**Cancer Research Journal**
2023; 11(2): 78-85
http://www.sciencepublishinggroup.com/j/crj
doi: 10.11648/j.crj.20231102.17
ISSN: 2330-8192 (Print); ISSN: 2330-8214 (Online)

# Effect of Angiotensin II on the Malignant Biological Behaviors of Colorectal Cancer Cells and Its Mechanism of Action

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## To cite this article:

Yingxue Yang, Xiaotian Yang, Qinghua Wang. Effect of Angiotensin II on the Malignant Biological Behaviors of Colorectal Cancer Cells and Its Mechanism of Action. *Cancer Research Journal.* Vol. 11, No. 2, 2023, pp. 78-85. doi: 10.11648/j.crj.20231102.17

**Received**: May 17, 2023; **Accepted**: June 2, 2023; **Published**: June 9, 2023

**Abstract:** *Objective* To explore the effects of angiotensin II (AngII) and the angiotensin II type I receptor (AT1R) antagonist Losartan on the malignant biological behaviors of colorectal cancer (CRC) cells including proliferation, invasion, migration and epithelial-mesenchymal transition (EMT), as well as the underlying mechanism of action. *Methods* The CRC cell line CT26 was cultured *in vitro*, and the expression of AT1R in CT26 cells was detected by immunofluorescence staining. Cells were divided into control group, AngII treatment group, and Losartan + AngII treatment group. We analyzed cell growth, proliferation, migration and invasion in the above three groups by adopting functional experiments. Meanwhile, ELISA was performed to detect the expression levels of AngII, TGF-β and TNF-α in cell supernatants, and the Western blotting (WB) assay was carried out to detect the expression of related proteins. In addition, the xenograft tumor nude mouse model was constructed to explore the impact of AT1R antagonist Losartan on the *in vivo* growth of CRC cells. *Results* Through experiments in vitro, immunofluorescence staining results verified that AT1R was significantly expressed in CT26 cells. Compared with control group, AngII treatment remarkably promoted the growth, proliferation, migration and invasion of CT26 cells. In the meantime, relative to AngII treatment group, Losartan + AngII treatment group dramatically suppressed the growth, proliferation, migration and invasion of CT26 cells ($P < 0.05$). Moreover, relative to control group, AngII treatment evidently up-regulated the expression levels of MMP-2, MMP-9, N-cadherin, Vimentin, Snail and p-Smad proteins in cells, and induced the down-regulated expression of E-cadherin protein. Compared with AngII treatment group, Losartan + AngII treatment group showed dramatically decreased expression levels of MMP-2, MMP-9, N-cadherin, Vimentin, Snail and p-Smad proteins in cells, whereas significantly elevated expression of E-cadherin protein ($P < 0.05$). After AngII treatment, the TGF-β level secreted in cells was higher than that in control group, while that in cells of Losartan + AngII treatment group markedly decreased relative to AngII treatment group ($P < 0.05$). As revealed by *in vivo* experimental results, the xenograft tumor growth was evidently suppressed in Losartan + AngII treatment group compared with control group and AngII treatment group, and the serum AngII, TGF-β and TNF-α levels in mice were markedly reduced ($P < 0.05$). *Conclusions* AngII promotes the proliferation, migration, invasion and EMT of CT26 cells, while Losartan can antagonize the effect of AngII on promoting malignant growth of CRC both *in vivo* and *in vitro*.

**Keywords:** Colorectal Cancer, Angiotensin II, Epithelial-mesenchymal Transition

## 1. Introduction

Colorectal cancer (CRC) is currently one of the most common causes of cancer-associated death in the world, which occupies approximately 10% of the global newly diagnosed cancer cases and cancer-related deaths annually [1, 2]. Around 20% of CRC cases have developed metastatic lesions when they are first diagnosed, while 25% of patients with locoregional lesions will eventually develop distant

metastasis [3]. Besides, such malignant metastasis is a major factor inducing the mortality of CRC cases. Consequently, it is urgently needed to further explore and understand the molecular mechanism underlying the occurrence and development of CRC, and develop the novel effective therapeutic targets and strategies on this basis to improve the clinical efficacy of CRC. Currently, the commonly used drugs for underlying diseases in the clinic may have potential anti-tumor property, which can serve as the adjuvant therapies to enhance the therapeutic efficacy of conventional antitumor chemotherapy. For instance, some research suggests that, the resin-angiotensin system inhibitors (RASIs) used for lowering blood pressure and protecting the kidney can also exert the antitumor activity [4].

