Long Non-Coding RNA (LncRNA)-ATB Promotes Inflammation, Cell Apoptosis and Senescence in Transforming Growth Factor-β1 (TGF-β1) Induced Human Kidney 2 (HK-2) Cells via TGFβ/SMAD2/3 Signaling Pathway

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Background: Renal fibrosis occurs in the end-stage of all chronic kidney disease. Transforming growth factor-β1 (TGF-β1) is a central contributor in fibrosis. Identifying effective biomarkers that targets TGF-β1 is necessary for the development of therapeutic agents for kidney disease. In this study, we investigated the effects and mechanism of long non-coding RNA (LncRNA)-ATB in TGF-β1 induced human kidney 2 (HK-2) cells.

Material/Methods: We investigated the effects of either overexpression or knockdown of LncRNA-ATB on inflammation, cell apoptosis, and senescence in TGF-β1 induced HK-2 cells. TGF-β1 induced HK-2 cells served as the cell model. The gene level was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) and protein expressions by western blot. Cell Counting Kit-8 (CCK-8) assay was performed for assessment of cell viability. Flow cytometry was applied for detection of cell apoptosis. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 were measured by corresponding kits.

Results: LncRNA-ATB was highly expressed in TGF-β1 induced HK-2 cells. Inflammation, cell apoptosis, and senescence were enhanced by TGF-β1 and these effects were all reduced by knockdown of LncRNA-ATB. Whereas overexpression of LncRNA-ATB had the opposite effects with knockdown of LncRNA-ATB. The TGFβ/SMAD2/3 signaling pathway was activated by TGF-β1 and this effect was further enhanced by LncRNA-ATB overexpression. Silencing LncRNA-ATB inhibited the TGFβ/SMAD2/3 signaling pathway in TGF-β1 induced cells. The effects of LncRNA-ATB overexpression aforementioned in TGF-β1 induced cells were abolished by blockage of the TGFβ/SMAD2/3 signaling pathway.

Conclusions: LncRNA-ATB overexpression have promoting effects on inflammation, cell apoptosis and senescence in TGF-β1 induced HK-2 cells via activating the TGFβ/SMAD2/3 signaling pathway. LncRNA-ATB act as a key downstream mediator via activating the TGFβ/SMAD2/3 signaling pathway and silencing LncRNA-ATB might be a new strategy for chronic kidney disease treatment.

MeSH Keywords: Apoptosis • Cell Aging • Inflammation • RNA, Long Noncoding

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Background

Kidney diseases have been increasing gradually and have become a health burden across the world. Over 10% of the population suffers from kidney diseases [1,2]. Current treatment is only effective in delaying kidney disease progression without the use of radical treatment and the survival rate is very poor [3,4]. Inflammation and cell apoptosis have been confirmed as the major factors that contribute to kidney diseases. Furthermore, inflammation in kidney disease has been confirmed to activate the apoptosis signaling pathway and trigger increased cell apoptosis [5,6]. Discovering therapeutic targets that have inhibiting effects on inflammation and cell apoptosis might be a promising kidney disease treatment.

In addition, chronic kidney disease is considered a form of renal ageing. Renal ageing leads to renal hypofunction, glomerular sclerosis, glomerular basement membrane thickening, interstitial fibrosis, vascular sclerosis, and capillary loop collapse [7–9]. Renal ageing plays a vital role in kidney disease, and once kidney disease occurs, there is no effective treatment to reverse it. Targeting of senescent cells (senotherapy) is a promising avenue for prevention and treatment of kidney disease. Identifying therapeutic targets in cell senescence and illuminating the corresponding mechanism is of great significance for prevention and treatment of kidney disease.

