Tripartite motif-containing 35 (TRIM35) is up-regulated in UUO-induced renal fibrosis animal model

Authors: Yu Chen, Yue Ding and Li-Ming Wang

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Tripartite Motif-Containing 35 (TRIM35) is Up-Regulated in UUO-Induced Renal Fibrosis Animal Model

Running title: TRIM35 is up-regulated in renal fibrosis

Yu Chen, Yue Ding, Li-Ming Wang*
Organ Transplantation Institute of PLA, Chang zheng Hospital, Naval Medical University, Shanghai, China

Corresponding to: Li-Ming Wang
Organ Transplantation Institute of PLA, Chang zheng Hospital, Naval Medical University, Fengyang Road 415, Shanghai 200003, China
Abstract

Renal fibrosis has been recognized as a serious health threat in the world because of the high cost of treatment and poor prognosis. However, the molecular mechanism of renal fibrosis is still largely unknown. In this study, we aimed at illustrating the role of TRIM35 in the renal fibrosis process. A UUO mouse model and a TGF-β1-induced tubulointerstitial fibrosis model were constructed for the research of renal fibrosis at animal and cell level, respectively. Hematoxylin-eosin and Masson staining were used for visualizing the pathological change. qRT-PCR, Western blot analysis and immunohistochemical staining were used to detect the expression of fibrosis-associated proteins and TRIM35. The results showed that, after the modeling, the expressions of α-SMA, Collagen I, Collagen III, Fibronectin and Snail1 were up-regulated, while the expression of E-cadherin was down-regulated, indicating the successful construction of animal and cell models. More importantly, TRIM35 was proved to be up-regulated in both animal and cell models. Therefore, this study demonstrates the potential promotional effect of TRIM35 in the renal fibrosis process, which may prove to be a new biomarker for the diagnosis and development of new treatments of renal fibrosis.

Keywords: Renal fibrosis; TRIM35; EMT; UUO model; TGF-β
Introduction

Renal fibrosis is represented by the increase of stromal cells and especially the promoted synthesis of stromal proteins, and the accumulation of extracellular matrix (ECM) resulting from inhibited degradation, which finally induces glomerular sclerosis and renal interstitial fibrosis under the interaction of various pathogenic factors such as inflammation, injury and drugs (Liu, 2006; Boor et al., 2010; Meng et al., 2014). With the increase in prevalence, the high cost of treatment and poor prognosis, this kind of disease is a serious threat to global public health. Therefore, it is urgent to accelerate the understanding and the development of effective prevention and control strategies for renal fibrosis (Wynn, 2004; Humphreys, 2017). Actually, renal fibrosis is a common histological feature of end-stage renal failure of all chronic progressive renal diseases, which plays a leading role in the process of chronic renal disease and acts as an important factor for evaluating the severity of renal dysfunction and prognosis (Hewitson, 2009; Humphreys, 2017). Renal fibrosis can occur after a variety of renal damage factors such as hypertension, inflammation, high glucose and high lipid concentration, and drug damage, but previous studies found that even after the effective control of these primary causes, the process of renal fibrosis is still difficult to contain (Liu, 2011; Farris et al., 2012). Therefore, it has become an important research direction in recent years to elucidate the molecular mechanism of fibrogenesis and to find a therapeutic target to directly inhibit fibrosis. Actually, the epithelial mesenchymal transition (EMT) which was first proposed by Strutz et al. at 1996 (Strutz et al., 1996), has been recognized as the morphological changes of epithelial cells which causes the expression up-regulation of fibroblast biomarker such as α-smooth muscle actin (α-SMA) thus the promotion of fibrosis process (Tomasek et al., 2002; Rodgers et al., 2003; Kassiri et al., 2009).

The tripartite motif (TRIM) family is also known as RBCC protein. TRIM proteins are highly conserved in structure, including a RING domain, one or two B-Box, and a coiled-coil region (Nisole et al., 2005). At present, more than 70 TRIM molecules have been identified in humans (Ozato et al., 2008). TRIM protein can participate in a variety of cellular biological processes and play a variety of functions, such as cell proliferation, differentiation, development and apoptosis (Nisole et al., 2005). Moreover, emerging evidence shows that a
large number of proteins in the TRIM family can interact with other proteins to form macromolecular complexes and participate in a large number of biological processes (Ozato et al., 2008; Hatakeyama, 2011). As a member in the family of TRIM, TRIM35 (also known as MAIR and HLs5) has been proved to have an apoptosis-inducing function in bone marrow macrophages (Kimura et al., 2003). Besides, previous studies have indicated that TRIM35 was able to act as a tumor suppressor in various malignant tumors such as hepatocellular cancer because of its activity in inhibiting cell proliferation, clonogenicity and tumorigenicity (Wang et al., 2015). Although the effect of TRIM35 in tumors has been studied to some extent, its role in renal fibrosis has never been reported.