Angiotensin converting enzyme (ACE) is one of the key enzymes in the renin-angiotensin system (RAS), which can convert angiotensin I into angiotensin II (AngII). The angiotensin II receptors belong to the G protein-coupled receptors, mainly including type 1 (AT1R) and type 2 (AT2R). Of them, AT1R has an important role in the development of human diseases. Previous studies have suggested that, AT1R possesses multiple biological functions, including blood pressure regulation and cardiovascular system homeostasis [5]. As discovered in recent research, the high expression of AT1R can promote the growth of breast cancer and accelerate epithelial-mesenchymal transition (EMT) [6]. In esophageal cancer, AngII can up-regulate AT1R expression, induce the activation of mammalian target of rapamycin (mTOR), and promote the proliferation of esophageal cancer cells [7]. As reported, the high expression of AT1R in liver cancer cells will activate ERK and Smad2, promote the TGF signaling pathway, and then induce EMT and the metastasis of liver cancer cells [8]. Consequently, this study aimed to further explore the impacts of AngII and its type 1 receptor (AT1R) antagonist Losartan on the proliferation, invasion, migration and EMT of CRC cells as well as the underlying mechanism, so as to provide theoretical and experimental foundation for the clinical application of AT1R antagonist Losartan in the treatment of CRC and provide a new thinking for the treatment of CRC.

# 2. Materials and methods

### *2.1. Major Experimental Materials and Cell Culture*

Cell line: The CRC cT26 cells were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences. Experimental animals: The 4-6-week-old SPF grade BALB/c female nude mice were provided by the Laboratory Animal Center of Yangzhou University). Reagents utilized in this work included AngII and Losartan (Sigma, USA); rabbit anti-AT1R antibody (Arigo, Taiwan, China); rabbit anti-TGF-β and anti-phospho-Smad3 antibodies (Cell Signaling, USA); mouse anti-MMP-2 and anti-MMP-9 antibodies (Santa Cruz Biotechnology, USA); mouse anti-Snail, anti-α-SMA and anti-β-actin antibodies (Abcam, USA); rabbit anti-E-cadherin and anti-N-cadherin antibodies and secondary antibodies (AB clonal, Wuhan, China). Additionally, DAPI cell nuclear dye (Beyotime Institute of Biotechnology), CCK-8 kit (Beijing Solarbio), ELISA kit (MultiSciences Co., Ltd), Transwell chamber (Corning, USA), and Matrigel (BD, USA) were also adopted.

The CT26 cell line was cultured in the RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin, and incubated in the constant temperature incubator at 37°C with 5% CO2.

### *2.2. Expression in CT26 Cells Detected by Immunofluorescence Analysis*

Cells were inoculated into the 24-well plates and cells growing on glass slides were prepared. To be specific, cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 10 min, and blocked with PBST that contained 1% BSA and 22.52 mg/ml glycine at room temperature for 30 min. Each slide was incubated with PBS-diluted anti-AT1R primary antibody (1: 500) at 4°C overnight. On the following day, slides were further incubated with PBS-diluted fluorescence secondary antibody (1: 500) at room temperature in dark for 1 h. Meanwhile, cell nuclei were stained with DAPI for 10 min at room temperature in dark, and the coverslips were sealed with the sealing medium. The expression of AT1R was observed under the fluorescence microscope and photos were taken.

### *2.3. Cell Proliferation Rate Detected by CCK-8 Assay*

CT26 cells at the logarithmic growth phase were harvested and inoculated into the 96-well plate at the density of 1×103/well. Then, cells were incubated in the incubator, after reaching cell adherence, the medium was replaced with RPMI 1640 medium containing AngII (1, 10, 100 nmol/L), and three replicate wells were set for each group. At 72 h after incubation, the CCK-8 solution was added for incubation in dark, and the absorbance at 450 nm was measured with the microplate reader. The cell survival rate was calculated, and the 100 nmol/L AngII concentration was adopted for later experiment. According to the above-mentioned steps, cells were treated with RPMI1640 medium that contained Losartan (10, 100, 1000 nmol/L) and AngII (100 nmol/L), respectively, for CCK-8 assay. The 1000 nmol/L Losartan concentration was selected for subsequent experiment.