Non-coding RNAs with 200 nucleotides are defined as long non-coding RNAs (LncRNAs) [10]. Nowadays, many LncRNAs have been confirmed as promising biomarkers and therapeutic targets involved in many diseases such as cancer, cardiovascular disease, neurodegenerative disease, respiratory disease, and so forth [11–16]. Identifying LncRNAs that target senescent cells, inflammation, and apoptosis might be a good strategy for prevention and treatment of kidney disease. Transforming growth factor-β1 (TGF-β1) has been confirmed to be a vital factor contributing to inflammation, apoptosis, and cell senescence [17,18]. A promising strategy would be to find the LncRNA that is closely related to TGF-β1. LncRNA-ATB (a LncRNA activated by transforming growth factor-β1) has been confirmed to be involved in hepatic fibrosis or pulmonary fibrosis [19,20]. LncRNA-ATB has also been confirmed to be upregulated by TGF-β1. LncRNA-ATB has also been reported to be upregulated in renal cell carcinoma and associated with poor prognosis [21]. LncRNA-ATB has been reported to contribute to renal cell carcinoma via promoting cell migration, metastases, and invasion [22]. In addition, LncRNA-ATB is involved in renal tubular epithelial-mesenchymal transition induced by TGF-β1 [23]. We speculated that LncRNA-ATB might play a certain role in cell senescence, inflammation, and apoptosis of the kidney.

In this research, TGF-β1 induced human kidney 2 (HK-2) cells served as the cell model and we further investigated the effects and possible mechanism of LncRNA-ATB in TGF-β1-induced HK-2 cells.

Material and Methods

Cell culture, treatment and transfection

HK-2 cells (ATCC® CRL-2190™) were cultured in Dulbecco’s medium supplemented with 10% fetal bovine serum (FBS), L-glutamine, bovine pituitary extract (BPE), and epidermal growth factor (EGF) and placed in an incubator containing 5% CO₂. The cells were cultured in 96-well plates at a density of 1×10⁴ for 24 hours and then the cells were treated with TGF-β1 (10 ng/mL) for 12 hours, 24 hours, and 48 hours, respectively. pcDNA-NC, pcDNA-LncRNA-ATB, ShRNA-NC, ShRNA-LncRNA-ATB-1, and ShRNA-LncRNA-ATB-2 were purchased from GenePharma (Shanghai, China). The cells after treatment with TGF-β1 were transfected with pcDNA-NC, pcDNA-LncRNA-ATB, ShRNA-NC, ShRNA-LncRNA-ATB-1, and ShRNA-LncRNA-ATB-2, respectively according to the manufacturer’s protocol, using Lipofectamine 2000 reagent (Invitrogen).

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

TRIzol reagent was used for isolation of the total RNA and high capacity RNA-to-cDNA Kit was used for reverse transcription into cDNA. QuantNovo SYBR Green PCR Kit was used for gene amplification for PCR assay. The primers for LncRNA ATB were: ACAAGCTGTGACGTCTCAGG (forward), CTAGGCCCAAAGACAATGGA (reverse). GAPDH and U6 served as the internal references. The conditions for PCR were as follows: 95°C for 30 seconds for predegeneration, subsequently, degeneration was performed under 40 cycles of 95°C, 5 seconds and 60°C, 30 seconds for annealing. The relative LncRNA-ATB level was determined by using 2–ΔΔCT method.

Cell Counting Kit-8 (CCK-8) assay

Cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assay. The cells were seeded in 96-plates and treated with TGF-β1 10 ng/mL for 12 hours, 24 hours, and 48 hours. Then the cells of different groups were incubated with CCK-8 agent (10 µL). The cell viability was determined by the optical density at 450 nm via using a microplate reader.

Assessment of cell apoptosis

The cells (1.0×10⁵ cells/mL) were plated in 6-well plates and the cells after treatments in different groups were harvested. After the cells were resuspended in buffer solution (500 µL), FITC-Annexin-V and propidium iodide (5 µL) were added into
the cells for 15 minutes in the dark, at room temperature. The cell apoptosis of different groups was evaluated by flow cytometry (BD Biosciences).

Measurement of inflammation level

The cells, after treatment in different groups, were lysed and the supernatants were collected. The levels of tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6 were detected by the corresponding kits. The whole detection process was performed according to the instructions of the manufacturers.

