the Angiotensin II receptor type 1 (AGTR1) and Angiotensin II receptor type 2 (AGTR2) play a pivotal role in the RAS. Among these, AGTR1 mediates most of Ang II's functions. Elevated levels of Ang II and AGTR1 are strongly associated with the initiation, progression, and metastasis of cancers, primarily by promoting tumor angiogenesis and aggressive tumor expansion. AGTR1 has been implicated in regulating angiogenesis across various malignant tumors and is directly correlated with poor patient prognosis.

Recent studies suggest that Ang II and AGTR1 play pivotal roles in tumor progression and metastasis. Ang II influences every stage of tumorigenesis, shielding tumor cells from adverse conditions, stimulating their growth, promoting migration, and invasion, and inducing angiogenesis [18]. Numerous investigations have shown the upregulation of Ang II in various cancer types including lung, breast, colon, liver cancer, and glioblastoma [19–21]. Additionally, recent studies have revealed that Ang II can enhance tumor metastasis by activating Matrix metalloproteinase 2 (MMP-2), $\alpha 5\beta 1$ integrin/integrin-related kinase/Akt, and GSK-3β/Snail/E-cad signaling pathways in tumor cells. Notably, serum Ang II levels were found to be significantly elevated in ESCC patients [22]. However, the precise mechanisms through which Ang II and AGTR1 promote ESCC growth and spread remain unclear. This study aims to investigate the roles of Ang II and AGTR1 in ESCC invasion and metastasis, utilizing human ESCC cell lines to elucidate their underlying mechanisms and potentially identify novel therapeutic targets for ESCC.

Materials and Methods

Cells and Major Reagents

The ESCC cell lines EC9706 (HTX2246) and ECA109 (JK-CS0338) were obtained from the Cell Resource Centre at the Shanghai Institutes for Biological Sciences, a part of the Chinese Academy of Sciences in Shanghai, China. Ang II was procured from Sigma (A9525, New York City, NY, USA), while *AGTR1* shRNAs were acquired from Dharmacon (4331182, Lafayette, CO, USA). TRIZOL reagent was purchased from Invitrogen Life Technologies (15596018CN, Carlsbad, CA, USA), and β-actin antibodies were obtained from Cell Signaling Technology (4967, Boston, MA, USA). The All-in-OneTM quantitative real-time polymerase chain reaction (qRT-PCR) assay kit was acquired from GeneCopoeia (QP001, Rockville, MD, USA). Transwell chambers were obtained from Corning (CLS3470, Corning, NY, USA), and the Cell cycle kit (C1052) was sourced from Shanghai Biyuntian Biotechnology Co., Ltd. in Shanghai, China. Primary (BIWB020) and secondary antibody diluents (BIWB021) for western blotting were acquired from Nanjing Senbeca Biotechnology Co., Ltd. in Nanjing, China. STAT3 inhibitor (NSC74859) purchased from Beyotime (SD4774-25mg, Shanghai, China).

The last Short Tandem Repeat (STR) authentication of the EC9706 cell line was conducted on June 8th, 2021. The genotyping results for STR and Amelogenin loci indicated that the DNA of this cell line perfectly matched cell number 69 corresponding to EC9706 in the cell line search database, with no multiple alleles detected. Similarly, the last STR verification for the ECA109 cell line was performed in September 2023. The STR genotyping results revealed that the DNA of this cell line completely matched the ECA-109 cell line with the number CVCL_6898 in the cell line search and EXPASY databases, with no detection of multiple alleles. Furthermore, the mycoplasma test report confirmed the absence of mycoplasma infection in all cells.

Cell Culture and Group Processing

The ESCC cell lines EC9706 and ECA109 were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% Fetal Bovine Serum (FBS) in a cell incubator at 37 °C with 5% CO_2. Cells were routinely passaged every 2–3 days.

For *in vitro* experiments, ESCC cells were divided into four groups: blank control (NC), Ang II, losartan, and Ang

II + losartan groups. The NC group received Phosphate buffer saline (PBS) only. In the Ang II group, a concentration of 60 µmol/L Ang II was added. In the losartan group, 70 µmol/L losartan was added to EC9706 cells and 100 µmol/L losartan was added to ECA109 cells. The cultures were then further incubated for 48 hours, after which the cells were harvested for subsequent experiments.

Clone Formation Assay

Each experimental group was seeded with 400–1000 cells per well on a 6-well plate, with 3 replicates per experimental group. After seeding, the cells were placed in an incubator, and the culture medium was replenished every 72 hours. Following a 14-day incubation period, the cell status was observed.

To assess cell proliferation, the cells were washed with PBS, fixed with 4% paraformaldehyde for 30 minutes, and then washed again with PBS. Subsequently, 1000 µL of pristine crystal violet staining solution was added to each well, and the cells were incubated for 20 minutes. After staining, the cells were rinsed three times with double-distilled water for 5 minutes each, followed by drying. Finally, images were captured using a digital camera (CKX53, Olympus, Tokyo, Japan).

Cell Counting Kit-8 (CCK-8) Assays

Cell growth was assessed using the CCK-8 assay. Initially, 5000 cells were seeded into each well of a 96-well plate. Cell viability, indicative of growth, was evaluated by measuring cell metabolic activity using a CCK-8 kit (C0038, Beyotime, Shanghai, China) from Dojindo, Kumamoto, Japan, following the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader (HBS-1096A, DeTie, Nanjing, China), with three wells designated for each sample to ensure accuracy and reproducibility.

