LncRNA np_5318 promotes renal ischemia-reperfusion injury through the TGF-β/Smad signaling pathway

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Abstract. Long noncoding (Lnc)RNA np_5318 has been proved to be involved in renal injury, while its functionality in renal ischemia-reperfusion (I/R) injury is unknown. Therefore, the present study aimed to investigate the role of LncRNA np_5318 in the development of renal I/R injury. Renal I/R injury model and I/R cell model were established \textit{in vitro}. The expression of np_5318 in I/R cell was inhibited by small interfering (si)-np_5318 and increased by pc-np_5318. Renal function was detected and evaluated by automatic biochemical tests. Immunohistochemical staining was performed to detect the expression cluster of differentiation (CD)31, transforming growth factor (TGF)-β1, Smad3 and phosphorylated (p)-Smad3 in renal tissue and renal cells. Expression of np_5318 in renal tissue and renal cells was detected by reverse transcription-quantitative PCR. Relative cell viability was confirmed by MTT assay. Renal function was impaired and pathological changes in renal tissue were observed in the renal I/R injury group, indicating the renal I/R injury model was successfully established. Compared with the sham group, the expression level of np_5318 significantly increased in the renal I/R injury group. ChIP data confirmed the interaction between np_5318 and Smad3 was verified by chromatin immunoprecipitation (ChIP). Western blotting was performed to detect the expression levels of TGF-β1, Smad3 and phosphorylated (p)-Smad3 in renal tissue and renal cells. Expression of np_5318 in renal tissue and renal cells was detected by reverse transcription-quantitative PCR. Relative cell viability was confirmed by MTT assay. Renal function was impaired and pathological changes in renal tissue were observed in the renal I/R injury group, indicating the renal I/R injury model was successfully established. Compared with the sham group, the expression level of np_5318 significantly increased in the renal I/R injury group. ChIP data confirmed the interaction between np_5318 and Smad3. The expression of TGF-β1, Smad3 and p-Smad3 in renal tissue was also significantly increased in the renal I/R injury group. Furthermore, the I/R cell model \textit{in vitro} was successfully constructed and np_5318 in I/R group was significantly increased compared with the control group. Cell growth was significantly suppressed in the I/R group compared with the control group. Additionally, transfection with pc-np_5318 significantly inhibited cell growth of I/R cells at 48 and 72 h. While inhibition of np_5318 by si-np_5318 significantly increased the cell growth of I/R cells at 48 and 72 h. Moreover, the level of TGF-β1, p-Smad3 and Smad3 was significantly increased in the I/R group compared with the control group, and transfection with pc-np_5318 significantly increased the level of TGF-β1, p-Smad3 and Smad3. While inhibition of np_5318 by si-np_5318 significantly suppressed the level of TGF-β1, p-Smad3 and Smad3.

LncRNA np_5318 may participate in the development of renal I/R injury through TGF-β1/Smad signaling pathway.

Introduction

Acute renal failure (ARF) of the kidney is an important cause of morbidity and mortality in hospitalized intensive care unit patients. Renal ischemia is a major cause of ARF, initiating a complex and interrelated sequence of events, resulting in injury and the eventual death of renal cells (1,2). The prognosis is complicated by the fact that reperfusion, although essential for the survival of ischemic renal tissue, causes additional damage (reperfusion-injury) (3), contributing to the renal dysfunction and injury associated with ischemia/reperfusion (I/R) of the kidney (4). I/R injury is an inevitable consequence of the procedure of kidney transplantation and has a negative impact on both short- and long-term graft survival (5-7). The pathophysiology of I/R injury is complex, with at least three major components contributing to the process of reperfusion injury: Molecular oxygen, neutrophils and components of the activated complement cascade (8-10).

Long noncoding RNA (LncRNA) is a group of non-coding RNAs with a length longer than 200 nucleotides (11). LncRNAs are not associated with protein synthesis and have previously been considered to be ‘noise’ of the transcriptome (11). However, in recent years, LncRNAs have been indicated to regulate the expression of protein-coding genes to participate in a number of pathological and physiological processes (11). It has been well established that LncRNAs are associated with the progression of a variety of human diseases, including renal ischemia-reperfusion injury, by regulating the expression of genes associated with the development and progression of the disease (12). LncRNA np_5318 has been proved to be closely correlated with the development of different kidney diseases such as transforming growth factor (TGF)-β3-mediated renal inflammation (13), which is common in renal ischemia-reperfusion (14),

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indicating that np_5318 may also be involved in the progression of renal ischemia-reperfusion injury. However, the role of np_5318 in renal I/R injury is still unknown.