Western blot

The cells, after treatment, were collected and lysed in RIPA lysis buffer. Then, the cell lysates of different groups were collected. Total protein was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Then the membranes, after blocking with 5% dry nonfat milk, were incubated with the following primary antibodies: VCAM-1 (#ab174279, Abcam), sE-selectin (Anyan, Shanghai), bcl2 (#ab182858,Abcam), pSmad2 (ab216482, Abcam), pSmad3 (#ab52903, Abcam), TGF-β3 (#ab32389, Abcam), p21 (#ab109520, Abcam), p16 (#ab51243, Abcam), TGF-βRI (#ab121024, Abcam), TGF-βRII (#ab186838, Abcam), Smad2 (#ab33875, Abcam), Smad3 (#ab40854, Abcam), pSmad2 (ab216482, Abcam), and pSmad3 (#ab52903, Abcam). Thereafter, the membranes were incubated with the secondary antibody goat anti-rabbit IgG H&L (Alexa Fluor® 488), #ab150077). Image Lab™ Software (Bio-Rad, Shanghai, China) was applied for quantifying the intensity of the bands.

Statistical analysis

SPSS 20.0 software (IBM, CA, USA) was used for data analysis. The results from 3 individual experiments are presented as mean ± standard deviation. Comparison between groups were performed using one-way or two-way ANOVA. P<0.05 was considered a significant difference.

Results

LncRNA-ATB was highly expressed in TGF-β1 induced cells

As seen in Figure 1A, the LncRNA-ATB level was elevated by TGF-β1 significantly in a time-dependent manner in contrast to the control group, indicating that LncRNA-ATB might play a vital role in TGF-β1 induced cells. The cell viability was decreased by TGF-β1 in a time-dependent manner in comparison to the control group, suggesting that TGF-β1 was a vital contributor to cell apoptosis (Figure 1B).

Overexpression of LncRNA-ATB contributed to cell apoptosis and knockdown of LncRNA-ATB reduced cell apoptosis in TGF-β1 induced cells

To verify the overexpression and knockdown effect of LncRNA-ATB, PCR was performed. As presented in Figure 2A, compared with the control, the LncRNA-ATB level was higher in TGF-β1 induced cells, which was consistent with our results shown in Figures 1A and 2B. Furthermore, the LncRNA-ATB level was higher in LncRNA-ATB overexpression group than that in the TGF-β1 induced group, confirming that LncRNA-ATB overexpression effect was achieved. As shown in Figure 2B, the LncRNA-ATB level was decreased dramatically by ShRNA-LncRNA-ATB-1, therefore, ShRNA-LncRNA-ATB-1 was used for knockdown of LncRNA-ATB. We further evaluated the effects of LncRNA-ATB on cell apoptosis in TGF-β1 induced cells (Figure 2C, 2D). Consistent with Figure 1B, the cell apoptosis was increased by TGF-β1 when compared with the control. As seen evidently in Figure 2C, overexpression of LncRNA-ATB
promoted cell apoptosis and knockdown of LncRNA-ATB inhibited cell apoptosis in TGF-β1 induced cells. The findings demonstrated that LncRNA-ATB was a vital biomarker in TGF-β1 induced cell apoptosis.

**Overexpression of LncRNA-ATB elevated senescence-associate proteins and decreased the anti-apoptosis protein, while, the results for knockdown of LncRNA-ATB was the opposite in TGF-β1 induced cells**

We further evaluated the effects of LncRNA-ATB on apoptosis-related proteins and senescence-associate proteins in TGF-β1 induced cells (Figure 3). We found that the bcl2 was downregulated and senescence-associate proteins including p53, p21, and p16 were upregulated by TGF-β1 compared to the control, demonstrating that TGF-β1 contributed to cell apoptosis and senescence. Furthermore, after overexpression of LncRNA-ATB, the effects of TGF-β1 on bcl2, p53, p21, and p16 were promoted and after knockdown of LncRNA-ATB, the opposite results were found. These results confirmed that LncRNA-ATB played a vital role in TGF-β1 induced cell apoptosis and senescence.

