The effects of Kangxianling on renal fibrosis as assessed with a customized gene chip

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OBJECTIVE: To determine the mechanisms by which Kangxianling (KXL) treats renal interstitial fibrosis using a customized gene chip.

METHODS: Twelve out of 18 specific pathogen-free sprague dawley (SPF SD) rats underwent a unilateral ureteral occlusion. These rats were then randomly assigned into either the model unilateral ureteral obstruction (UUO) or Kangxianling (KXL) group. The other six rats were assigned to the sham-operated group. The UUO and sham-operated groups were given normal saline via intragastric administration, whereas the KXL group was given KXL via intragastric administration. All rats were sacrificed for renal tissue collection (i.e. left nephridial tissue), and the detection of genetic changes with the customized chip.

RESULTS: Compared to the sham-operated group, transforming growth factor-β1 (TGF-β1), Smad2, and Smad3 genes were significantly up-regulated in the UUO group, with >1.5-fold rise (P<0.01). The Smad7 gene was significantly reduced in the UUO versus sham-operated group, with a down-regulation of >1.5-fold (P<0.01). In the KXL group, TGF-β1, Smad2, and Smad3 genes were significantly reduced compared to the UUO group, with a down-regulation of >1.5-fold (P<0.01), whereas the Smad7 gene was significantly increased compared to the UUO group, with an up-regulation of >1.5-fold (P<0.01).

CONCLUSION: It was found that KXL can significantly reduce the gene levels of TGF-β1, Smad2, and Smad3. Immunohistochemistry findings also revealed significantly lower TGF-β1/Smads-mediated gene transcription activity. These findings suggest that KXL may negatively regulate the TGF-β1/Smads signal pathway to inhibit the occurrence of renal fibrosis.
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**Key words**: Unilateral ureteral obstruction animal model; Kangxianling; Customized gene chip; immunohistochemistry; TGF-β1/smads; Renal fibrosis

### INTRODUCTION

Kangxianling (KXL) granule is a Chinese herbal compound clinically proven to be effective for the treatment of early and mid-stage renal fibrosis via long-term practice at the Department of Nephrology, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine. Many years of clinical practice revealed that KXL has a positive effect in preventing and curing chronic kidney disease\(^1\)\(^{-}\)\(^5\). It can significantly improve renal function and quality of life for patients with chronic kidney disease. A series of studies\(^6\)\(^{-}\)\(^9\) demonstrated that KXL can inhibit the development of chronic kidney disease via systemic therapeutic effects. KXL induces is therapeutic effects by improving proteinuria and lipid metabolism, as well as chronic glomerular sclerosis. KXL can reduce renal fibrosis in animal models, as well as inflammatory cell infiltration and extra-cellular matrix accumulation. Previous findings suggest that KXL has a multi-channel, multi-target, and multi-gene mechanism of action. Based on previous studies, there may be mechanisms by which KXL inhibits renal fibrosis that affect a group of related genes, which may result in a variety of cell function changes that: 1) inhibit tubular epithelial cell (TEC) apoptosis; 2) block fibroblasts and their phenotypic switch into myofibroblasts; 3) inhibit the influx and/or proliferation of lymphocytes/macrophages, fibrocytes (circulating fibroblast precursors), and fibroblasts; and 4) reduce the epithelial-to-mesenchymal transition (EMT) of TECs. A customized array gene chip was used to investigate the mechanisms by which KXL inhibits renal fibrosis in early and mid-stage chronic kidney disease.

### MATERIALS AND METHODS

**Animals**

Eight-week old, male Sprague-Dawley (SD) rats, weighing 180±20 g, were supplied by the Center of Experimental Animals, Shanghai University of Traditional Chinese Medicine. The experimental animal license number was SYXK (HU) 2004-0005 and the certificate number was 0058668. The animals were kept in a specific pathogen-free environment. The experiments were performed according to the institutional guidelines and were approved by the Shanghai University of Traditional Chinese Medicine.

**Renal interstitial fibrosis induced by UUO**

UUO or sham surgery was performed under 3% pentobarbital sodium anesthesia. The left ureter was ligated with 4-0 silk at two points, and then severed between the ligatures to prevent retrograde urinary tract infection. The control rats underwent a sham-operation. The animals were sacrificed one week later via exsanguination through a cardiac puncture under general anesthesia. The left kidneys were harvested and sectioned longitudinally. Half of the kidney was snap frozen immediately for gene analysis, and the other half was formalin-fixed and paraffin-embedded for immunohistochemical analyses.

**Main reagents**

The cell and tissue total protein extraction kit (KangChen, KC-415), BCA protein assay kit (KangChen, KC-430), and biotin-labeled protein molecular weight standards (KangChen, KC-410) were purchased from KangChen (Shanghai, China). The rabbit anti-rat CHIP antibody were purchased from Santa Cruz (CA). The rabbit anti-rat TGF-β1, smad2, smad3, and smad7 antibodies were purchased from Abcam (UK). The horseradish peroxidase (HRP) combination of two antibodies and beta-actin antibody were obtained from the Shanghai Zhaorui Bio-Engineering Company (Shanghai, China).