Taken together, this study aimed to illustrate the role of TRIM35 in renal fibrosis by using a unilateral ureteral occlusion (UUO) mice model and TGF-β1-induced cell model. The exploration of the effect of TRIM35 on the process of EMT and renal fibrosis could increase the overall understanding of the occurrence and development of renal fibrosis, and lay the foundation to seek accurate treatment targets of renal fibrosis.

Materials and Methods

Materials

The following materials were used: HK-2 cells (Chinese Academy of Sciences Cell Bank), TGF-β1 (Amyjet Sci. Technology Co., Ltd. China), α-SMA antibody (Sigma-Aldrich LLC.), TRIM35 antibody (Abcam, Cat. # ab87169), Snail1 antibody (Sigma-Aldrich LLC.), E-Cadherin antibody (Cell Signaling Technology, Inc. Cat. # 3195s), GAPDH antibody (Bioworld Technology, Inc., Cat. # AG019), Fibronectin antibody (Abcam, Cat. # ab2413), Collagen I and Collagen III antibody (Abcam, Cat. # ab7778, ab34710), β-Actin (Cell Signaling Technology, Inc. Cat. # AF7018).

6- to 8-week-old male C57BL/6 mice were purchased from Shanghai Jake BIO Technology Co. LTD. and divided into two Groups (UUO group and sham operated group) randomly. All mice were housed under standard housing conditions.
**Cell Culture**

HK-2 cells were grown in six-well plates using low glucose DMEM supplemented with 10% FBS at 37°C in humidified 5% CO₂ in air. Cell culture medium was changed every 72 h. The cells were subcultured at 80% confluence using 0.05% trypsin with 0.02% EDTA. The experiment was then performed on cells following a 24 h incubation in DMEM medium without FBS. All experiments were performed under serum-free conditions in which the cells remained viable in a nonproliferating state.

HK-2 cells were inoculated in 24-well culture plates with DMEM medium containing 30 mmol/l glucose and TGF-β1 (0, 2, 5 and 10 ng/mL) and cultured, cell morphology were observed at 1, 3, 8 and 24 h after TGF-β1 were added.

**Unilateral ureteral obstruction model of fibrosis**

All animal experiments performed were approved by the Committee on Ethics of Biomedicine of the Second Military Medical University. Mice underwent ligation of the left ureter during surgery and UUO was performed under aseptic conditions. Before surgery the mice were anesthetized via face mask delivering sevoflurane. The left ureter was exposed through a midline abdominal incision and was completely obstructed 1 cm below the renal pelvis with 5.0 silk with ligated group mice, and sham operated group mice were manipulated similarly but not ligated. Mice regained consciousness quickly under postoperative supervision and were returned to fresh cages. Fourteen days after surgery the kidneys were collected, rinsed with isotonic saline, dissected and stored in liquid nitrogen for further analysis.

**H&E staining**

The kidney tissues were fixed with neutral formalin, sliced up, and then baked at 70°C for 4 h, dewaxed, hydrated in distilled water, stained with hematoxylin (2 min), differentiated in hydrochloric acid alcohol (2 min), blued in ammonia water (5 min), counterstained with eosin (10 s), dehydrated with ethanol at different concentrations (75%, 90% and anhydrous
ethanol), transparentized with xylene I and xylene II (10 min), and finally mounted in neutral gum and were observed under a microscope.