**Overexpression of LncRNA-ATB increased the levels of inflammatory factors and adhesion factors, while, the opposite results for knockdown of LncRNA-ATB in TGF-β1 induced cells**

TGF-α, IL-1β, and IL-6 are vial inflammatory factors and adhesion factors including VCAM-1 and E-selectin closely relate to inflammation are upregulated under the inflammation stimulation. As seen in Figures 4 and 5, inflammatory factors and adhesion factors were upregulated by TGF-β1 versus the control, indicating that TGF-β1 contributed to inflammation. Overexpression of LncRNA-ATB had promoting effects on inflammatory factors and adhesion factors induced by TGF-β1. Furthermore, after knockdown of LncRNA-ATB, the effects of TGF-β1 on inflammatory factors and adhesion factors were reduced. These results confirmed that LncRNA-ATB played a pivotal role in TGF-β1 induced inflammation.

**Overexpression of LncRNA-ATB activated TGF-β1/Smad2/3 signaling pathway and knockdown of LncRNA-ATB had the opposite effects with LncRNA-ATB overexpression in TGF-β1 induced cells**

Since TGF-β1 plays a vital role in the TGF-β1/Smad2/3 signaling pathway, in this study, we further evaluated the effects of LncRNA-ATB on the TGF-β1/Smad2/3 signaling pathway in TGF-β1 induced cells. The results demonstrated that TGF-β1...
activated the TGF-β1/SMAD2/3 signaling pathway compared with the control and this effect was further enhanced by overexpression of LncRNA-ATB (Figure 6). By contrast, the knockdown of LncRNA-ATB inhibited the effects of TGF-β1 on the TGF-β1/SMAD2/3 signaling pathway. Taken together, all the findings indicated that effects of LncRNA-ATB in TGF-β1 induced cells might be through the TGF-β1/SMAD2/3 signaling pathway.

The effects of LncRNA-ATB on inflammation, cell apoptosis, as well as senescence in TGF-β1 induced cells was achieved via the TGFβ1/SMAD2/3 signaling pathway

We further investigated the relation between the TGFβ1/SMAD2/3 signaling pathway and the effects of LncRNA-ATB in TGF-β1 induced cells. As seen in Figure 7, LncRNA-ATB overexpression...
activated the TGF-β1/SMAD2/3 signaling pathway and this effect was further inhibited by TGF-β1 receptor inhibitors (LY 3200882), indicating that after LncRNA-ATB treatment, LY 3200882 was still an effective inhibitor of the TGFβ/SMAD2/3 signaling pathway. After blocked the TGFβ/SMAD2/3 signaling pathway, whether overexpression of LncRNA-ATB still contributed to inflammation, cell apoptosis, as well as senescence or not in TGF-β1 induced cells was further investigated. As seen in Figures 8–11, consistent with the results aforementioned, the inflammation, cell apoptosis, as well as senescence were all enhanced by overexpression of LncRNA-ATB compared to the TGF-β1 group. In addition, after the TGFβ/SMAD2/3 signaling pathway was blocked, the effects of LncRNA-ATB overexpression on inflammation, cell apoptosis, as well as senescence were abolished. The results presented herein strongly support that LncRNA-ATB contributed to inflammation, cell apoptosis,
Discussion

TGF-β1, as an abundant member in TGF-β superfamily, exists in all infiltrated inflammatory cells and renal cells. Accumulative evidence has confirmed that TGF-β1 plays a central role in inflammation, apoptosis, and senescence [18,24–26]. The profibrotic activity of TGF-β1 is well known and TGF-β1 is considered a common inducer in end-stage of renal fibrosis. The study on the mechanism of TGF-β1 as involved in renal diseases is needed to inform effective diagnosis and therapeutic methods.

Furthermore, LncRNA has been reported to play a dynamic and crucial role in different pathologic conditions. In this study, we found that LncRNA-ATB contributed to inflammation, cell apoptosis, and senescence in TGF-β1 induced HK-2 cells via the TGFβ/SMAD2/3 signaling pathway.