Migration Assays

For the upper and lower chambers of the kit, 500 µL of serum-free medium was added to each, followed by the addition of 100 µL of cell suspension to the upper chamber and 600 µL of medium containing 10% FBS to the lower chamber. The chambers were then placed in an incubator set at 37 °C. To remove non-migratory cells, the medium was aspirated by inverting the petri dish onto absorbent paper. Any remaining non-migrated cells were gently removed using a cotton swab. The dish underwent three washes with PBS. Each chamber was treated with 4% paraformaldehyde for 30 minutes and then air-dried for an additional 30 minutes. Subsequently, a 24-well plate (without the chamber) was prepared by adding 500 µL of 0.1% crystal violet solution to each well. The chamber was placed into the wells, allowing the membrane to be immersed in the solution, and incubated for 30 minutes. Following incubation, the membrane was carefully removed from the chamber, washed three times with PBS, and photographed. For cell counting, five random microscope fields (at 100× magnification) were selected for each experiment. Three samples were taken for each group, and the experiment was repeated three times for statistical reliability.

Transwell Assays

500 µL of serum-free medium was added to both the upper and lower chambers of the kit to rehydrate the Matrigel matrix layer. The chambers were then transferred to a fresh well plate, and the liquid in the upper chamber was aspirated and replaced with 100 µL of cell suspension. The subsequent procedures were identical to those of the cell migration tests.

Propidium Iodide (PI) Staining Fluorescence Activating Cell Sorter (PI-FACS) Cell Cycle Detection

The assay focused on cells in the logarithmic growth phase. After digestion and centrifugation, the supernatant was discarded, and 1.0 mL of medium was added to resuspend the cells, resulting in a concentration of approximately 4×10^6 cells/mL. The cells were then cultured in a 5% CO_2 incubator at 37 °C until they adhered to the culture surface. After 48 hours, the cells were harvested and transferred to a centrifuge tube as a single-cell suspension. Subsequently, the cells were centrifuged at 1500 revolutions per minute for 10 minutes. After removing the supernatant, the cells were washed once more with PBS.

Next, the samples were suspended in 250 µL PBS, and 95% ethanol precooled to –20 °C was slowly added to achieve a final concentration of 70%. The suspension was then incubated on ice for 30 minutes before undergoing flow analysis. Additionally, 0.2 mL of RNaseA was placed in a water bath at 37 °C for 30 minutes. Following this, 0.3 mL of staining solution was added to the samples in a dimly lit room and allowed to incubate for 20 minutes. Finally, the fluorescence intensity was measured by FlowCytometry (CytoFLEX, BECKMAN, Beckman Coulter Co., Ltd., Miami, FL, USA) with an excitation wavelength of 488 nm and the data were analyzed using cell quest and modified software (FlowJo10.9, Dako company, Santa Clara, CA, USA).

Flow Cytometry

First, tumor cells (5×10^5 cells) were collected and centrifuged twice with PBS. Subsequently, the cancerous cells were suspended in a 500 µL solution of 1× Binding Buffer. Then, 5 µL of Annexin V-FITC and 10 µL of propidium iodide (PI) were added to each tube (AP101, Hangzhou Lianke Biotechnology Co., Ltd., Hangzhou, China). The mixture was gently mixed and incubated in the dark for 5 minutes. Analysis was then performed using flow cytometry (CytoFLEX, BECKMAN, Beckman Coulter Co., Ltd., Miami, FL, USA).

qRT-PCR

Total RNA was extracted from tissues and cells using TRIzol reagent, followed by cDNA synthesis through reverse transcription. The reaction system was set up according to the manufacturer's instructions, utilizing glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as internal reference genes. Quantitative detection of the corresponding genes was performed using the AceQ Universal SYBR qPCR Master Mix (Q511, Vazyme, Nanjing, China). The forward primer for *AGTR1* was 5′-ATTTAGCACTGGCTGACTTATGC-3′ and the reverse primer was 5′-CAGCGGTATTCCATAGCTGTG-3′. For *GAPDH*, the forward primer was 5′-AGAAGGCTGGGGCTCATTTG-3′ and the reverse primer was 5′-AGGGGCCATCCACAGTCTTC-3′. PCR cycling conditions comprised an initial denaturation step at 95 °C for 50 s, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.

RNA Transfection

In Connecticut, United States, RNA interference was utilized to suppress the *AGTR1* gene, employing the targeting sequence provided as 5′-CUGUAGAAUUGCAGAUAUU-3′. Lipofectamine 3000 was employed to transfect target cells with 20–40 pmol/well of shRNA in 6-well plates during transfection. After 24 hours, the cells were harvested for subsequent tests.

Western Blotting

The expression of associated proteins was assessed via western blotting, with β-actin serving as the reference protein. Protein samples were carefully loaded into the wells using a pipette gun. Following electrophoresis, the proteins were transferred onto a membrane.

Subsequently, the membrane sections were blocked with 5% skim milk at room temperature for 1 hour on a shaking device. After a single wash with tris buffered saline (TBS), the membrane sections were incubated overnight with the primary antibody on a shaker at 4 °C. The next day, after removing the primary antibody, the membrane sections were washed three times with TBS for 10 minutes each. Following this, the membrane sections were incubated with a secondary antibody (1:5000) at room temperature and washed three times with TBS for 10 minutes each.

The details of the antibodies used were as follows:

- E-Cadherin Mouse mAb (A18135, 1:1000, ABclonal, Wuhan, China)

- N-Cadherin Rabbit pAb (A0432, 1:1000, ABclonal, Wuhan, China)

- Vimentin Rabbit pAb (A11423, 1:1000, ABclonal, Wuhan, China)

- Bax Rabbit mAb (A19684, 1:1000, ABclonal, Wuhan, China)

- B-cell lymphoma-2 (Bcl-2) Rabbit pAb (A0208, 1:1000, ABclonal, Wuhan, China)

- Active Caspase-3 Rabbit mAb (A11021, 1:1000, ABclonal, Wuhan, China)

- Cyclin B1 (*CCNB1*) gene Rabbit mAb (A22435, 1:1000, ABclonal, Wuhan, China)

