**Original Article**

**Astragaloside IV attenuates renal fibrosis through repressing epithelial-to-mesenchymal transition by inhibiting microRNA-192 expression: in vivo and in vitro studies**

Yaochen Cao¹, Li Zhang², Yu Wang², Qingchun Fan¹, Yakun Cong¹

¹Department of Nephrology, Daqingshi No. 4 Hospital, Daqing 163000, Heilongjiang, China; ²Department of Nephrology, The First Affiliated Hospital of Jilin University, Changchun 130000, Jilin, China. *Equal contributors.

Received July 19, 2019; Accepted July 25, 2019; Epub August 15, 2019; Published August 30, 2019

**Abstract:** The aim of this study was to investigate the effect of Astragaloside IV (AS-IV) on renal fibrosis in vivo and in vitro, and further to explore the underlying mechanism. To investigate the effect of AS-IV treatment on renal fibrosis in vivo, mouse renal fibrosis model was established by performing unilateral ureteral occlusion (UUO). The mice in the intervention group of AS-IV were given AS-IV 20 mg/(kg/d) on the day after surgery for 7 consecutive days. Then renal sections were stained with hematoxylin and eosin (H&E) to evaluate the degree of fibrosis. For in vitro study, human kidney tubular epithelial cells induced by (TGF-β1) were performed to research the protective role of AS-IV in anti-fibrosis. Results form the in vivo study showed that AS-IV treatment in UUO mice significantly reduced parenchymal loss and tubular atrophy, indicating that AS-IV treatment attenuated renal fibrosis caused by UUO. TGF-β1 treatment significantly increased the expression of α-SMA, vimentin, collagen I, miR-192 and decreased E-cadherin expression in HK-2 cells, suggesting that TGF-β1 stimulated renal tubulointerstitial fibrosis. Moreover, in TGF-β1 stimulated HK-2 cells, AS-IV clearly inhibited the expression levels of α-SMA, vimentin, collagen I, and miR-192 in a dose-dependent fashion while increased the expression level of E-cadherin in the same manner, indicating that AS-IV functioned the inhibitory role in renal interstitial fibrosis. Interestingly, we noted that ZEB2 was a direct target of miR-192. The effects of AS-IV on the expression of α-SMA, vimentin, collagen I and E-cadherin were inhibited by miR-192 mimic and aggravated by miR-192 inhibitor. Taken together, our results provided evidence that AS-IV could effectively protect kidney against epithelial fibrosis, and this renoprotective effect involved miR-192. Therefore, AS-IV might be considered as a potential and promising candidate drug for the treatment of renal epithelial fibrosis.

**Keywords:** Astragaloside IV, HK-2 cells, unilateral ureteral occlusion, miR-192, renal fibrosis

**Introduction**

Chronic kidney disease (CKD) has threaten the worldwide public health with the incidence of CKD increasing yearly, and more and more young men suffer from CKD. Renal interstitial fibrosis (RIF) is known as the final common pathway for most of CKD, which progressively develops to end-stage renal failure. Epithelial-to-mesenchymal transition (EMT) plays an important role in the progression of RIF [1]. Tubular EMT is a phenotypic conversion of renal tubular epithelial cells into mesenchymal cells when kidney damage or partial activation. Numerous cytokines and autocrine growth factors are involved in EMT, among which, transforming growth factor beta (TGF-β) has been recognized as a major mediator in the genesis of chronic kidney diseases (CKD), and also been accepted as the most important inducer of EMT [2]. TGF-β signaling pathway involved in EMT are intricate. There is no doubt that inhibiting the progression of interstitial fibrosis could be a promising strategy to protect the kidney from the deleterious effects of CKD. Therefore, it is important and urgent to search for proper pharmacologic interventions to prevent renal tubulo-interstitial fibrosis following kidney injury.
Astragaloside IV ameliorates renal fibrosis