### *2.4. Cell Grouping and Treatment*

Based on the CCK-8 assay results, CT26 cells were divided into control group (routine culture), AngII treatment group (100 nmol/L AngII treatment for 72 h), and Losartan + AngII treatment group (after 2-h pretreatment with Losartan, 100 nmol/L AngII was added to treat cells for 72 h).

### *2.5. Clone Formation Assay*

CT26 cells were inoculated into the 6-well plates at the density of 1×103/well. After grouping, cells were cultured in the incubator till visible clones under the naked eyes were observed, fixed with 4% paraformaldehyde for 20 min, and

stained with crystal violet for 30 min. Then, clones were photographed and clone number was counted, with 3 replicate wells being set for each group.

### *2.6. Cell Scratch Assay*

CT26 cells at the logarithmic growth phase were inoculated into the 6-well plates and cultured to 90% cell confluence. Afterwards, a scratch was made with the 200 μL pipette tip, the original medium was discarded, cells were washed with PBS thrice and photographed under the microscope (0 h). In addition, cells at the same position were recorded and photographed at 24 and 48 h of culture with the microscope. The scratch area was calculated by the Image J software.

### *2.7. Transwell and Matrigel Transwell Assays*

After digestion of CT26 cells, the serum-free RPMI 1640 medium was added to resuspend cells into the cell suspension at 3×105 cells/mL. Thereafter, 200 μL cell suspension was added into the upper Transwell chamber covered with no Matrigel (Transwell migration assay) and with Matrigel (Matrigel Transwell invasion assay), while complete medium containing 20% FBS was added into the lower chamber. Cells were grouped for different treatments, three replicate wells were set for each group, and the assays were repeated thrice. Cells were further cultured for 24 h, then the chambers were taken out, washed with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min, and stained with crystal violet dye at room temperature for 20 min. The non-migrating cells on the upper Transwell chamber were wiped with a cotton swab, and cell number was counted under the microscope. Three fields of view (FOVs) were selected from each sample to take the average value.

### *2.8. Western Blotting (WB) Assay*

Cells in different groups were collected separately and lysed to extract the total cellular proteins. Proteins were then separated through the 12% Tris-glycine SDS-PAGE separation gel electrophoresis, and transferred onto the membranes by the wet-transfer method. Then, membranes were blocked with 5% defatted milk for 1 h, incubated with primary antibody at 4°C overnight, and then with secondary antibody for 2 h at room temperature. After washing thrice with TBST, the ECL color developing solution was added, protein blots were observed under the chemiluminescence imager and photos were taken.

### *2.9. Animal Experiments*

An equivalent amount of CT26 cells (1×106/mouse) were subcutaneously injected into the armpit of each 6-week-old female BALB/c mouse to construct the xenograft tumor nude mouse model. Subsequently, mice were randomly divided into two groups. When the xenograft tumor volume reached 100 $mm^3$ (Day 0), 800mg/L Losartan and AngII were added into the drinking water for mice of Losartan + AngII treatment group, while for mice in control group, the ordinary drinking water without Losartan or AngII was given. The mouse tumor size was measured with a vernier caliper every three days. Mice were sacrificed on day 23, the tumor tissue was separated and the tumor size was measured. All animal experiments were approved by the Institutional Laboratory Animal Protection and Use Committee of Jiangsu University.

The expression levels of AngII, TGF-β and TNF-α in the serum of tumor-bearing mice were determined by the ELISA kit.

### *2.10. Statistical Analysis*

Each assay was carried out independently for three times at least, and at least three replicate wells were set for intra-group experiments. GraphPad Prism 5.0 statistical software was applied in data analysis and graph drawing. Measurement data were expressed as means ± standard deviation (means ± ea), LSD-t test was adopted for inter-group comparison of means, and a difference of $P < 0.05$ stood for statistical significance.

## 3. Results

### *3.1. AngII Promotes AT1R Expression in CTC CT26 Cells*

First of all, we observed the expression of AT1R in CRC CT26 cells. As revealed by our immunofluorescence staining results, AT1R (green fluorescence) was significantly expressed in CT26 cells (Figure 1A). Relative to control group, the addition of AngII dramatically up-regulated AT1R expression ($P < 0.05$) in a dose-dependent manner (Figure 1B).