- Cyclin-Dependent Kinase 4 (CDK4) Rabbit mAb (A23522, 1:1000, ABclonal, Wuhan, China)

- Cyclin D1 Rabbit pAb (A11022, 1:1000, ABclonal, Wuhan, China)

- Phospho-Janus kinase (JAK) Rabbit pAb (AP0531, 1:1000, ABclonal, Wuhan, China)

- JAK Rabbit pAb (A7694, 1:1000, ABclonal, Wuhan, China)

- Phospho-signal transducer and activator of transcription 3 (STAT3) Rabbit pAb (AP0070, 1:1000, ABclonal, Wuhan, China)

- STAT3 Rabbit pAb (A16975, 1:1000, ABclonal, Wuhan, China)

- GAPDH Rabbit pAb (A19056, 1:1000, ABclonal, Wuhan, China)

- HRP-conjugated Goat anti-Rabbit IgG (AS014, ABclonal, Wuhan, China)

- HRP polymer-conjugated Goat Anti-Mouse Anti-Rabbit IgG H (AS080, ABclonal, Wuhan, China)

The proteins were visualized using electrochemiluminescence. Western blotting strips were analyzed using ImageJ (1.48, National Institutes of Health, Rockville, MD, USA) to calculate gray statistics.

Statistical Analysis

Data analysis for this study was conducted using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) and the R programming language. The measured data were presented as Mean $\pm$ Standard Deviation (SD). An unpaired Student's *t*-test was employed to compare two groups of data with a normal distribution. The Shapiro-Wilk test was used to verify whether the data fit the normal distribution. One-way Analysis of Variance (ANOVA) was utilized to compare data between multiple groups followed by post hoc Tukey's test. IC50 curves were generated using GraphPad Prism 9, and repeated measures design analysis of variance was employed to analyze the optimal concentration. A significance level of $p < 0.05$ was considered statistically significant.

Results

Effects of Ang II and AGTR1 on Proliferation of ESCC Cells

According to the CCK-8 assay, Ang II concentration gradients of 0, 10, 30, 60, and 90 µmol/L were designed. Losartan concentration gradients of 0, 12.5, 25, 50, 100,

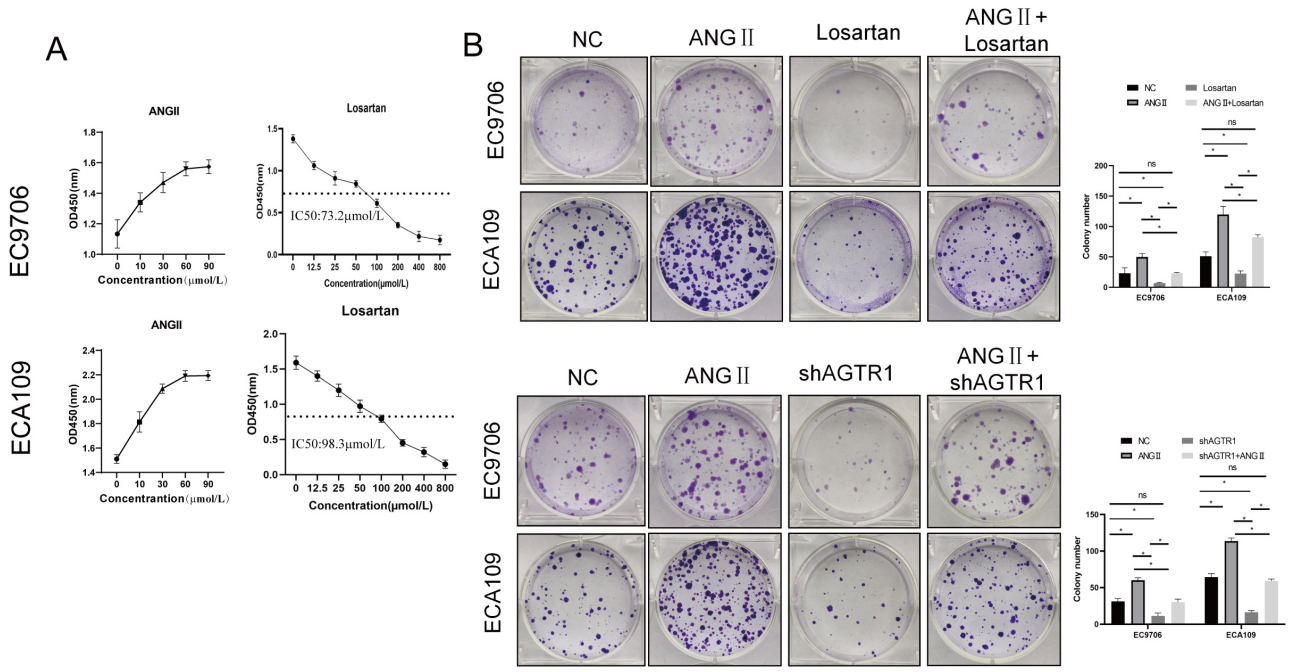

Fig. 1. Promoting effects of Angiotensin II (Ang II) and Angiotensin II receptor type 1 (AGTR1) on esophageal squamous cell carcinoma (ESCC) cell proliferation. (A) Impact of Ang II/Losartan on the viability of EC9706 and ECA109 cells. (B) Colony formation tests demonstrate the influence of Ang II/Losartan on the number of clones formed by EC9706 and ECA109 cells. Each experiment was conducted three times (n = 3). ns, no statistical significance; *, $p < 0.05$.

200, 400, and 800 μmol/L were also designed. We found that the promoting effect of Ang II on the activity of both cell lines reached its peak at a concentration of 60 μmol/L. After treatment with Losartan, the IC50 value for EC9706 cells was approximately 70 μmol/L, while the IC50 value for ECA109 cells was approximately 100 μmol/L. Based on these data, we selected the following treatment conditions for subsequent experiments: 60 μmol/L Ang II for EC9706 and ECA109 cells, 70 μmol/L Losartan for EC9706 cells, and 100 μmol/L Losartan for ECA109 cells (Fig. 1A).