The current study aimed to explore the expression and function of np_5318 in renal I/R and investigate the potential interaction between the np_5318 and TGF-β/Smad signaling pathway in this disease. In the present study, the roles of np_5318 in renal I/R were explored by establishing I/R animal and cell models and the related mechanism was also verified using transfection experiments. It was demonstrated that lncRNA np_5318 may participate in the development of renal I/R injury through the TGF-β/Smad signaling pathway.

Materials and methods

Animal, grouping and model construction. Specific pathogen free grade female Balb/C mice (n=20; age, 3 weeks; weight, 20±5 g) were obtained from Jinan Jinfeng Experimental Animal Breeding Co., Ltd., [license number: SCXK (lu) 2014-0006]. Animals were reared under specific pathogen-free conditions (12 h dark/light cycles; 25°C) with 95% humidity with access to food and water ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Yucheng People's Hospital. Mice were randomly divided into the sham group, renal I/R 4, 9, 24 and 48 h model groups, 6 mice in each group. Mice were fasted for 12 h before model construction. Intraperitoneal injection of 4% chloral hydrate at a dose of 400 mg/kg was performed for anesthesia. An incision was made along ventral midline to expose the kidneys. Both renal pedicles were clamped using a non-invasive vessel clip until the kidneys became purple and black. Clips were removed 45 min later to restore perfusion. Reperfusion was achieved when kidneys turned pink. Then, kidneys were collected at 4, 24 and 48 h for further analysis. Mice in the sham group were treated with the same procedure but the renal pedicles were not clamped.

Detection and evaluation of renal function. Serum creatinine (Scr) level and blood urea nitrogen (BUN) were detected using automatic biochemical test (Hitachi 7180; Hitachi, Ltd., Tokyo, Japan). Each experiment was repeated 3 times.

Cell transfection. Vector pc-np_5318 for overexpression of lncRNA np_5318 in I/R cell was constructed by inserting the coding oligonucleotides of lncRNA np_5318 into a pcDNA3.1 vector (Invitrogen: Thermo Fisher Scientific, Inc.). Small interfering (si)RNAs targeting lncRNA np_5318 (si-np_5318) were constructed for inhibition of lncRNA np_5318. For cell transfection, I/R cells as mentioned before were cultured in a six-well plates (10^4 cells per well) for 24 h and transfected with 10 nM pc-np_5318, si-np_5318 (5'-CCUGUGCAACGUGCUCAUCAUA-3'), and their corresponding controls [pc-NC and si-NC(5'-GCGUGUACGUGUACGUGUACGAG-3') respectively] using Lipofectamine 2000® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Cells continued to be incubated for another 48 h before subsequent experiments.

MTT assay. Using an MTT colorimetric assay, cell viability was assessed. In brief, different transfected cells at logarithmic stage were grown into a 96-well plate. After 24, 48 and 72 h of transfection, 20 ml MTT was added into each well to incubate cells for another 4 h. Then 150 ml dimethylsulfoxide was added into each well to dissolve the formazan precipitates for 10 min. The absorbance (490 nm) was measured under an absorption spectrophotometer (Olympus Corporation, Tokyo, Japan). Each experiment was repeated 3 times.

RT-qPCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.). RNA concentration was determined using NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, Inc.) and only the ones with a ratio of A260/A280 between 1.8 and 2.0 were used for reverse transcription to synthesize cDNA using ploy (T) as primer and SuperScript III Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.) through following conditions: 55°C for 15 min and 75°C for 10 min. SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Inc.) was used to prepare the PCR reaction system. Following primers were used: 5'-AAC
TCGCCCAAGAATCCCA-3' (forward) and 5'-ACAACC CCAAAACAAGCTGTC-3' (reverse) for np_5318; 5'-TGCTGA GTATGTCGAGCTCA-3' (forward) and 5'-AGTGGG AGTTGCTGTGAAATC-3' (reverse) for GAPDH. PCR reaction conditions were: 95˚C for 40 sec, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 45 sec. Cq values were processed using 2-ACTing method (16). Relative expression level of each gene was normalized to endogenous control GAPDH.