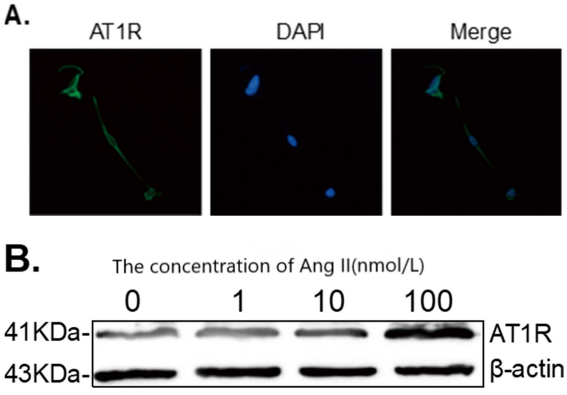



***Figure 1.*** *AngII promotes AT1R expression in CRC CT26 cells.*

A: AT1R expression (green fluorescence) in CT26 cells. B: The AT1R protein expression in CT26 cells treated with AngII (0, 1, 10, 100 nmol/L) detected by WB assay.

### *3.2. Losartan Antagonized the Effect of AngII on Promoting the Growth and Proliferation of CRC CT26 Cells*

Subsequently, we observed the impacts of AngII on the growth and proliferation of CRC CT26 cells. Firstly, CT26 cells were treated with AngII at gradient concentrations (0, 1, 10, 100 nmol/L), and cell growth activity was then detected by CCK-8 assay. The results suggested that compared with

control group, AngII treatment significantly enhanced cell growth in a dose-dependent manner (Figure 2A). Compared with AngII treatment group, Losartan + AngII treatment remarkably inhibited the growth of CT26 cells (Figure 2B) dose-dependently. Based on the above results, the experiment was divided into control group, AngII treatment group (100 nmol/L AngII), and Losartan + AngII treatment group (pretreatment with 1000 nmol/L Losartan for 2 h, and then treatment with 100 nmol/L AngII). As verified through clone formation assay, the clone formation rate in AngII treatment group was higher than that in control group, besides, AngII treatment evidently promoted cell proliferation, while Losartan + AngII treatment remarkably decreased cell proliferation relative to AngII treatment (Figure 2C).

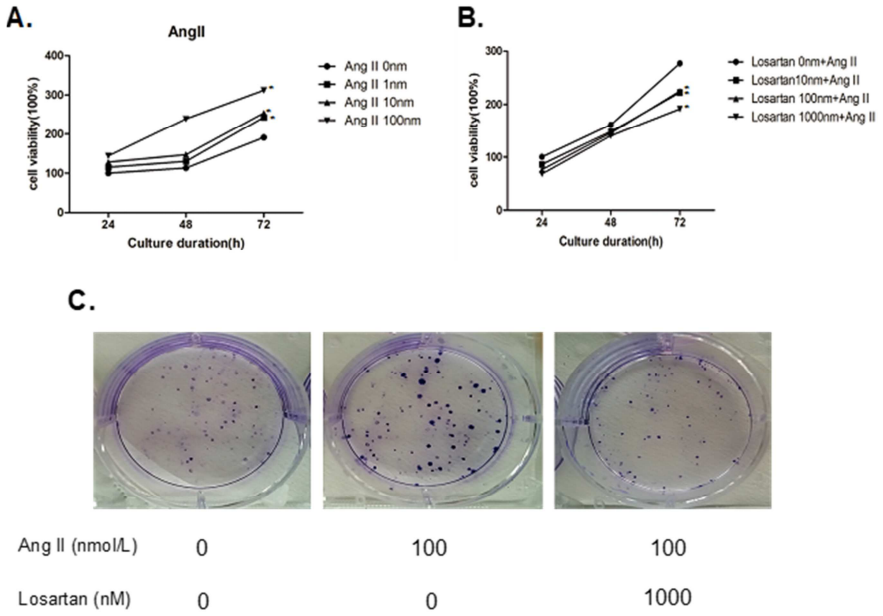
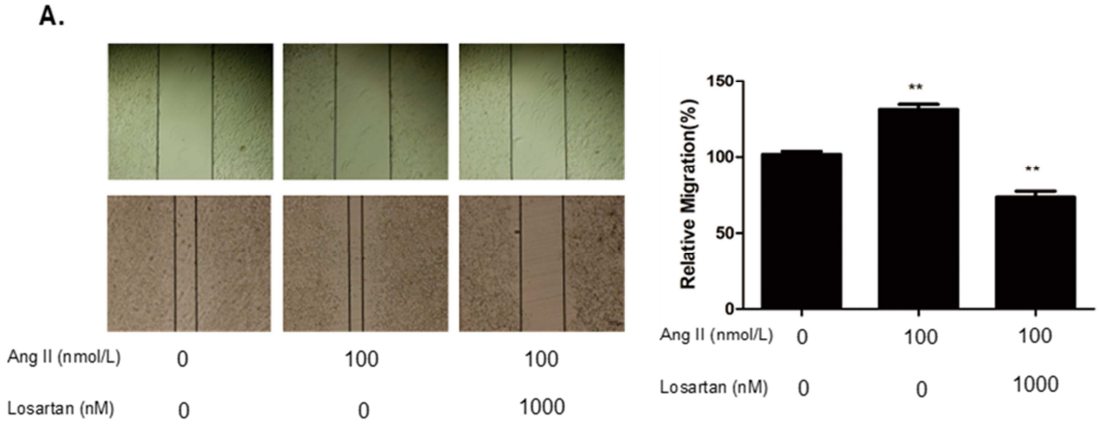