In the clone formation assay results, the Ang II group exhibited significantly higher proliferation activity compared to the Negative Control (NC) group. Conversely, the losartan group displayed significantly lower cell proliferation activity than the NC group. However, there was no significant disparity in cell proliferation activity between the Ang II + losartan group and the NC group. These findings suggest that Ang II greatly increases cellular growth, while losartan, an antagonist of AGTR1, effectively diminishes the impact of Ang II. After suppressing AGTR1 with shRNA (Fig. 1B, $p < 0.05$), the growth rate of ESCC cells was notably reduced compared to the NC group. The transfection efficiency of silencing AGTR1 by RT-PCR and WB was shown in **Supplementary Fig. 1A,B**.

Angiotensin II and its receptor AGTR1 facilitated the migration and invasion of ESCC cells. Upon treatment with Ang II, the Transwell assay demonstrated a significant increase in the quantity of migratory and invasive EC9706 and ECA109 cells compared to the NC group. Conversely, the number of migratory and invasive cells experienced a significant decrease following losartan treatment. Following the suppression of AGTR1 levels in both types of cells using shRNA, there was a notable decrease in the number of cells migrating and invading compared to the NC group (Fig. 2A,B, $p < 0.05$).

Following Ang II treatment, western blot analysis indicated a significant decrease in the expression levels of E-cadherin in EC9706 and ECA109 cells compared to the NC group. Additionally, there was a significant increase in the levels of N-cadherin and vimentin compared to the NC group. Following the administration of losartan, the levels of E-cadherin expression in EC9706 and ECA109 cells exhibited a significant increase compared to the NC group, along with a significant decrease in the levels of N-cadherin and vimentin. Following AGTR1 activation (Fig. 2C, $p < 0.05$), there was a notable rise in the expression levels of E-cadherin, N-cadherin, and vimentin.

Ang II and AGTR1 Inhibited Apoptosis of ESCC Cells

Flow cytometry results indicated that the Ang II group exhibited a significantly reduced percentage of apoptosis compared to the NC group, whereas the losartan group showed a significantly elevated proportion of apoptosis compared to the NC group (Fig. 3A, $p < 0.05$). The proportion of apoptosis in the Ang II + losartan group was not