**Masson staining**

The kidney tissues were fixed with neutral formalin, sectioned, deparaffinized in water, stained with Wiegert iron hematoxylin stain for 5 min, washed and differentiated in 1% acid alcohol, then stained with acid fuchsine (8 min), washed (with tap water?), then washed with 5% phosphomolybdic acid (5 min), and stained with bright green staining solution without washing for 5 min, washed with 1% acetic acid solution (1 min), dehydrated in absolute alcohol, transparentized in xylene, and mounted with neutral gum and were observed under a microscope.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was isolated from kidney tissues and culture cells using Trizol® (Thermo Fisher Scientific (China) Co., Ltd.) according to the manufacturer's instructions. After the total RNA was dissolved in ultrapure water pretreated with DEPC, the OD values at 260 nm and 280 nm were detected by Nanodrop 2000/2000C ultraviolet-visible spectrophotometer (Thermo, America) to evaluate the quality of total RNA, followed by the adjustment of RNA concentration. Reverse Transcription Kit (Takara, Dalian, China) was used to synthesize cDNAs. The cDNA was stored in a -20°C freezer. The reaction system for qRT-PCR was prepared following the kit instructions (Fermentas, America). The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, denaturation at 94°C for 10 s, annealing at 60°C for 20 s, extension at 70°C for 20 s, 35 cycles in total. The detection was done using Bio-Rad iQ5 qRT-PCR apparatus (TAKARA BIO INC.). GAPDH was used as the internal reference. The expressions of genes were calculated and $2^{-\Delta\Delta Ct}$ represented relative expression of each target gene. Each experiment was repeated 3 times.
Western blot analysis

The total protein specimens of kidney tissues and cells were extracted and added into 1 × SDS loading buffer. A total of 20 µL loading buffer was subjected to 12% polyacrylamide gel electrophoresis, transferred to membrane and blocked in decolorizing shaking table by Tris-Buffered Saline and Tween 20 (TBST) containing 5% bovine serum albumin (BSA) at room temperature for 1 h. Then the confining liquid was removed, the membrane was placed into a plastic groove, and antibodies at certain concentrations prepared with 5% BSA were added, including α-SMA, Snail1, E-cadherin, TRIM35 and GAPDH. The impression surface was put on the front, and the membrane was stored in a 4°C freezer overnight. Next day, the membrane was washed with TBST 3 times (10 min each time), incubated at 4°C for 4 ~ 6 h with the addition of diluted second antibody (Abcam Inc., Cambridge, UK), and finally washed in TBST again (3 × 15 min). Chemiluminescence reagent A and reagent B (Santa Cruz Biotech, Santa Cruz, USA) were mixed at a proportion of 1:1 and dropwise dripped on the nitrocellulose (NC) membrane. The protein bands were developed by developing solution. Relative OD analysis was performed in all Western blotting bands.

Immunohistochemical staining

The expression of TRIM35 was detected by immunohistochemistry in both kidneys of UUO mice model. Tissue sections from the mice were deparaffinized. After citrate antigen repair and blocking, the samples were incubated with the TRIM35 antibody (1/200, abcam, # ab87169) at 4°C overnight in an incubator. Tissue sections were stained with DAB, and again stained with hematoxylin. Images were captured using a photomicroscope and analyzed.

Statistical analysis

The data were expressed as mean ± SD and analyzed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). T-test were used to compare the differences. A p value < 0.05 was considered statistically significant.
Results

TRIM35 was up-regulated in UUO mice model

In order to investigate the role of TRIM35 in the development of renal fibrosis, a UUO mice model, which is a widely used model for the research of renal fibrosis, was constructed. As shown in Fig. 1A, the kidney subjected to UUO modeling exhibited distinct enlargement compared with either the mice in sham group or the right kidney of the same mouse in UUO group. Subsequently, hematoxylin-eosin (H&E) and Masson staining were utilized to visualize the pathological features of the treated kidney after UUO modeling (Fig. 1B). After 14 days of UUO modeling, the left renal tubules showed moderate dilatation, atrophy of some distal renal tubules, lumen occlusion, tubular epithelial cell swelling and degeneration, and renal interstitial cells were significantly donated, in which a large number of fibrous tissue proliferation and renal interstitial fibrosis were observed. Moreover, compared with the corresponding right kidney, the results of Masson staining showed renal tubular atrophy, with dilatation of renal tubular lumen, renal interstitial enlargement, renal interstitial inflammatory cells increased and there was obvious deposition of blue collagen fiber. All the above results indicated the successful construction and the typical pathological features of the UUO mice model. Next, we detected the mRNA expression of various fibrosis biomarkers, including cytoskeletal protein such as α-SMA, ECM ingredients such as Collagen I and Collagen III, mesenchymal markers such as Fibronectin, and transcription factors such as Snail1, and TRIM35. The results showed distinctly up-regulated expression of α-SMA, Collagen I, Collagen III, Fibronectin, Snail1 and TRIM35, which was consistent with the successful construction of UUO modeling and indicated the up-regulated expression of TRIM35 during the fibrosis process (Fig. 2A). Accordingly, the immunohistochemical staining also revealed up-regulated TRIM35 expression in the UUO treated kidney (Fig. 2B). More importantly, Western blot analysis exhibited the up-regulation of α-SMA and Snail1 which was consistent with the PCR results, and the down-regulation of E-cadherin which is a well-known epithelial biomarker (Fig. 3). Combined with the above results, the increase of protein expression of TRIM35 detected by Western blot analysis indicated the potential promotional role of TRIM35 in EMT and renal fibrosis process.
TRIM35 was up-regulated in TGF-β1 treated HK-2 cell model