***Figure 2.*** *Losartan antagonizes the effect of AngII on promoting CT26 cell growth and proliferation.*

A: CT26 cell growth was detected by CCK-8 assay after AngII (0, 1, 10, 100 nmol/L) treatment, $P < 0.05$; B: CT26 cell growth was detected after gradient concentrations of Losartan (0, 10, 100, 1000 nmol/L) combined with AngII (100 nmol/L) treatment, $P < 0.05$; C: Cell proliferation in control group, AngII treatment group, and Losartan (1000 nmol/L) + AngII (100 nmol/L) treatment group detected by clone formation assay.

### *3.3. Losartan Suppressed the Impact of AngII on Promoting CT26 Cell Migration*

he aforementioned results preliminarily revealed that Losartan dramatically suppressed the role of AngII in promoting CT26 cell growth and proliferation. Subsequently, we further verified the impact of Losartan on the function of AngII in promoting CTC CT26 cell migration. As confirmed by results of scratch assay (Figure 3A) and Transwell assays (Figure 3B), compared with control group, AngII treatment alone markedly enhanced the CT26 cell migration, while relative to AngII treatment alone group, Losartan combine with AngII treatment remarkably suppressed cell migration.



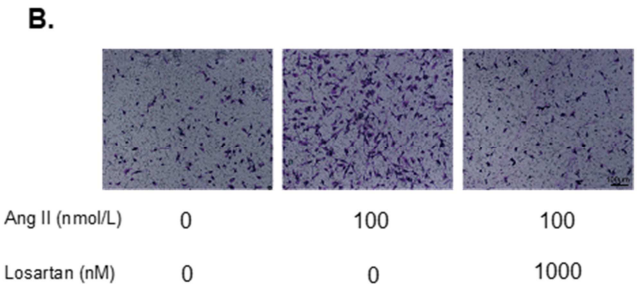



***Figure 3.*** *Losartan suppresses the role of AngII in promoting CRC CT26 cell migration.*

Scratch assay (A) and Transwell cell migration assay (B) conducted to detect the CT26 cell migration in control group, AngII (100 nmol/L) treatment group, and Losartan (1000 nmol/L) + AngII (100 nmol/L) treatment group (* $P < 0.05$; ** $P < 0.01$).

### *3.4. Losartan Suppressed the Function of AngII in Promoting CRC CT26 Cell Invasion*

As verified by cell invasion assay, compared with control group, the CT26 cell invasion ability in AngII treatment group evidently elevated, while relative to AngII treatment alone group, Losartan combined with AngII treatment remarkably suppressed the CT26 cell invasion capacity (Figure 4).