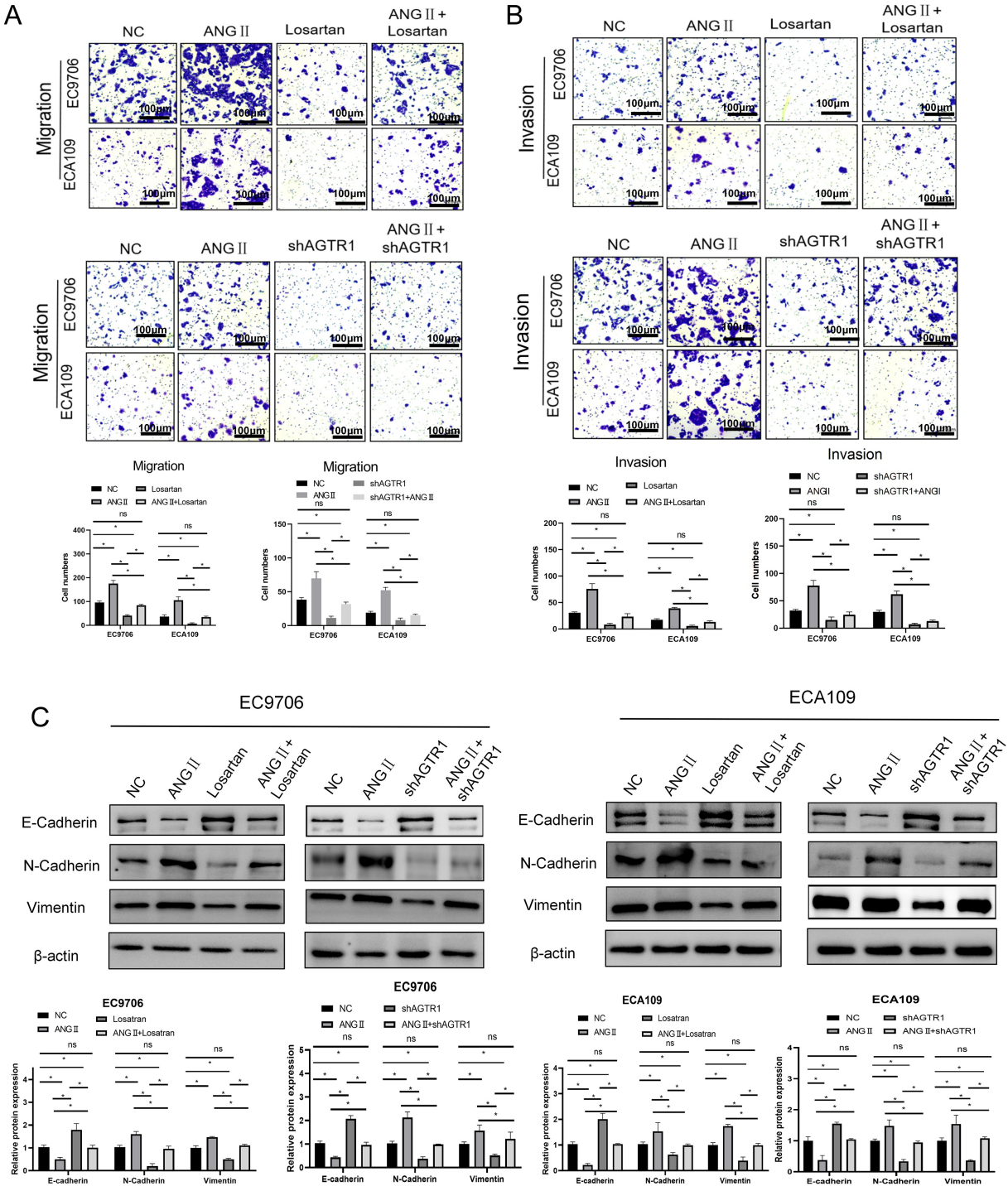

Fig. 2. Promoting effects of Ang II and AGTR1 on migration and invasion of ESCC cells. (A) Impact of Ang II/Losartan on the metastasis of EC9706 and ECA109 cells. (B) Influence of Ang II/Losartan on the invasion of EC9706 and ECA109 cells. (C) Protein levels of E-cadherin, N-cadherin, and vimentin were examined using Western blotting in various groups and cell types. Each experiment was conducted three times (n = 3). ns, no statistical significance; *, $p < 0.05$.

significantly different from that in the NC group. Following the suppression of AGTR1 through shRNA, the ESCC cells exhibited a greater apoptosis ratio compared to the NC group (Fig. 3B, $p < 0.05$).

Following Ang II treatment, Western blot analysis indicated that Bcl-2 associated X protein (BAX) and Caspase 3 were reduced and Bcl-2 was elevated in the expression levels of apoptotic proteins in EC9706 and ECA109 cells compared to the NC group. Following the administration

of losartan, there was a notable reduction in the levels of BAX and Caspase 3 in EC9706 and ECA109 cells, accompanied by a significant increase in the level of Bcl-2. Consequently, the impact of Ang II administration was eradicated. Blocking AGTR1 led to an elevation in Bcl-2 levels and a reduction in levels of BAX and Caspase 3 (Fig. 3C, $p < 0.05$).

Effects of Ang II and AGTR1 on ESCC Cell Cycle

Flow cytometry results showed that the Ang II group had a significantly higher proportion of cells in the synthesis (S) phase compared to the NC group, whereas the losartan group had a significantly lower proportion of cells in the S phase compared to the NC group. On the other hand, the Ang II group exhibited a notably reduced proportion of cells in the first gap (G1) phase compared to the NC group. Conversely, the losartan group showed a significantly elevated proportion of cells in the G1 phase compared to the NC group. Additionally, there was no significant difference in the proportion of cells in the S and G1 phases between the Ang II + losartan group and the NC group (Fig. 4A, $p < 0.05$). Following the suppression of AGTR1 through shRNA, the percentage of ESCC cells in the S phase decreased compared to the NC group, while the percentage of ESCC cells in the G1 phase showed a notable increase compared to the NC group (Fig. 4B, $p < 0.05$). Western blot analysis showed that following Ang II treatment, the levels of CDK4, CCNB1, and cyclinD1 expression in EC9706 and ECA109 cells were markedly elevated compared to the NC group. Following the administration of losartan, there was a notable reduction in the levels of CDK4, CCNB1, and cyclinD1 expression in EC9706 and ECA109 cells. Levels of CDK4, CCNB1, and cyclinD1 showed a notable reduction following AGTR1 blockade (Fig. 4C, $p < 0.05$).

Ang II and AGTR1 Triggered Janus Kinase-Signal Transducer and Activator of Transcription 3 (JAK/STAT3) Signalling Pathway in ESCC Cells

To determine the signal transduction mechanism of Ang II and AGTR1 in ESCC cells, we evaluated the related JAK/STAT3 signaling pathway. This pathway has been shown to be activated by Ang II and AGTR1 in other cancer models, promoting cell proliferation, survival, and metastasis. Western blot results showed that compared to the NC group, there was no change in the expression of JAK and STAT3 in the Ang II group. And the expression of p-JAK and p-STAT3 increased. And the p-JAK/JAK and p-STAT3/STAT3 ratios increased (Fig. 5, $p < 0.05$). There was no statistically significant difference in the expression of JAK and STAT3 between the Losartan group and the NC group ($p > 0.05$). Compared with the NC group, shAGTR1 showed a decrease in p-JAK and p-STAT3 expression (Fig. 5, $p < 0.05$), and a decrease in p-JAK/JAK and p-STAT3/STAT3 ratios. Compared with the NC group, there was no statistically significant difference in the expression of JAK, STAT3, and p-STAT3 between the ANGII+shAGTR1 group and the ANGII+losartan group ($p > 0.05$). There is a significant difference in the expression of p-JAK between the two groups ($p < 0.05$). The CCK-8 test indicated a notable decrease in the proliferative activity of ESCC cell lines EC9706 and ECA109 as the concentration of stat inhibitors increased (**Supplementary Fig. 1C**). The IC50 of STAT inhibitors in EC9706 cells was 71 μM, the IC50 of STAT inhibitors in ECA109 cells was 72.51 μM. Cloning and formation experiment results indicated that cell proliferation in the Ang II group was notably higher than the NC group, whereas cell proliferation in the stat inhibitors group was notably lower than the NC group, with no significant difference observed between the Ang II + stat inhibitors group and the NC group (**Supplementary Fig. 1D**). These results suggest that Ang II can significantly promote cell proliferation, and the impact of Ang II is greatly reduced by stat inhibitors.

Discussion

Esophageal cancer entails an intricate regulatory system involving multiple gene-mediated and enzyme responses, including the abnormal activation of various proto-oncogenes and the loss or elimination of tumor suppressor genes. Currently, esophageal cancer resection combined with chemoradiotherapy remains the primary treatment, yet the mortality rate remains high. Therefore, exploring therapeutic strategies and novel drug targets to supplement esophageal cancer resection is imperative.