Western-blotting. Total protein extraction was performed using a Proteome Mammalian Protein Prep kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Primary antibodies used were rabbit anti TGF-β1 (1:2,000; cat. no. ab92486; Abcam), Smad3 (1:2,000; cat. no. ab28379; Abcam), p-Smad3 (1:2,000; cat. no. ab52903; Abcam) and α-tubulin (1:2,000; cat. no. ab18251; Abcam). After that, membranes were washed three times with TBST. Membranes were then incubated with secondary antibody at room temperature for 2 h. The secondary antibody was HRP-Goat Anti-Rabbit (IgG) secondary antibody (1:2000; cat. no. ab6721; Abcam). After washing twice with TBST, 15 min for each time, ECL™ Blotting Reagents GE Healthcare (Sigma-Aldrich; Merck KGaA) was added to detect the signals. Images were processed using Bandscan 5.0 software (Nuohebio) to calculate the relative expression level of each protein.

Statistical analysis. SPSS 10.0 was used for all statistical analysis (SPSS, Inc., Chicago, IL, USA). Experimental data were expressed as the mean ± standard deviation. Comparisons within a group were performed using single factor analysis of variance and comparisons between two groups were performed using independent t test. Multiple comparison corrections were performed using Bonferroni multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

Renal I/R injury group shows obvious renal function impairment. Compared with the levels of SCr and BUN in the sham group (24.56±5.94 and 7.44±3.99 µmol/l, respectively), levels of SCr and BUN were increased in I/R group at 4 (41.61±4.79 and 14.45±0.83 µmol/l, respectively), 24 (94.42±53.89 and 39.35±15.88 µmol/l, respectively) and 72 h (36.34±4.78 and 17.42±11.48 µmol/l, respectively) after reperfusion. Those data suggest that I/R can increase the levels of SCr and BUN in mice, which in turn impairs renal function (Table I).

As shown in Fig. 1, the results of HE staining showed the structure of renal tissue. The sham group was normal and only local renal tubular epithelial cell degeneration was observed. Serious tubular epithelial cell swelling, vacuolar degeneration, deep nuclear staining, cell necrosis, damaged basement membrane and renal tubular lumen expansion were observed in I/R groups, indicating the successfully constructed I/R model.

Changes of microvessel density after renal I/R injury. Cluster of differentiation (CD)31 mediates the penetration of leukocytes into blood vessel walls to cause tissue damage and compensatory hyperplasia (3). The increased staining of CD31 in the vascular tissue of the sham group and I/R groups was detected by immunohistochemical staining. Results showed that the signal of CD31 in I/R groups was significantly increased at 24 and 72 h after reperfusion compared with the sham group (P<0.05). These data suggest that renal I/R can stimulate the compensatory hyperplasia of microvessels (Table II).

| Table I. Comparison of levels of SCr and BUN among groups. |
| Group | SCr (µmol/l) | BUN (µmol/l) |
|-------|-------------|--------------|
| Sham  | 24.56±5.94  | 7.44±3.99    |
| I/R 4 h | 41.61±4.79  | 14.45±0.83   |
| I/R 24 h | 94.42±53.89 | 39.35±15.88  |
| I/R 72 h | 36.34±4.78  | 17.42±11.48  |

*P<0.05 vs. the sham group. BUN, blood urea nitrogen; SCr, serum creatinine; I/R, ischaemia/reperfusion.

| Table II. Comparison of positive rate of CD31 among groups |
| Group | CD31 |
|-------|------|
| Sham  | 12.04±0.78 |
| I/R 24 h | 21.43±0.22 |
| I/R 72 h | 23.51±0.69 |