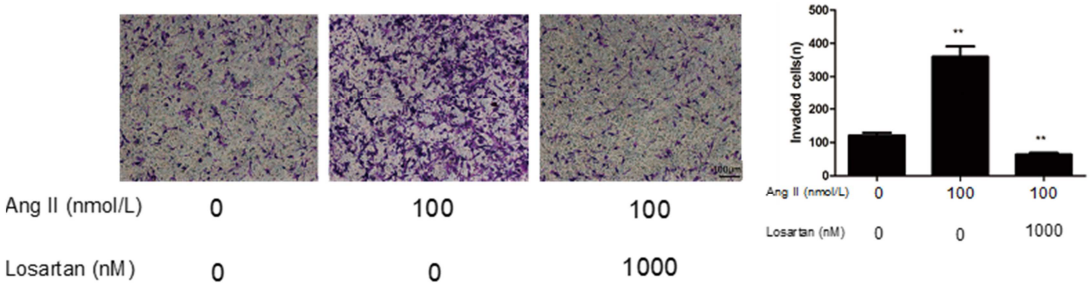



***Figure 4.*** *Losartan inhibits the role of AngII in promoting CRC CT26 cell invasion.*

Matrigel Transwell assay conducted to detect the CT26 cell invasion capacities in control group, AngII (100 nmol/L) treatment group, and Losartan (1000 nmol/L) + AngII (100 nmol/L) treatment group (* $P < 0.05$; ** $P < 0.01$).

### *3.5. Losartan Suppressed the Effect of AngII on Promoting Migration-Related Protein Expression in CRC CT26 Cells*

Thereafter, we further verified the expression levels of migration-related proteins in the above groups. According to our experimental results, relative to control group, MMP-2 and MMP-9 expression in AngII treatment group was up-regulated in CT26 cells, and their expression decreased in Losartan + AngII treatment group compared with AngII treatment group (Figure 5).

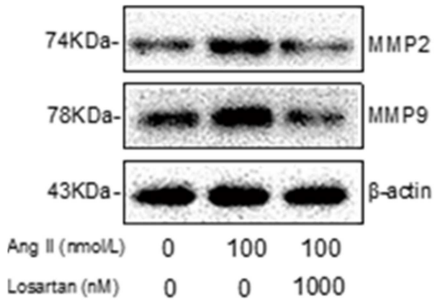



***Figure 5.*** *MMP protein expression in CT26 cells detected by WB assay.*

### *3.6. Losartan Inhibited the Effect of AngII on Promoting EMT-Related Protein Expression in CRC CT26 Cells*

According to WB results, relative to control group, N-cadherin and Snail expression increased, while E-cadherin expression dramatically decreased in AngII treatment group; meanwhile, compared with AngII treatment alone group, Losartan + AngII treatment dramatically suppressed the N-cadherin and Snail expression, and increased the E-cadherin expression (Figure 6).

### *3.7. Losartan Inhibited the AngII-Induced Activation of TGF-β/Smad Signal in CRC CT26 Cells*

Based on our experimental results, compared with control group, the TGF-β and phosphos-Smad 3 expression in AngII treatment group was up-regulated, while that in Losartan + AngII treatment group decreased relative to that in AngII treatment group ($P < 0.05$). In addition, the total Smad expression was not significantly different among different groups, revealing that Losartan inhibited the AngII-induced TGF-β/Smad signal activation in CRC CT26 cells (Figure 6).