RAS plays a pivotal role in all stages of cancer cell conversion, particularly in neoplastic angiogenesis. *In vitro* experiments have shown that vascular endothelial growth factor (VEGF) significantly influences tumor angiogenesis through RAS. Furthermore, Ang II has been demonstrated to enhance VEGF expression. The specific mechanism of signal transduction may involve the stimulation of Extracellular Regulated Protein Kinases 1/2 (ERK1/2), Protein Kinase C (PKC), Activator Protien-1 (AP-1), Nuclear Factor Kappa-B (NF-κB), and other pathways by Ang II via AGTR [23].

Some studies have indicated that candesartan inhibits tumor cell growth in hepatocellular carcinoma by reducing angiogenesis, with the expression of AGTR1 being directly related to VEGF-A and microvessel density. Our results are consistent with these findings. This study demonstrates a notable increase in the growth rate of ESCC cells with elevated Ang II levels, while a decrease in losartan levels significantly reduces their proliferation activity. Ang II enhances cell proliferation, whereas losartan suppresses it, countering the impact of Ang II. Following the inhibition of AGTR1, ESCC cell proliferation activity was notably reduced compared to the NC group, effectively neutralizing the impact of Ang II treatment. These results suggest that RAS is implicated in the development of ESCC and that

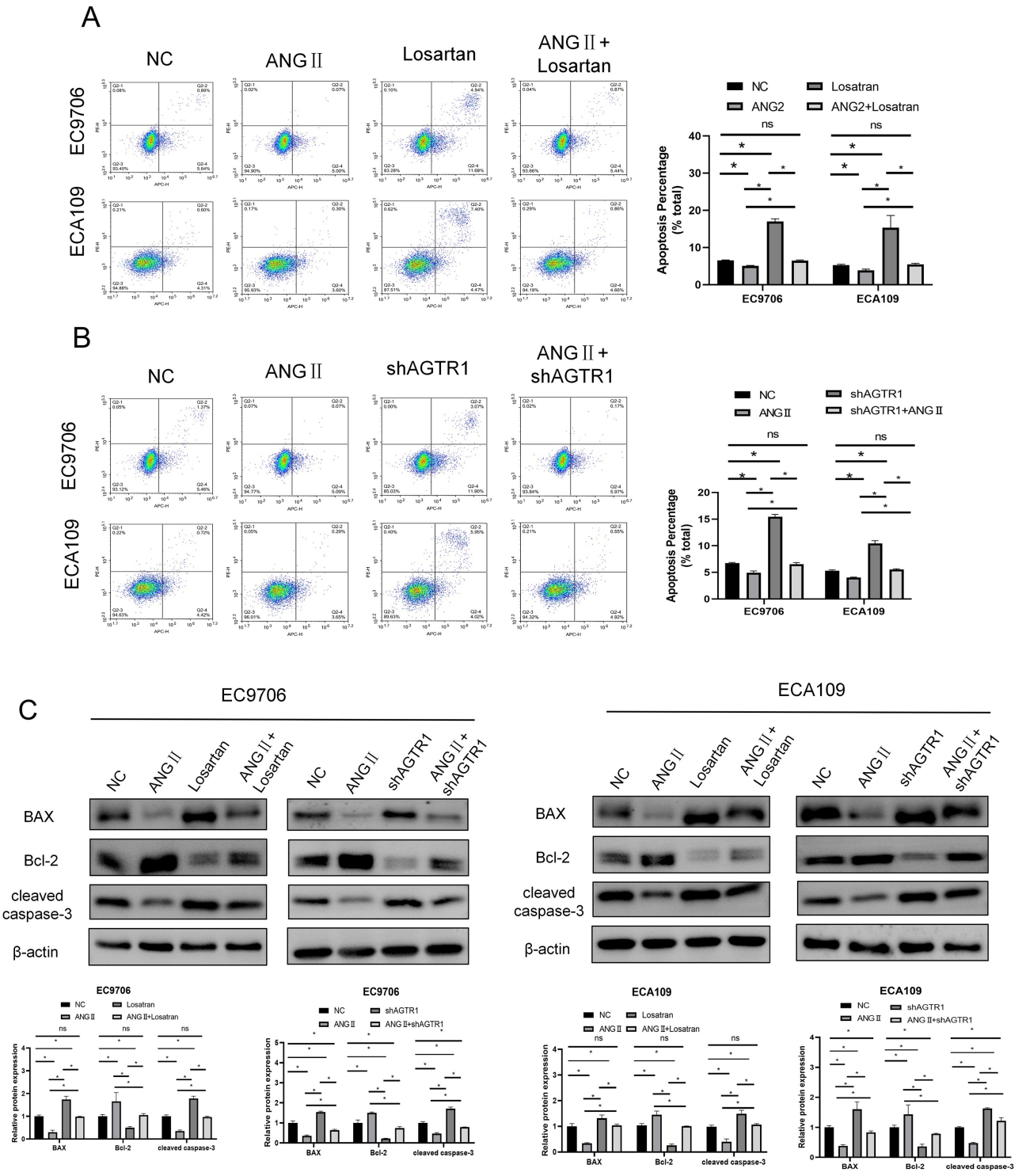

Fig. 3. Ang II and AGTR1 inhibited ESCC cell apoptosis. (A,B) Effects of Angiotensin II and Losartan on apoptosis in EC9706 and ECA109 cells were studied. (C) Protein levels of Bcl-associated X (BAX), B-cell lymphoma-2 (Bcl-2), and Caspase-3 were examined using Western blotting in various groups and cell types. Each experiment was conducted three times (n = 3). ns, no statistical significance; *, $p < 0.05$.

Ang II enhances cell proliferation through AGTR1. Losartan, an antagonist of AGTR1, effectively mitigates the impact of Ang II.