Although some pathways including TGF-β have been suggested to be involved in the progress of renal tubular epithelial, the mechanisms underlying these structural and functional changes still remain unclear. As we all known, TGF-β could promote accumulation of renal extracellular matrix proteins in the kidney in disparate conditions including diabetes and ureteral obstruction by both promoting their synthesis and inhibiting their degradation. Moreover, accumulating evidences have been identified that microRNAs (miRNAs) also play an important role in regulating target gene expression [3]. These non-coding, approximately 22 nucleotide-long RNAs regulate gene expression by binding directly to the 3'-UTR of target gene mRNA, resulting in translational repression or degradation; they may also influence other steps in the initiation and elongation phases of mRNA translation [4]. miRNAs have been emerged as key players in renal physiology. Among them, miRNA-192 plays an important role in EMT of cancer and fibrosis of tissue and organ. Recent studies have shown that miR-192 is over-expressed in diabetic mouse and mesangial cells treated with TGF-β or exposure to high-glucose ambience [4]. Podocyte-specific deletion of Dicer, a key enzyme for miRNA production, leads to glomerular and tubular lesions with massive proteinuria and accelerated death by 4 weeks of age [1]. It has been reported that TGF-β/Smad3 axis alters the expression of miRNAs through facilitating their maturation [5]. miR-21 mediates pro-fibrotic effects of TGF-β/Smad3 axis in ureteral obstruction associated renal fibrosis [6]. However, TGF-β-induced fibrosis through inhibiting the secretion of miR-192 from injured renal tubular epithelial cells has not been explored.

Astragaloside IV (AS-IV) is a natural triterpene glycoside extracted from astragalus membranaceus Bge (AM), a traditional Chinese herb that has been widely used in the treatment of kidney disease. AS-IV is one of the major active components in AM and displays various pharmacological effects, such as vasodilating effect, preventing endothelial dysfunction, improving cardiac cell energy metabolism, anti-inflammatory effect, and antioxidant effects [5, 7, 8]. Previous studies have proved that AS-IV could inhibit apoptosis and inflammation in two rodent models: renal ischemic-reperfusion injury and contrast-induced nephropathy. However, whether AS-IV has protective effects on CKD still remain clear. The previous work identified that AS-IV protected renal tubular cells from high-glucose-induced injury via inhibiting tubular cell apoptosis [8, 9]. In order to define the function of AS-IV on the process of kidney fibrosis, mouse renal fibrosis model was established by performing UUO, and TGF-β-induced injury in renal tubular epithelial cells were conducted.

In the present study, renal fibrosis in vivo and in vitro models were established to explore the protective effects of AS-IV on renal fibrosis. We hypothesized that AS-IV would provide renoprotection against fibrosis in TGF-β-induced HK-2 cells, and that the protective effects were unleashed via inhibiting the miR-192 pathway.

Materials and methods

Animals and treatment

Animal experiments were approved by the Ethics Committee of Daqingshi No. 4 Hospital. A total of 15 male C57BL/6 mice were purchased from the Animal Core Facility of Nanjing Medical University. Under specific pathogen-free conditions, male mice (8 weeks old, 20-25 g) were housed in standard rodent cages. Mice were divided into three groups (n = 5) for treatment: sham group [phosphate-buffered saline (PBS) treatment and sham surgery]; model group (PBS treatment and induction of renal fibrosis); AS-IV group (AS-IV treatment and induction of renal fibrosis). The mice in the intervention group of AS-IV were given AS-IV 20 mg/(kg × d) on the day after surgery for 7 consecutive days. Mice from the sham group and model group were given the same amount of normal saline.