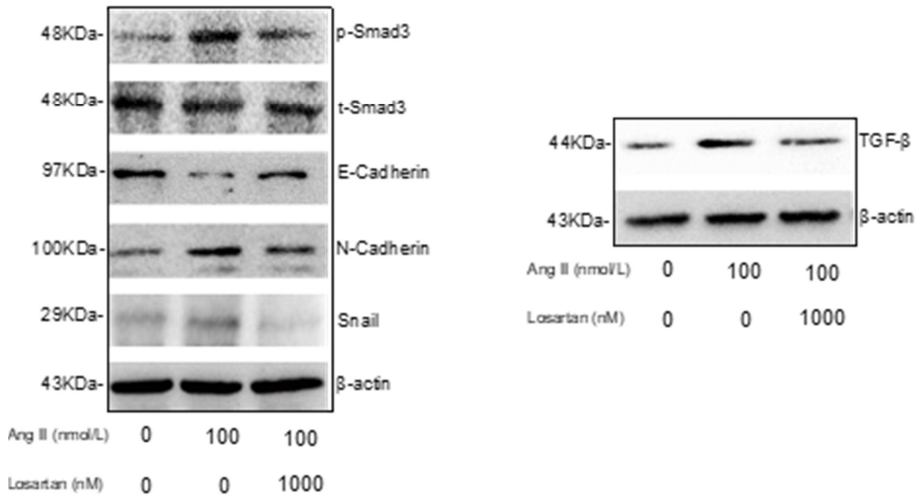



***Figure 6.*** *Expression of the above-mentioned proteins in CT26 cells measured by WB assay.*

### *3.8. Losartan Dramatically Restrained the in Vivo Growth of CT26 Cells*

To further determine the role of Losartan in antagonizing CRC cell growth, the xenograft tumor nude mouse model was constructed to analyze the function of Losartan in antagonizing CRC cell growth in vivo. No any adverse reaction or body weight change was observed in mice of every treatment group. Compared with tumor-bearing mice in control group, mice treated with Losartan had markedly decreased xenograft tumor volume (Figure 7A-B) and evidently reduced body weight (Figure 7C). In addition, the serum levels of AngII, TGF-β and TNF-α in mice were determined by ELISA, and the results suggested that relative to control group, mice in Losartan treatment group exhibited apparently decreased serum levels of AngII, TGF-β and TNF-α ($P < 0.05$) (Figure 7D-F).

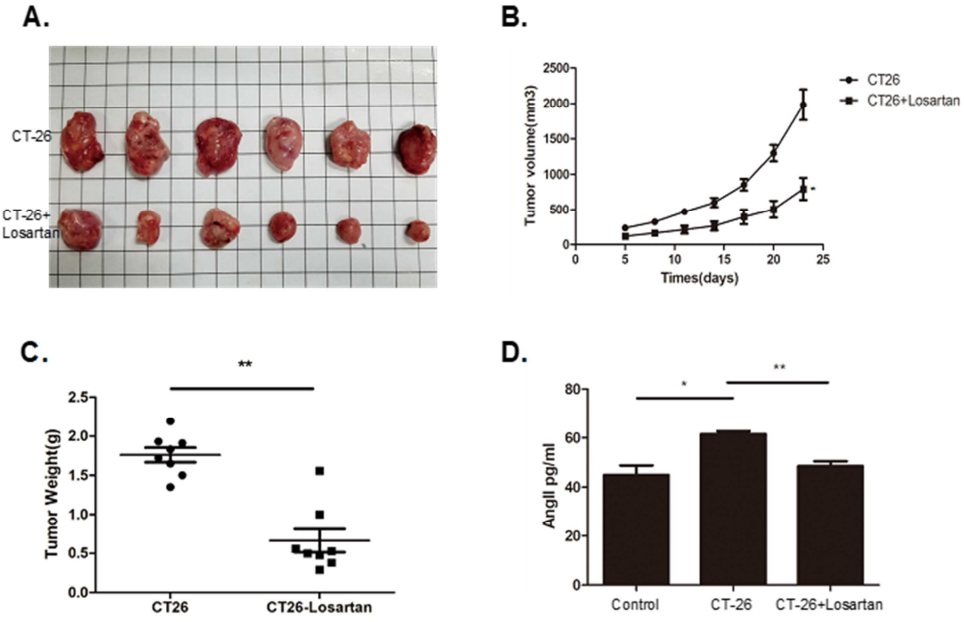



***Figure 7.*** *Losartan significantly inhibits the in vivo growth of CT26 cells.*

A: Mice were sacrificed on day 23, and the xenograft tumors were isolated; B: recording of mouse xenograft tumor volume; C: measurement of mouse xenograft tumor weight in both groups; D-H: ELISA conducted to determine serum levels of AngII, TGF-β and TNF-α (* $P < 0.05$; ** $P < 0.01$).

## 4. Discussion

Colorectal cancer (CRC) is a common malignant tumor associated with high morbidity and mortality rates. The current clinical treatments targeting CRC, including chemotherapy, surgery, radiotherapy, molecular targeted therapy and the advanced early screening and diagnostic

techniques, have greatly improved the prognosis and 5-year overall survival of CRC patients. But the prognosis of advanced, metastatic and recurrent CRC patients remain unfavorable, therefore, it is of crucial importance to explore the molecular pathological mechanism of CRC and to develop new therapeutic strategies on this basis [1].