*P<0.05 vs. the sham group. CD, cluster of differentiation; I/R, ischaemia/reperfusion.
Expression level of np_5318 in vivo and in vitro. To investigate the level of np_5318 in the mice, RT-qPCR was used in the sham and I/R groups with GAPDH as endogenous control. As shown in Fig. 2A, compared with the sham group, the expression level of np_5318 was increased at 24 h after reperfusion. For the RNA interference experiment, three siRNA sequences were synthesized and the transfection efficiency was detected. The sequence was chosen with the highest transfection efficiency for demonstration (Fig. 2B). Si-np_5318 significantly decreased np_5318 level (P<0.05) and pc-np_5318 significantly increased np_5318 level compared with the Ctrl (control; P<0.01; Fig. 2B). Furthermore, an I/R cell model in vitro was constructed and these cells were divided into six groups, including I/R, I/R+si-NC, I/R+si-np_5318, I/R+pc-NC, I/R+pc-np_5318, and control groups as shown in Fig. 2B. The expression of np_5318 in these groups were confirmed by RT-qPCR. Results indicated that np_5318 in the I/R group was significantly increased compared with the control group (P<0.05). In addition, the present study confirmed that si-np_5318 significantly decreased np_5318 level in I/R group and pc-np_5318 significantly increase np_5318 level in I/R group (P<0.05). Then, relative cell viability at 24, 48 and 72 h in these groups were investigated by MTT assay (Fig. 2C). The results of the present study suggested that cell growth was significantly suppressed in the I/R groups compared with the control group (P<0.01) and transfection with pc-np_5318 significantly suppressed the cell growth of I/R cells at 48 and 72 h (P<0.01). While inhibition of np_5318 by si-np_5318 significantly increased the cell growth of I/R cells at 48 and 72 h (P<0.01). Those data suggested that the expression of np_5318 increased in I/R groups and np_5318 may promote renal I/R injury.

LncRNA np_5318 can bind to Smad3. As shown in Fig. 3A, np_5318 contained conserved Smad3 binding sites in both human and mouse genomes. To confirm this finding, a ChIP assay was used to determine the interaction of Smad3 with the promoter regions of np_5318 in mouse embryonic fibroblast. The antibody against Smad3 could successfully immunoprecipitate the DNA fragments from MEFs containing the potential SBSs in the promoter regions of np_17856 and np_5318, supporting that Smad3 could physically interact with their promoter regions (Fig. 3B).

Expression of TGF-β1 and Smad3 in different groups. To determine TGF-β1 and p-Smad3 expression in the kidney, immunohistochemical staining was performed. The sham operated controls did not show any TGF-β1 and p-Smad3 expression. However, there was significant increase in TGF-β1 and p-Smad3 expression in I/R model (P<0.01) compared with the sham mice (Fig. 4). Levels of TGF-β1, Smad3 and p-Smad3 were also detected by western blotting in the renal tissue of the different groups. As shown in Fig. 5A and B, compared with the sham group, levels of TGF-β1, Smad3 and p-Smad3 were significantly increased in renal tissue of I/R group (P<0.001).

Then levels of TGF-β1, Smad3 and p-Smad3 in cells were detected by western blotting, including I/R, I/R+si-NC, I/R+si-np_5318, I/R+pc-NC, I/R+pc-np_5318, and the control groups as shown in Fig. 5C and D. It was demonstrated that the level of TGF-β1, p-Smad3 and Smad3 was significantly increased in I/R group compared with the control group, and transfection with pc-np_5318 significantly increased the level of np_5318 compared with in the I/R group. While inhibition of np_5318 by si-np_5318 significantly suppressed the level of TGF-β1, p-Smad3 and Smad3 compared with the I/R group. Those data suggest that I/R can increase the expression levels of TGF-β1, Smad3 and p-Smad3.

Discussion

Renal IR is a process of the reduced blood supply to kidneys followed by re-oxygenation and restoration of blood flow (17). Reperfusion is critical for the recovery of renal function. However, this procedure sometimes can cause renal damage. The blocking of renal blood flow is usually required for some surgical operations, such as nephrolithotomy, surgical resection of renal tumors and renal transplantation. Renal reperfusion during those operations can also cause renal damage (18). BUN and SCr are two traditional biomarkers of kidney (19). Decreased renal function caused increases in levels of BUN and SCr (12).