To further verify the results obtained by the animal model, a cellular fibrosis model was constructed by treatment of TGF-β1 based on Human renal tubular epithelial HK-2 cells. The changes in cellular morphology were observed by microscopy after the treatment of TGF-β1 with various concentrations, which exhibited loose contact between cells and elongated to spindle shape, indicating that the cells have been or are being transformed into fibroblasts (Fig. 4). Subsequently, the results of RT-PCR and Western blot analysis revealed the up-regulated expression of α-SMA, Collagen I, Collagen III, Fibronectin, Snail1 and down-regulation of E-cadherin upon the treatment of TGF-β1 (2 ng/mL), which were in accordance with the results from UUO animal model and proved the successful construction of the cell model (Fig. 5). Notably, it was also demonstrated that both the mRNA and protein expression of TRIM35 were up-regulated in the TGF-β1 treated HK-2 cells. Collectively, the cellular study proved the up-regulated expression and possible promotion effect of TRIM35 in EMT and renal fibrosis.

Discussion

Renal fibrosis is one of the major causes of end-stage renal failure, and all chronic kidney diseases eventually develop into renal fibrosis. Therefore, how to effectively prevent and treat renal fibrosis has become an important problem encountered by nephrologists. EMT is the basic physiological mechanism of embryonic development and tissue repair, and it is also an important pathological mechanism of tissue fibrosis and tumor metastasis (Iwano, 2010; Carew et al., 2012). The EMT process of renal tubular epithelial cells and podocytes is an important pathological process to promote renal fibrosis (Yao, 2011). A large number of studies on the mechanisms of EMT and renal fibrosis have found that, among many cytokines, humoral, metabolic and hemodynamic factors, TGF-β and Smad signaling pathway mediated by EMT play very important roles in renal fibrosis, and are closely related to cell proliferation, activation, migration and extracellular matrix synthesis (Iwano, 2010; Wei et al., 2013). TGF-β over-expression can cause a series of changes and eventually mediate the transformation of
renal tubular epithelial cells into mesenchymal cells (EMT) (Yang et al., 2001; Siegel et al., 2003). At present, although most of the treatments for renal fibrosis are still in the experimental stage, it was found that the mechanism of some effective drugs against renal fibrosis is anti-TGF-β (Gross et al., 2004). However, the prognosis of renal fibrosis is still poor because of the late diagnosis. Therefore, the exploration of more biomarkers for EMT and renal fibrosis is of great significance for patients with chronic kidney disease or renal fibrosis.

TRIM35 is a member of the tripartite motif-containing family which has been proved to possess apoptosis-inducing function (Kimura et al., 2003). However, it should be noted that a majority of studies on TRIM35 mainly focus on the field of malignant tumors. For example, He et al. reported that TRIM35 can interact with pyruvate kinase isoform M2 (PKM2) with the involvement of the coiled-coil domain (Chen et al., 2015). They also demonstrated that the coiled-coil domain induced the decreases in the Warburg effect and cell proliferation ability of hepatocellular cancer (HCC) cells. Accordingly, TRIM35 was defined as a tumor suppressor in HCC. Moreover, He et al. further indicated that the PKM2/TRIM35 combination was an independent and significant risk factor for recurrence and survival in HCC, and can be a biomarker for the treatment against HCC (Chen et al., 2015). Wang et al. demonstrated that miR-4417 promoted cell proliferation and inhibited cell apoptosis of HCC cells through the regulation of PKM2 phosphorylation by targeting TRIM35 (Song et al., 2017). Besides, studies of Sun et al. revealed the negative role of TRIM35 in the regulation of Toll-like receptor (TLR)7/9-mediated interferon (IFN) production during the immune response induced by DNA virus infection (Wang et al., 2015). To the best of our knowledge, the effect of TRIM35 in EMT and renal fibrosis is still unclear and rarely reported, which is why it was selected as the aim of this study.