The renin-angiotensin system (RAS) plays an essential role in the progression of different malignant tumors, and mainly participates in regulating blood pressure and body fluid equilibrium, while AngII is the major effector peptide in RAS, which acts as the vasoconstrictor substance in controlling cardiovascular function and kidney homeostasis [9]. In 1998, Lever et al. reported that, the tumor incidence apparently decreased in hypertensive patients receiving RASIs treatment [10]. Thereafter, an increasing number of studies have indicated that, RAS exists in numerous tumors, and its effector peptide AngII can activate AT1R to promote tumor growth and metastasis [11-12]. According to our results in this study, AngII up-regulated AT1R expression in CRC cells, while Losartan suppressed the AngII-induced up-regulation of AT1R expression. The angiotensin receptor blockers mainly include Losartan, Telmisartan, Valsartan and Candesartan.

The infinite proliferative and migrating abilities of tumor cells are the major causes leading to the malignant progression of tumor and the main factors affecting patient prognosis [13]. As preliminarily confirmed in this study, AngII promoted CRC cell proliferation and migration, while Losartan dramatically inhibited the effect of AngII on promoting malignant growth of CRC. Extracellular matrix (ECM) is the natural barrier blocking tumor migration and invasion, therefore, ECM degradation is needed in tumor metastasis, while matrix metalloproteinases (MMPs) have a critical function in this process. MMP-2 and MMP-9 can degrade type IV collagen, enable tumor cells to break through the basilar membrane, and metastasize to the distant organ. Based on our results, AngII accelerated CRC cell invasion, furthermore, it also remarkably promoted MMP-2 and MMP-9 expression in CT26 cells, indicating that AngII enhanced the CRC invasiveness, highly consistent with our observations in esophageal cancer and breast cancer [6, 7].

EMT was first proposed by Greenburg and Hay in 1982 [14]. After the occurrence of EMT, tumor cells are endowed with the ability to break through the basilar membrane and migrate across the blood vessels. It has been discovered that EMT is tightly associated with the development of multiple tumors. The reduction or loss of E-cadherin is the most important hallmark change of EMT, which is also an important manifestation promoting tumor metastasis. The levels of N-cadherin and Vimentin apparently elevate during EMT, which can promote tumor cell migration [15, 16]. We discovered that AngII enhanced the expression of N-cadherin, Vimentin and Snail in CRC cells and decreased that of E-cadherin, while Losartan antagonized the above effect, revealing that Losartan antagonized the role of AngII in promoting EMT of CRC cells.

TGF-β is closely related to the maintenance of CRC tissue homeostasis, and the abnormal activation of TGF-β signal can promote the occurrence of inflammatory diseases and exert an important effect on CRC genesis and development [17]. TGF-β can promote EMT in tumor cells through the TGF-β/Smad, Wnt and MAPK/ERK pathways [18-19]. The EMT process is accompanied by changes in multiple molecular markers, the phosphorylated Smad2/3 bind to the intracellular Smad4 to form a trimer, which then transfers into the cell nucleus and binds to the target gene DNA, thereby accelerating the transcription of multiple oncogenes [19]. In this study, AngII promoted TGF-β expression in CRC cells, while Losartan suppressed its effect. According to previous study, the combined application of TGF-β inhibitor and 5-fluorouracil can effectively postpone tumor growth and suppress lymphatic metastasis of tumor [20]. As a kind of hypotensive agent, Losartan has been extensively applied in clinic. It was preliminarily verified in this study that, Losartan suppressed the AngII-induced TGF-β/Smad3 signal activation, indicating that Losartan inhibited TGF-β/Smad activation to efficiently restrain CRC cell growth and migration.

## 5. Conclusion

AngII can promote the proliferation, migration, invasion and EMT of CRC CT26 cells, while Losartan can suppress the activation of TGF-β/Smad signal to antagonize the pro-carcinogenic effect of AngII. This study provides the preliminary theoretical foundation for Losartan as one of the candidate therapeutics for the clinical intervention of CRC.

## Acknowledgements

This work was supported by Clinical Medical Science and Technology Development Fund of Jiangsu University (Grant no. JLY20180113).

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