Unilateral ureteral occlusion (UUO) mouse model conduction [10]

The mice were anesthetized with 3% pentobarbital sodium (80 mg/kg), and then placed on the test bench in the prone position. We opened the middle of the abdomen of the mouse, cut the skin and muscles, separated the left ureter, freed the ureter at the 1/3 of the ureter, and ligated 2 lines with the 6th line to cut the ureter. The wounds of the mice were cleaned and
sutured. Mice were then given penicillin by intramuscular injection and were allowed to recover in standard rodent cages. The sham operation group only separated the left ureter, and did not ligature and not cut. Mice were sacrificed at 14 days, and renal tissue samples were collected for further analysis.

**Histological analysis**

Renal tissues were fixed in 4% formaldehyde, dehydrated, embedded, and sectioned at 4 μm thickness. Renal sections were stained with hematoxylin and eosin (H&E) (Cloud-clone-Crop, Wuhan, China) to evaluate the degree of fibrosis.

**Renal cell culture and treatment**

Immortalized human proximal tubular cells (HK-2 cells) were purchased from the American Type Culture Collection (Manassas, VA, USA). HK-2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA)/Nutrient Mixture F-12 (Gibco) containing 10% fetal bovine serum (Gibco).

In order to induce HK-2 cell fibrosis in vitro, HK-2 cells were seeded in 6-well plates at 1 × 10^6 cells/ml and stimulated with 5 ng/ml TGF-β1 for 72 h [11]. Cells were divided into 2 groups: control group, TGF-β group.

AS-IV was dissolved in dimethyl sulfoxide (DMSO) and preserved at -20°C until use. For AS-IV treatment, HK-2 cells were seeded in 8-well plates at 1 × 10^6 cells/ml. After 30 minutes of treatment of the cells with various concentrations of AS-IV (0, 7.5, 15, 30 mg/ml), TGF-β1 stimulation (5 ng/ml TGF-β1 for 72 h) was performed.

**Western blot assay**

Total proteins were obtained from HK-2 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s protocol. RNA yield and purity were determined by measuring the absorbance at 260 and 280 nm. Total RNA was reverse transcribed into cDNA using Reaction Ready First-strand cDNA synthesis kit (SABiosciences, Valencia, CA, USA). 50 ng of cDNA samples was used for PCR analysis using SYBR green master mix (SABiosciences). cDNAs were analyzed by qPCR assay with the SYBR Premix Ex TaqTM II (TliRNaseH Plus) kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer’s protocol. U6 for miRNA and GAPDH for mRNA was used as internal control. Primer sequences for PCR were listed as following: GAPDH, forward 5’-TGTGTCATCAATGACCCCTT-3’; reverse 5’-CTCCACAGCTACCTCACGGC-3’; U6, forward 5’-GCTTCACGGATTTGCGTGTCAT-3’; reverse 5’-GGTTGGCTTCCAGTTTTCG-3’; miR-192, forward 5’-GGGAGTTTGCGTCATCATGC-3’; reverse 5’-GACGCTAAGTGATATCATCC-3’; reverse 5’-CAAGAGGGTCCAGGGTCCAG-3’; ZEB2, forward 5’-CAAGAGGGTCCAGGGTCCAG-3’; reverse 5’-GGTTGGC-
Astragaloside IV ameliorates renal fibrosis

Am J Transl Res 2019;11(8):5029-5038

AATACCGTCATCC-3'; α-SMA, forward 5'-TTCTGTTACTACTGCTGAGCGTGA-3'; reverse 5'-AA AGATGGCTGGAAGAGGTC-3'; E-Cadherin, forward 5'-AGCCATGTACGTTGCTATCC-3'; reverse 5'-CTAGACAGCCTCCTCCTTAAT-3'; Vimentin, forward 5'-CTGCAGGCCAGCCAGATTCA-3'; reverse 5'-TTCACTGCTGCGGCACAT-3'; collagen I, forward 5'-CAATGGCACGGCTGTGTGCG-3'; reverse 5'-CACTCGCCCTCCCGTCTTTGG-3'. Relative mRNA expression was calculated using the DDCt method [9].