TGF-β1 is an important factor that induce tissue fibrosis in many organs [27]. TGF-β1 is capable of inducing cell apoptosis in human renal cells [18,28]. TGF-β1 induced HK-2 cells were used as the cell model in this study. Consistent with previous studies, we found that the cell viability was reduced by TGF-β1 in a time dependent manner [18,28]. In addition, LncRNA-ATB was reported to be upregulated by TGF-β1 in papillary thyroid carcinoma cells [29]. In this study, the LncRNA-ATB level

as well as senescence in TGF-β1 induced cells through the TGFβ/SMAD2/3 signaling pathway.
was elevated by TGF-β1 in a time dependent manner, indicating that LncRNA-ATB might play a central role in TGF-β1 induced HK-2 cells.

We then further evaluated the effects of LncRNA-ATB in TGF-β1 induced HK-2 cells. Apoptosis and inflammation are 2 known major contributors in kidney disease. TGF-β1 is the vital contributor of inflammation and apoptosis in kidney disease [24]. In this study, both inflammation and apoptosis were elevated by TGF-β1 in HK-2 cells. Furthermore, this effect was enhanced by LncRNA-ATB overexpression and inhibited by knockdown of LncRNA-ATB. Silencing LncRNA-ATB might be an effective
It is activated by TGF-β1 in renal disease. The TGF-β/SMAD2/3 signaling pathway and has a pro-fibrotic role in kidney disease [38]. The TGF-β/SMAD2/3 signaling pathway, the link between LncRNA-ATB and cellular senescence in TGF-β1 induced cells. The findings in this study provide a new direction for prevention and treatment of kidney disease, providing a reference for future researches.

Conclusions

TGF-β1 has been confirmed as a promising therapeutic target in kidney disease. In the current research, we found that LncRNA-ATB was a critical biomarker in TGF-β1 induced cells. LncRNA-ATB was proven to promote inflammation, cell apoptosis, and senescence in TGF-β1 induced cells; and thus LncRNA-ATB might be a promising therapeutic target in kidney disease treatment.

Cellular senescence has been confirmed to possess acceleration effects on renal fibrosis and renal disease progression [30–32]. TGF-β1 has been confirmed to induce cell senescence in a variety of types of cells [25,33,34]. It is reported that p53, p21, and p16 are the pro-senescence factors that induced cell senescence [35–37]. In this research, the cellular senescence related proteins, including p53, p21, and p16, were increased by TGF-β1. In addition, silencing LncRNA-ATB blocked this effect and LncRNA-ATB overexpression of LncRNA-ATB still had promoting effects on inflammation, cell apoptosis, and senescence in TGF-β1 induced HK-2 cells.

Since TGF-β1 is an important protein in the TGF-β/SMAD2/3 signaling pathway, the link between LncRNA-ATB and TGF-β1 receptor activation, Smad proteins, as downstream mediators, are phosphorylated, and after a series of events, regulate gene expression by translocating into the nucleus [38]. The TGF-β/SMAD2/3 signaling pathway is a pivotal signaling pathway and has a pro-fibrotic role in kidney disease. The TGF-β/SMAD2/3 signaling pathway is elevated in polycystic kidney disease [39]. It is activated by P311 in renal fibrosis [40]. In this research, overexpression of LncRNA-ATB activated the TGF-β1/SMAD2/3 signaling pathway, whereas, silencing LncRNA-ATB had the opposite effects, indicating that the effects of LncRNA-ATB in TGF-β1 induced cells might be realized through the TGF-β1/SMAD2/3 signaling pathway.

In order to further verify the effects of LncRNA-ATB, we blocked the TGF-β1/SMAD2/3 signaling pathway to investigate whether overexpression of LncRNA-ATB still had promoting effects on inflammation, cell apoptosis, and senescence in TGF-β1 induced cells or not. We found that after blockage of the TGF-β1/SMAD2/3 signaling pathway, the effects of LncRNA-ATB on inflammation, cell apoptosis, and senescence in TGF-β1 induced cells was abolished, demonstrating that the effects of LncRNA-ATB are achieved by activating the TGF-β1/SMAD2/3 signaling pathway.
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