Previous studies have revealed that Ang II, in a dose-dependent manner, increases the expression of AGTR1 and VEGF-A in hepatocellular carcinoma cells, thereby amplifying tumor angiogenesis and metastasis. Additionally, it

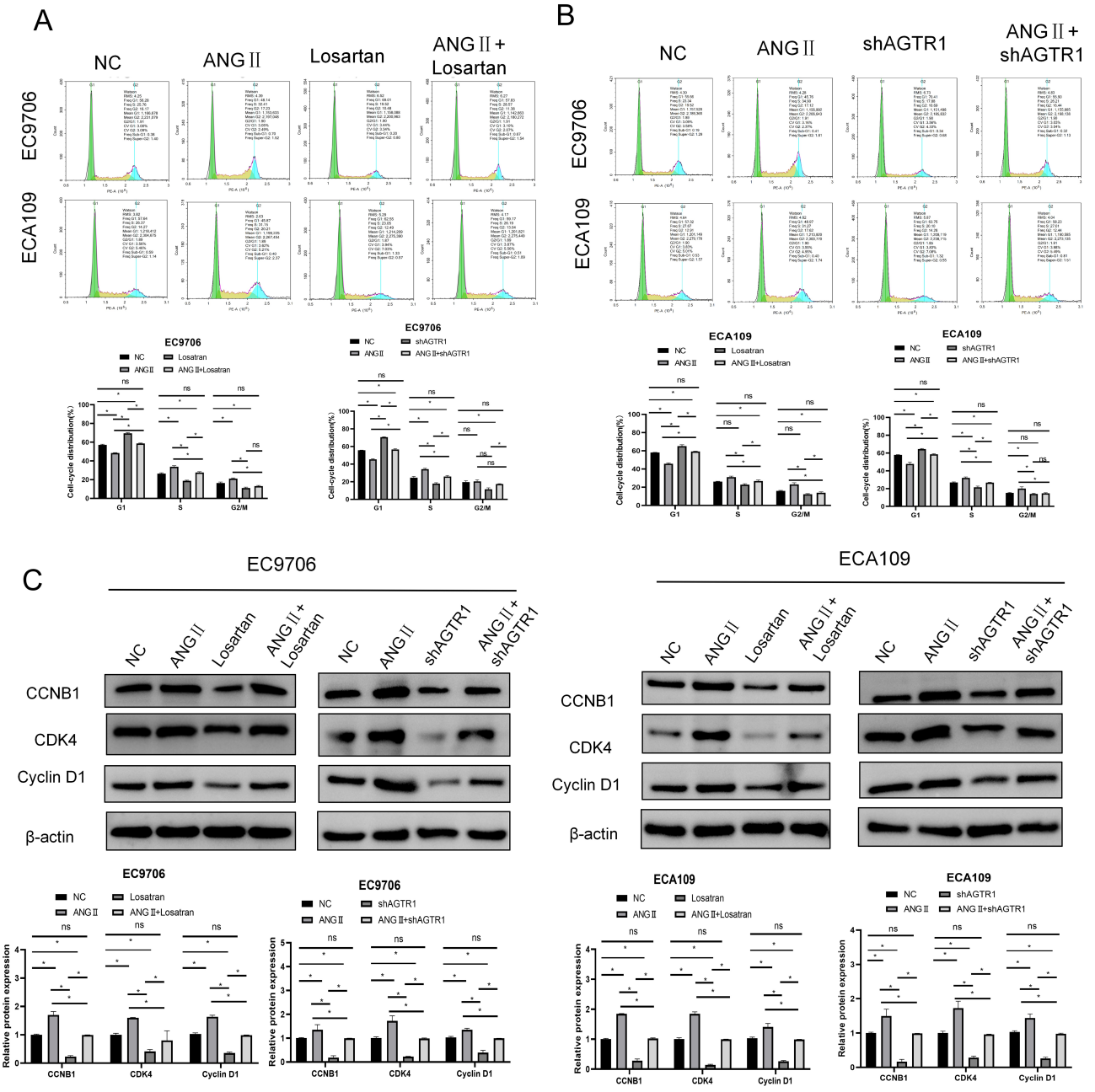

Fig. 4. Effects of Ang II and AGTR1 on ESCC cell cycle. (A,B) Effects of Angiotensin II and losartan on the cell cycle of EC9706 and ECA109 were examined. (C) Protein levels of Cyclin B1 (CCNB1), Cyclin-Dependent Kinase 4 (CDK4), and Cyclin D1 were assessed using Western blotting in various groups and cell types. Each experiment was conducted three times (n = 3). ns, no statistical significance; *, $p < 0.05$.

was found that candesartan, in a dose-dependent manner, counteracts this impact, as stated in reference [24]. According to reports, the levels of Ang II in the peripheral blood are elevated in early ESCC compared to the hyperplasia group. Additionally, the invasive ESCC group exhibited the highest levels of Ang II among all the groups. It is worth noting that the level of Ang II is associated with both carcinogenesis and the progression of ESCC [25]. Our results are consistent with those findings. The findings of our study indicate that Ang II has the ability to enhance the migration and invasion of ESCC cells. However, the administration of losartan significantly decreased the number of cells that migrated and invaded, effectively neutralizing the impact of Ang II treatment. Following the inhibition of AGTR1, the ESCC cells exhibited notably reduced migration and invasion compared to the NC group. The findings suggest that Ang II increases the migratory and invasive capacity of AGTR1, potentially facilitating tumor metastasis. Losartan, an antagonist of AGTR1, effectively mitigated the impact of Ang II. The findings for proteins related