Cell transfection

miR-192 mimic, miR-192 inhibitor or the negative control (NC) were obtained from GeneChem Co., Ltd. (Shanghai, China) and transfected into HK-2 cells with Lipofectamine 3000 transfection reagent in accordance with the manufacturer’s instructions. 24 h after cell transfection, transfection efficiency was determined using qRT-PCR.

Dual luciferase reporter assay

We used TargetScan bioinformatics software to predict the potential targets of miR-192, and results showed the binding sites between miR-192 and 3’UTR of ZEB2. Then, the binding sites between miR-192 and ZEB2 were confirmed by dual luciferase reporter assay. The wild type (WT ZEB2) and mutant (Mutant ZEB2) 3’UTR of ZEB2 was cloned into a pmiR RB ReportTM dual luciferase reporter gene plasmid vector (Guangzhou Ribobio Co., Ltd., Guangzhou, China) in line with the manufacturer’s instructions. HK-2 cells were co-transfected with WT ZEB2 or Mutant ZEB2 and miR-192 mimic or mimic control using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, luciferase activity was detected using the dual luciferase assay system (Promega Corporation, Madison, WI, USA) and normalized to the renilla luciferase activity.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Comparison among groups was made with Student’s t-test or one-way ANOVA followed by Tukey’s post-hoc test. Multiple-comparison tests were applied only when the difference as determined by ANOVA was significant (P < 0.05). The statistical analysis was carried out by the SPSS 18.0 software (IBM Corp., Armonk, NY, USA). P values < 0.05 were considered to be statistically significant.

Results

AS-IV treatment attenuated renal fibrosis caused by unilateral ureteral occlusion (UUO)

To investigate the effect of AS-IV treatment on renal fibrosis in vivo, mouse renal fibrosis model was established by UUO. The mice in the intervention group of AS-IV were given AS-IV 20 mg/(kg/d) on the day after surgery for 7 consecutive days. Mice form the sham group and model group were given the same amount of normal saline. Then renal sections were stained with hematoxylin and eosin (H&E) to evaluate the degree of fibrosis. As shown in Figure 1, AS-IV treatment in UUO mice significantly reduced parenchymal loss and tubular atrophy, indicating that AS-IV treatment attenuated renal fibrosis caused by UUO.

Expression of EMT markers and miR-192 in TGF-β1-induced HK-2 cells

More and more evidences have suggested that TGF-β can trigger kidney damage response and cause irreversible pathological change in tubular epithelial cells, including necrosis, apoptosis...
Figure 2. Expression of miR-192 in HK-2 cells induced by TGF-β1. A. Western blot assay detected the protein expression of α-SMA, vimentin, E-cadherin and collagen I in HK-2 cells with or without TGF-β1 treatment; B-F. Verification of α-SMA, vimentin, E-cadherin, collagen I mRNA expression, and miR-192 expression in HK-2 cells with or without TGF-β1 treatment by using qRT-PCR. Data were presented as mean ± SD for 3 independent experiments. **P < 0.01 vs. Control.
Astragaloside IV ameliorates renal fibrosis

sis and phenotype transformation, resulting in renal interstitial fibrosis [12]. As expected, TGF-β1 exposure increased the protein expressions of α-SMA, vimentin, collagen I and decreased the expression of E-cadherin in HK-2 cells (Figure 2A).

qRT-PCR findings were consistent with the results of western blot assay. The mRNA expression of α-SMA, vimentin and collagen I were also up-regulated in HK-2 cells exposed to TGF-β1. In contrast, the mRNA expression level of E-cadherin significantly decreased in HK-2 cells treated with TGF-β1 (Figure 2B-E).

Our results also showed that compared with the control group, the expression of miR-192 was increased in HK-2 cells by TGF-β1 treatment (Figure 2F). Thus, we predicted that miR-192 might be involved in the development of renal fibrosis.