In this study, in order to investigate the effect of TRIM35 in EMT and renal fibrosis, we constructed a UUO mice model, which is the most widely used experimental animal model for the study of renal fibrosis (Eleni et al., 2016). It causes obstruction of the renal drainage system by ligating the unilateral ureter, resulting in changes in acute renal function and chronic renal structural damage, eventually leading to renal fibrosis and renal failure (Klahr et
al., 2002; Sun et al., 2017). In our study, the up-regulated expression of α-SMA, Collagen I, Collagen III, Fibronectin, Snail1 and the down-regulation of E-cadherin proved the successful construction of a UUO mice model. Among the proteins, Snail1, which belongs to the Snail superfamily, is a transcription factor containing zinc finger protein. Its main function is to down-regulate epithelial markers such as E-cadherin, up-regulate mesenchymal markers such as Fibronectin, and regulate matrix metalloproteinases, such as MMP1 and MMP2 (Lin et al., 2008; Humphreys et al., 2010). More importantly, the up-regulated mRNA and protein expression of TRIM35 in the UUO group compared with the sham group indicated its potential role in the renal fibrosis process.

Accumulating evidence has proved that TGF-β is a key mediator in the induction of progressive renal failure in a variety of kidney injuries, and it is considered to be a strong stimulant for renal fibrosis. TGF-β1 is the most widely expressed and studied subtype in the TGF-β family and is recognized as the most important profibrotic factor (Vega et al., 2016; Yuen et al., 2016). Active TGF-β1 is a key regulator of fibrosis by inducing EMT and apoptosis. Therefore, in this work, we utilized the TGF-β1-induced tubulointerstitial fibrosis model as the research object at the cellular level in vitro. Similar to the results of the animal model, the same trend in the expression changes of fibrosis-associated proteins and the observed morphological change of HK-2 cells proved the successful construction of the cell model. Again, the up-regulation of TRIM35 in the TGF-β1 treated cells suggested the promotion effect of TRIM35 in EMT and renal fibrosis process. Despite all the results, a more detailed mechanism of the effect of TRIM35 on EMT and renal fibrosis is still not clear, and thus will be the focus of our future work.

In conclusion, this study showed that TRIM35 was up-regulated in a UUO mice model and TGF-β1 induced tubulointerstitial fibrosis model, and may act as a promoter during the EMT and renal fibrosis process. This study can provide a new molecular marker for the study of renal fibrosis, and provide a new basis for the diagnosis and the development of new treatment of renal fibrosis.
Acknowledgements

Not applicable.

Conflicts of interest

The authors declare no conflicts of interest.
References:


Figure legends

Figure 1. Construction of UUO mice model. (A) The images of the kidneys removed from mice of sham group and UUO group. (B) After 14 days of UUO modeling, H&E and Masson staining were used to visualize the pathological features of the treated kidney after UUO modeling.

Figure 2. mRNA expression of related proteins after UUO modeling. (A) qRT-PCR was used to detect the mRNA expression levels of α-SMA, Collagen I, Collagen III, Fibronectin, Snail1 and TRIM35. (B) Immunohistochemical staining was used to detect the expression of TRIM35 in kidney tissues. Data are presented as the mean ± SD. *P<0.05, **P<0.01.

Figure 3. Change of the expression level of related proteins after UUO modeling. Western blot analysis was used to detect the protein expression levels of α-SMA, Snail1, E-cadherin and TRIM35.

Figure 4. Morphology change of HK-2 cells after treatment of TGF-β1. The morphological change of HK-2 cells was observed after the treatment of TGF-β1 with various concentrations.

Figure 5. mRNA and protein expression of related proteins in HK-2 cells after TGF-β1 treatment. qRT-PCR and Western blot analysis were used to detect the mRNA and protein levels of α-SMA, Collagen I, Collagen III, Fibronectin, Snail1, E-cadherin and TRIM35 in HK-2 cells treated with 2 ng/mL TGF-β1. Data are presented as the mean ± SD. *P<0.05, **P<0.01.