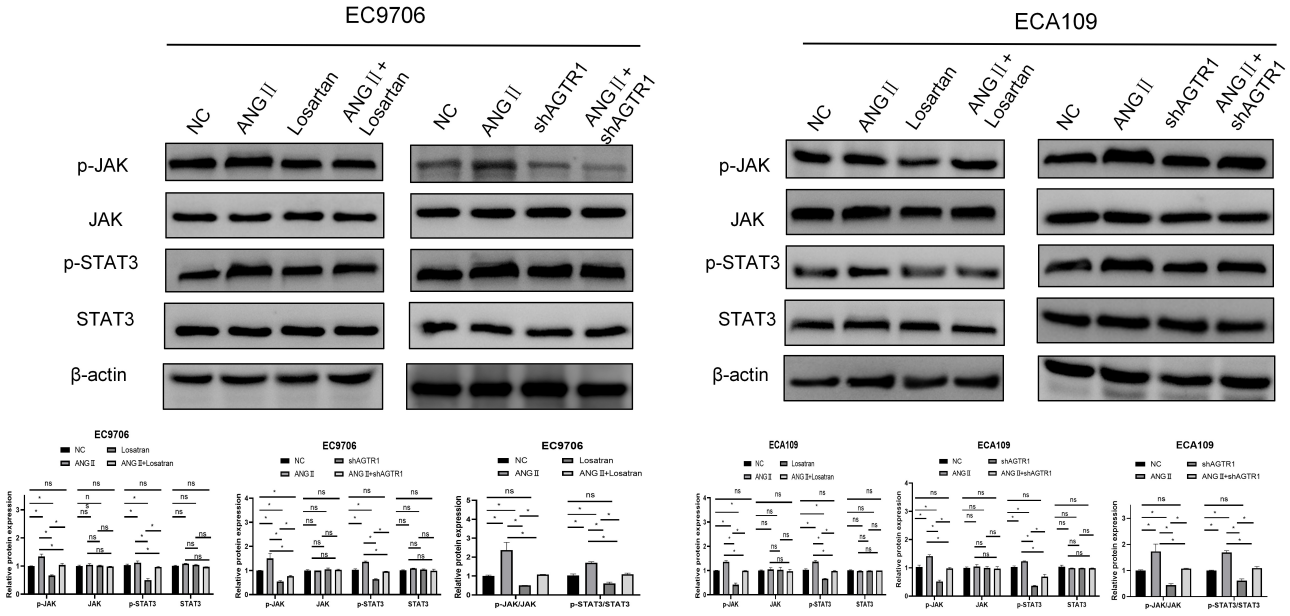

Fig. 5. Activation of the Janus kinase-signal transducer and activator of transcription 3 (JAK/STAT3) signaling pathway in ESCC cells initiated by Ang II and AGTR1. The protein content of P-JAK, JAK, p-STAT3, and STAT3 was examined using Western blotting across various groups. Each experiment was conducted three times (n = 3). ns, no statistical significance; *, $p < 0.05$.

to epithelial-mesenchymal transition (EMT) also demonstrated that esophageal squamous cell carcinoma (ESCC) cells undergo EMT via AGTR1 when stimulated by Ang II.

It has been found that losartan promotes myocardial cell apoptosis after acute myocardial infarction by inhibiting ANGII-induced JAK/STAT pathway activation. Additionally, our findings revealed that Ang II has the ability to impede the process of apoptosis in ESCC cells, while the administration of losartan can counteract the impact of Ang II treatment. Following the inhibition of AGTR1, there was a notable increase in the rate of ESCC cell apoptosis compared to the NC group. Similar results were obtained for apoptotic proteins. Furthermore, this investigation demonstrated that Ang II has the ability to facilitate the transition of ESCC cells from the G1 to S phase, while losartan has the capacity to impede this progression. Similarly, the inhibition of AGTR1 can inhibit this process. Similar results were obtained in a cell cycle-associated protein assay. The findings suggest that Ang II hinders the cell death of cancer cells and promotes the transition of ESCC cells from G1 to S phase via AGTR1, thereby fostering tumor progression.

The JAK/STAT3 pathway plays a crucial role in controlling growth and programmed cell death in various types of cancer cells [26]. Numerous studies have demonstrated that the JAK/STAT3 signaling pathway can promote the upregulation of downstream cell cycle-related proteins, such as survivin (a survival protein), anti-apoptotic proteins, and other factors post-activation, enabling cancer cells to resist apoptosis induced by immune cells. The survival and growth of tumors are significantly influenced by STAT3, which is a pivotal factor [27].

JAK is an intracellular tyrosine kinase that directly phosphorylates STAT3. Upon activation, STAT3 can form homodimers and translocate into the nucleus to interact with DNA, thereby increasing the expression of specific genes and contributing to the proliferation and metastasis of cancer cells [28]. Following treatment with Ang II, the protein levels of the JAK/STAT3 signaling pathway were found to be significantly increased in EC9706 and ECA109 cells. However, administration of losartan led to a significant decrease in these protein levels, effectively neutralizing the impact of Ang II treatment. Moreover, inhibition of AGTR1 resulted in a notable decrease in the protein levels of the JAK/STAT3 signaling pathway in both cell types. These findings suggest that Ang II activates the JAK/STAT3 signaling pathway via AGTR1, indicating that Ang II could potentially serve as a therapeutic target for the treatment of ESCC. Nevertheless, further comprehensive investigations involving a significant number of subjects are necessary to validate this assumption.

Conclusion

This study validates that Ang II/AGTR1 stimulates the growth, migration, and invasion of ESCC cells, suppresses their programmed cell death, facilitates the transition of ESCC cells from G1 to S phase, and activates the JAK/STAT3 signaling pathway via AGTR1 in ESCC. The findings of this research provide a basis for understanding the significance of Ang II/AGTR1 in the progression of ESCC. Targeting Ang II and AGTR1 could potentially serve as a therapeutic approach for treating ESCC.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

BX, YYG, JL, HW, and YYW performed the research. YYG collected and analyzed the data. BX, JL, HW, and YYW have been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors have given final approval for the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This work was supported by Grants from the Natural Science Research Project of Anhui Educational Committee Province (KJ2021A0726), the Health Research Program of Anhui (AHWJ2023A20341) and the Natural Science Project of Bengbu Medical University (2020byzd057).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537193.32.

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