AS-IV treatment inhibited TGF-β1 mediated EMT in HK-2 cells

In order to explore whether AS-IV could protect against TGF-β1 mediated EMT, we inspected the effects of AS-IV on regulating EMT related markers in HK-2 cells. After 30 minutes of treatment of cells with various concentrations of AS-IV (0, 7.5, 15, 30 mg/ml), TGF-β1 stimulation was performed. The mRNA expression of α-SMA, vimentin, E-cadherin, collagen I, and miR-192 expression in HK-2 cells were detected using qRT-PCR. Data were presented as mean ± SD for 3 independent experiments. *P < 0.05, **P < 0.01 vs. 0 mg/ml AS-IV group.

Effect of AS-IV on TGF-β1 induced HK-2 cells involved miR-192

In order to examine whether AS-IV could protect against TGF-β1 mediated EMT through regulating miR-192 expression, HK-2 cells were transfected with miR-192 mimics, miR-192 inhibitor, or the negative controls for 24 h, then the cells were treated with 5 ng/ml TGF-β1 with 30 mg/ml AS-IV for additional 72 hours. We found that the effect of AS-IV on the protein (Figure 4A)
and mRNA (Figure 4B-F) expression of ZEB2, α-SMA, vimentin, E-cadherin, and collagen I were inhibited by miR-192 mimics and aggravated by miR-192 inhibitor.

ZEB2 was a direct target of miR-192

Then, the following experiments were performed to confirm that ZEB2 was indeed a target of miR-192. As shown in Figure 5A, ZEB2 was considered to be a theoretical target gene of miR-192 through three algorithms (miRanda, TargetScan and DianamicroT). Then we used dual luciferase reporter assay to confirm the relationship between ZEB2 and miR-192. The data indicated that the luciferase reporter activity of cells co-transfected with WT-ZEB2 and miR-192 mimics was significantly reduced, while cells co-transfected with Mutant-ZEB2 and miR-192 mimics did not (Figure 5B). Besides, qRT-PCR assay was performed to determine the expression of miR-192 after HK-2 cells transfected with miR-192 mimics, miR-192 inhibitor and the corresponding negative controls. The results indicated that miR-192 mimics significantly up-regulated the expression of miR-192 (Figure 5C), whereas miR-192 inhibitor decreased the level of miR-192 in HK-2 cells (Figure 5D). Our data demonstrated miR-192 directly targeted ZEB2 in HK-2 cells.

Discussion

The present study demonstrated that AS-IV treatment attenuated renal fibrosis caused by UUO in mice. Besides, AS-IV inhibited TGF-β1 induced EMT in HK-2 cells. It has been reported that TGF-β is considered as an important mediator in interstitial fibrosis [13]. Various studies have identified that TGF-β is excessively generated by fibroblasts in many kidney diseases following interstitial fibrosis [14, 15]. The activation of TGF-β could regulate the cellular differentiation, proliferation, migration, as well as the protein expression level of ECM [16]. Up-regulated TGF-β could result in renal fibrosis while down-expressed TGF-β could ameliorate renal fibrotic lesions [2]. The main components of ECM, including α-SMA, vimentin, fibronectin, and collagen I, are closely associated with renal fibrosis. The synthesis and degradation of these proteins are in a state of dynamic balance under normal physiological condition. Our results showed that TGF-β1 treated HK-2 cells had higher expressions of α-SMA, vimentin, fibronectin, and collagen I, whereas had lower expression of E-cadherin. While these effects were reversed by co-treatment with TGF-β1 and AS-IV. Our findings suggested that the renoprotective effects of AS-IV could be mediated by inhibition of TGF-β1 induced EMT.