Reverse transcriptase 2 (RT2), ribonucleic acid (RNA), quantitative competitive (QC), polymerase chain reaction (PCR) Arrays. Tissue from the left kidney underwent RNA isolation, isolation of small quantity RNA, RNA clean up, assessments of RNA yield and quality, concentration and purity measurements, denaturing agarose gel electrophoresis, first strand cDNA synthesis; real-time PCR, and data analysis using the ΔΔΔCt method. Specifically, the ΔCt was calculated for each pathway-focused gene in each experimental group, where ΔCt (group 1) = average Ct - average of HK genes’ Ct in the control group (group 1) array, and ΔCt (group 2) = average Ct - average of HK genes’ Ct in the experimental group (group 2) array. Then, ΔΔCt was calculated for each gene across the two PCR Arrays (or groups) using the following equation: ΔΔCt = ΔΔCt (group 2) – ΔΔCt (group 1), where group 1 is the control and group 2 is the experimental group. The fold-change was calculated for each gene from group 1 to group 2 as 2-ΔΔCt. If the fold-change was greater than 1, the result was reported as a fold up-regulation. If the fold-change was less than 1, the result was reported as a fold down-regulation. The experiment was repeated five times. The criteria adopted were in accordance with international practices. A P-value less than 0.05, and a fold-change greater than 1 or less than 1 were considered statistically significant.

**Immunohistochemical analyses**

A portion of the kidney tissue was excised promptly af-
ter the animals were sacrificed, and immediately placed into 10% neutral-buffered formalin. The tissue was fixed overnight in formalin, processed for paraffin embedding following standard protocols, and then sectioned for antibody staining. Staining for TGF-β1, Smad2, Smad3, and Smad7 was performed after the sections were deparaffinized and hydrated. Heat-induced antigen retrieval was performed using 5 mM of ethylenediaminetetra-acetic acid (EDTA) solution (pH=8.0) and 10 mM of citrate solution (pH=6.0) at 25 psi for 2 min in a decloaking chamber. Non-specific staining was blocked using Sniper (Biocare Medical, Concord, CA, USA) for 9 min. Slides were incubated overnight with TGF-β1, Smad2, Smad3, and Smad7, and then washed and incubated with 3% hydrogen peroxide for 30 min. A HRP polymer detection system and 3, 3’-diaminobenzidine (DAB) substrate were used to tag and stain the first primary antibody brown. The tissue sections were washed in distilled water, counterstained with hematoxylin, dehydrated through an ethanol series, and mounted with cover slips. Cortical staining intensity was scored on a scale of 0-3, where 0 indicated no staining, 1 indicated mild staining, 2 indicated moderate staining, and 3 indicated intense staining for TGF-β1, Smad2, Smad3, and Smad7. Five high magnification fields were evaluated, and the results were expressed as mean±standard error.

Statistical analyses
Data are expressed as mean±SD. Data were analyzed using one-way analysis of variance (ANOVA) test for comparisons, and a P<0.05 was considered statistically significant. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad, San Diego, CA).

RESULTS

Key regulatory genes of renal interstitial fibrosis detected with a customized array
The key regulatory genes included 84 genes that are primarily involved in the dogma signaling pathway related to renal interstitial fibrosis. In the UUO model group, four genes were screened and found to be significant. Specifically, TGF-β1, Smad2, and Smad3, which are responsible for promoting fibrosis, and Smad7, which is responsible for inhibiting fibrosis, were identified. After KXL treatment, TGF-β1, Smad2, and Smad3 were down-regulated by >1.5-fold, whereas Smad7 was up-regulated by >1.5-fold. (Table 1 and Figure 1). Thus, KXL not only down-regulates fibrosis-promoting genes, but also up-regulates fibrosis-inhibiting gene, suggesting that it has multi-target, multi-role characteristics.

Immunohistochemistry
Using a customized array, it is found that the genes involved in renal interstitial fibrosis were TGF-β1, Smad2, and Smad3, which are the fibrosis-promoting genes, and Smad7, which are the fibrosis-inhibiting genes. Additionally, using immunohistochemistry, the protein expression of these genes was consistent with the results of the customized array chip. Differences in TGF-β1 expression between the UUO group and sham-operated group were significant (P<0.01), and differences in TGF-β1 expression between the KXL group and UUO group were also significant (P<0.05). The expressions of the corresponding genes involved in the TGF-β1/Smads signaling pathway regulating genes, specifically, Smad2 and Smad3, were significantly up-regulated in the UUO group. Differences in Smad2 and Smad3 expressions between the UUO group and sham-operated group were significant (P<0.01), and the differences between KXL group and UUO group were also significant (P<0.05). While the expression of the anti-fibrosis gene, Smad7, significantly decreased in the UUO group, it was significantly increased in the KXL group. Differences in Smad 7 expression between the UUO group and sham-operated group was significant (P<0.01), and differences between the KXL group and UUO group were also significant (P<0.01) (Figure 2).