Numerous studies have shown that the onset, development and progression of ischemia-reperfusion injury are closely related to the function of different lncRNAs (12-21). Yu et al (12) showed that, as a HIF-1α dependent lncRNA, expression level of psoriasis associated non-protein coding RNA induced by stress (PRINS) was upregulated under
hypoxia conditions caused by I/R and PRINS can interact with RANTES to promote I/R injury. In another study, lncRNA TapSAKI was found to be upregulated in the plasma of patients with acute kidney infarction (AKI) and the expression level of TapSAKI was positively correlated with the disease severity, indicating that TapSAKI can serve as a specific biomarker for
the prognosis of AKI (20). In contrast, lncRNA AK139328 was found to be downregulated in mice with liver I/R injury. In addition, AK139328 knockdown mediated by siRNA silencing was found to be able to inhibit the activation of caspase-3, reduce the expression of IP-10, inhibit the activity of nuclear factor-kB signaling and reduce the expression of various inflammatory cytokines during the development of liver I/R injury (21). It has been reported that expression level of lncRNAs np_5318 is increased during renal inflammation (13). However, to the best of our knowledge the involvement of lncRNAs np_5318 in renal I/R injury still hasn't been reported. In the present study, I/R mice models were successfully constructed and expression of lncRNAs np_5318 was found to be increased in mice with renal I/R injury compared with mice in the sham group, and the increased expression of lncRNAs np_5318 was also confirmed in I/R cells, indicating the possible involvement of np_5318 in this procedure.

The TGF-β/Smad signaling pathway plays pivotal roles in various renal diseases. The activation of TGF-β can regulate a variety of cellular functions including proliferation,
differentiation, apoptosis and inflammation (22). As a member of the TGF-β super family, TGF-β1 was found to be able to play a profibrotic role by stimulating the proliferation of fibroblasts, extracellular matrix synthesis and process of epithelial-to-mesenchymal transition (23). Members of the Smad family play central roles in TGF-β signaling pathway (24). In the present study, compared with mice in the sham group, expression levels of TGF-β1 and Smad3 were increased in mice of the I/R model group, indicating that I/R injury can upregulate the expression of TGF-β1 and Smad3. In addition, the level of p-Smad3, which is the activated form of Smad3, is also increased in mice with I/R injury. Then the results in vitro also confirmed that TGF-β1, Smad3 and p-Smad3 was increased in I/R cells. Those data suggested that I/R could increase the expression levels of TGF-β1 and Smad3 and induce the activation of Smad3. Furthermore, it was demonstrated that the inhibition of IncRNAs np_5318 level in I/R cells could enhance the cell growth of I/R cells. Additionally, TGF-β1, Smad3 and p-Smad3 levels were also inhibited by the inhibition of IncRNAs np_5318 level in I/R, while the increase of IncRNAs np_5318 level provided the opposite results.

In conclusion, expression level of IncRNA np_5318 was increased in mice with renal I/R and I/R cells. np_5318 could regulate the expression of Smad3 by binding to its promoter region. In addition, levels of TGF-β1, Smad3 and p-Smad3 were increased by renal I/R. Therefore, IncRNA np_5318 may participate in the development of renal I/R injury through the TGF-β/Smad signaling pathway. The present study provides new insights into the mechanism and treatment of I/R injury. However, the present study is still limited by the small sample size. Further studies with bigger sample size are still needed to verify the conclusions in this study.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Figure 5. Levels of TGF-β1, Smad3 and p-Smad3 are determined in vivo and in vitro. (A) Representative results of western blotting for TGF-β1, Smad3 and p-Smad3 in renal tissue of different group. (B) Normalized expression levels of TGF-β1, Smad3 and p-Smad3. ***P<0.001 vs. the sham group. (C) Representative results of western blotting for TGF-β1, Smad3 and p-Smad3 in these I/R cell groups (I/R, I/R+si-NC, I/R+si-np_5318, I/R+pc-NC, I/R+pc-np_5318 and control groups). (D) Normalized expression levels of TGF-β1, Smad3 and p-Smad3. **P<0.05 and ***P<0.001. p-Smad3, phosphorylated-mothers against decapentaplegic homolog; TGF, transforming growth factor; I/R, ischaemia/reperfusion; NC, negative control.
Authors' contributions

JS conceived and designed the study. JL and JM performed the experiments. JL and JM wrote the paper. JL and JS reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Yucheng People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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