Accumulating evidences have shown that AS-IV has many pharmacological effects including antidiabetes [3], antifibrotic, antioxidative [17], and anti-inflammatory stress [18] in various diseases. Our study indicted that AS-IV co-treatment with TGF-β1 could reverse TGF-β1 mediated EMT in HK-2 cells, suggesting that AS-IV modulated TGF-β pathway to reduce EMT thus relieving renal fibrosis. However, it is impossible that the modulation of TGF-β pathway is the only pathophysiologic mechanism that can reduce the renal fibrosis. Fibrosis is a common pathologic feature of the chronic inflammatory, thus it is associated with various inflammatory cytokines such as IL-1, IL-6, and TNF-α [16]. Due to the anti-inflammatory activity of AS-IV, we assumed that these anti-inflammatory effects might be connected with the anti-fibrotic effect of AS-IV. Unfortunately, we did not research on inflammatory cytokines in this study, but it is necessary to identify the relationship between the anti-inflammatory and anti-fibrotic effect of AS-IV.

In addition, we further explored the exact underlying regulation mechanisms between AS-IV and TGF-β1 induced fibrosis. Our findings demonstrated that after treated with AS-IV, the level of miR-192 was also inhibited in HK-2 cells. Moreover, we found that ZEB2, which was shown to be over-expressed in the kidney [20], was a direct target of miR-192. It has been reported that miRNAs not only can reduce protein expression (translational inhibition), but also can occur by mRNA degradation and destabilization [19]. These reports support our data demonstrating that miR-192 negatively regulated ZEB2 levels in HK-2 cells. More importantly, we confirmed that the effects of AS-IV on the expression of α-SMA, vimentin, collagen I and E-cadherin were inhibited by miR-192 mimic and aggravated by miR-192 inhibitor.

Therefore, our results revealed that AS-IV treatment could be applied to restrain renal fibrosis, at least partly, via targeting TGF-β/Smad3 path-
Astragaloside IV ameliorates renal fibrosis

Figure 4. Expression of EMT markers in HK-2 cells treated with TGF-β1 and AS-IV. The HK-2 cells were transfected with miR-192 mimic, miR-192 inhibitor, or the negative controls for 24 h, then the cells were cultured in medium containing TGF-β, or TGF-β+AS-IV for additional 72 hours. (A) The protein expressions of ZEB2, α-SMA, vimentin, E-cadherin and collagen I were investigated by Western blot assay and the data were quantified. The relative mRNA expression levels of ZEB2 (B), α-SMA (C), collagen I (D), E-cadherin (E) and vimentin (F) were determined by qRT-PCR. GAPDH was used as endogenous control. Data were presented as mean ± SD. Experiments were performed at least three times. **P < 0.01.
Astragaloside IV ameliorates renal fibrosis

5037
Am J Transl Res 2019;11(8):5029-5038

Our study has a limitation as far as the investigation of AS-IV deficiency in TGF-β stimulated HK-2 cells. Clinically, there is no asymptomatic decrease in AS-IV expression levels in healthy individuals [20]. Moreover, the level of AS-IV was not a representative of the functional activity of AS-IV, because the inactivated forms of AS-IV were also determined by immunoblotting or enzyme-linked assay. In addition, it is revealed that the interstitial myofibroblasts which play essential role in extracellular matrix remodeling and renal fibrosis are originated from bone marrow, pericytes, as well as endothelial cells [21, 22]. To further explore the reliable mechanism of AS-IV on preventing renal fibrosis, more researches with many possible sources of myofibroblasts shall be performed to verify our findings.

In conclusion, the data indicated that AS-IV could attenuate renal fibrosis caused by UUO in mice, and it inhibited the TGF-β1 induced EMT in HK-2 cells in vitro. The results also showed that AS-IV inhibited renal fibrosis, at least partly through the repression of the TGF-β1/ZEB2 signaling. Taken together, our study showed for the first time that AS-IV played a protective role in renal fibrosis.

Disclosure of conflict of interest

None.

Address correspondence to: Li Zhang, Department of Nephrology, The First Affiliated Hospital of Jilin University, No. 71 Xinmin Street, Changchun 130000, Jilin, China. E-mail: zhangli190131@163.com

References


Astragaloside IV ameliorates renal fibrosis