In Table 1 and Figure 1, it is evident that both the KXL and UUO groups demonstrated significant differences in the expression patterns of the four genes. Compared to the UUO group, TGF-β1, Smad2, and Smad3 were down-regulated >1.5-fold, whereas Smad7 was up-regulated 1.5-fold (P<0.01). Comparing the UUO group to the sham-operated group, TGF-β1, Smad2, and Smad3 gene expression increased 1.5-fold, whereas Smad7 was down-regulated >1.5-fold (P<0.01).

<table>
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<th>Genebank accession No.</th>
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<th>Fold up- or down-regulation</th>
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<th>KXL/UUO</th>
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Notes: If the fold change is positive, then the gene was up-regulated. If the fold change was negative, then the gene was down-regulated.
**DISCUSSION**

Unilateral ureteral obstruction fits into the category of kidney collateral disease in TCM, and its pathogenesis is considered to be a result of a deficiency in origin and excess in superficiality. This condition exhibits the in-

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**Figure 1** Comparisons of gene fold up-regulation or down-regulation via a 3D profile and scatter plot.

**Figure 2** Protein expression levels of TGF-β1, Smad2, Smad3, and Smad7. *P*<0.01, UUO vs. Sham; †*P*<0.05, UUO vs. KXL; and ‡*P*<0.05, UUO vs. KXL.
ternal accumulation of dampness, heat, blood stasis, and toxin. Thus, treatment should focus on both the root and branch aspects, supporting healthy qi and removing pathogenic factors. KXL is composed of radix salviae miltiorrhiae (Dan Shen), prepared rhubarb (Zhi-Da Huang), angelica (Dang Gui), Achyranthes (Niu Xi), and peach kernel (Tao Ren). It has obvious curative effects on activating blood and removing blood stasis, reinforcing body resistance, and eliminating poisons to restore the renal function. Miltiorrhiae (Dan Shen), angelica (Dang Gui), achyranthes (Niu Xi), and peach kernel (Tao Ren) are used to strengthen body resistance and promote blood circulation by removing blood stasis, and rhubarb is used for reinforcement and elimination. Previous research confirmed that KXL can inhibit renal tissue TGF-β1 over-expression in rats with chronic renal failure, reduce the accumulation of ECM to improve glomerular sclerosis, and reduce the glomerular cell apoptosis index [4-7]. KXL can also inhibit protein and fiber coupling type IV collagen in renal tissue with excessive sedimentation, improve the glomerular sclerosis index, and decrease angiotensin I and II, laminin, type III collagen, and tumor necrosis factor-α. It can decrease proteinuria and adjust lipid metabolism to suppress renal fibrosis and improve renal function [8-10]. In order to accurately understand the role of KXL, "signal pathway finder" (Pathway Finder) gene chips, designed by the SuperArray Bioscience Corporation, were used. Using customized arrays, it was found that the TGF-β1/Smads signaling pathway was significantly affected by KXL. That is, KXL is capable of regulating TGF-β1, Smad2, Smad3, and Smad7 in EMT and kidney fibrosis.

Using the customized array, it was found that TGF-β1, Smad2, and Smad3 were up-regulated by >1.5-fold, and Smad7 was down-regulated by >1.5-fold in the UUO group. These genes are closely related to the occurrence of renal fibrosis (Figure 1, Table 1). The UUO rat model of renal interstitial fibrosis is mediated via the TGF-β/Smads signaling pathway. Thus, KXL was not only involved in the regulation of fibrosis genes, specifically, TGF-β1, Smad2, and Smad3, but an anti-fibrosis gene, Smad7 [8-10]. In the present study, we found that TGF-β1, Smad2, and Smad3 protein levels were significantly reduced, and Smad7 was significantly increased, in the KXL group (Figure 2). These findings suggest that KXL influences the levels of Smads proteins to regulate the TGF-β signaling pathway, and inhibit renal fibrosis. To further confirm that KXL can regulate TGF-β1, Smad2, Smad3, and Smad7, which play a vital role in anti-fibrosis, we verified TGF-β1, Smad2, Smad3, and Smad7 protein expression via immunohistochemistry. The findings from the immunohistochemistry experiments were consistent with the data from the customized array (Figure 2). Thus, KXL acts via the inhibitory TGF-β signaling pathway to inhibit kidney fibrosis. Our findings are of great importance in the understanding of the molecular mechanisms involved in inhibiting renal fibrosis.

REFERENCES