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Influence of lncRNA HOTAIR on acute kidney injury in sepsis rats through regulating miR-34a/Bcl-2 pathway

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Abstract. – OBJECTIVE: To study the regulatory effect of long non-coding ribonucleic acid (lncRNA) HOX transcript antisense RNA (HOTAIR) on acute kidney injury (AKI) in sepsis rats and its regulatory mechanism.

MATERIALS AND METHODS: The sepsis-induced AKI model was established in Sprague-Dawley (SD) male rats through cecal ligation puncture. A total of 30 SD rats were randomly divided into the control group, model group and lncRNA HOTAIR mimic group, with 10 rats in each group. Relative levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in kidney tissues were detected via enzyme-linked immunosorbent assay (ELISA). Apoptosis of kidney tissues was detected via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Moreover, the target gene of miR-34a was searched using the miRNA online database. The messenger RNA (mRNA) expression levels of miR-34a and B-cell lymphoma-2 (Bcl-2) were detected via Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

RESULTS: Compared with those in the control group, the rats in the model group showed injured pathological morphology of kidney, elevated contents of TNF-α and IL-1β, and apoptosis in kidney tissues. The target gene of miR-34a was Bcl-2, according to the miRNA online database. MiR-34a level in kidney tissues was upregulated, while the mRNA level of Bcl-2 significantly decreased in the model group. Compared with those in the model group, the pathological morphology of kidney tissues was improved, the content of TNF-α and IL-1β markedly declined, and the apoptotic rate of kidney tissues also reduced in lncRNA HOTAIR mimic group. The miR-34a level in kidney tissues decreased, while the Bcl-2 mRNA level remarkably increased in lncRNA HOTAIR mimic group.

CONCLUSIONS: LncRNA HOTAIR overexpression can alleviate AKI in sepsis rats by inhibiting the apoptosis of kidney tissues by downregulating the miR-34a/Bcl-2 signaling pathway.

Key Words:
LncRNA, HOTAIR, MiR-34/Bcl-2, Sepsis, AKI.

Introduction

Sepsis is a kind of systemic inflammatory response syndrome, which is characterized by high morbidity and mortality rates, and has been well concerned in severe clinical medicine. Sepsis places a huge burden on health-care workers in the world, seriously affecting the life quality of patients and causing irreversible damage to the cognitive function[1,2]. In 2016, European Society of Intensive Care Medicine redefined sepsis as the uncontrolled response of hosts to infections, causing life-threatening organ dysfunction[3]. Acute kidney injury (AKI) is dominated in the organ dysfunction, and it will eventually develop into the end-stage renal disease in most patients, leading to over 30% of deaths[4]. Studies have demonstrated that the pathogenesis of sepsis-induced AKI is related to the renal hemodynamic abnormality and inflammatory response[5]. Inflammatory response abnormality is a research hotspot, and its abnormality in the kidney will lead to the release of a large number of inflammatory factors, causing apoptosis of kidney cells and aggravating kidney injury.

Micro-ribonucleic acids (miRNAs) are a kind of endogenous non-coding small RNAs mainly involved in gene transcription and protein translation[6]. Studies[7] have suggested that miRNAs are involved in cell proliferation, differentiation and apoptosis. B-cell leukemia/lymphoma 2 (Bcl-2) family protein is an important regulatory target for apoptosis. The apoptosis caused by abnormal

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inflammatory responses in AKI has attracted the attention of researchers. Searching for miRNAs that can regulate the expression of Bcl-2 in a targeted way will provide a novel therapy of AKI.

Long non-coding RNAs (lncRNAs) are a kind of RNA without protein-encoding function, which is about 200 nt in length and involved in various life activities. It is also closely related to the occurrence and development of many diseases[8]. LncRNA HOX transcript antisense RNA (HOTAIR) was the first RNA found to possess the trans-regulatory effect. Jiang et al[9] found that lncRNA HOTAIR is up-regulated in non-small cell lung cancer tissues or cell lines. Yang et al[10] proved that the high expression of lncRNA HOTAIR relieves TRAIL-induced apoptosis, indicating that lncRNA HOTAIR plays an important role in apoptosis. Therefore, this work aims to study the influence of lncRNA HOTAIR in sepsis rats with AKI, and further investigates its regulatory pathway.

Materials and Methods

Experimental Animals

A total of 30 specific pathogen-free Sprague-Dawley (SD) male rats weighing 220-250 g were purchased from the Laboratory Animal Center of Guangzhou University of Chinese Medicine (certificate No. 44005800004475). This study was approved by the Animal Ethics Committee of the Fudan University Animal Center.

Experimental Reagents

The hematoxylin-eosin (HE) staining kit was purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and enzyme-linked immunosorbent assay (ELISA) kit from Wuhan Elabscience Biotechnology Co., Ltd. (Wuhan, China), 4% paraformaldehyde fixative and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay kit from Beijing Solarbio Life Science Co., Ltd. (Beijing, China), and TRIzol and First Strand complementary deoxyribose nucleic acid (cDNA) Synthesis Kit from Thermo Fisher Scientific (Waltham, MA, USA).

Experimental Instruments

The microplate reader was purchased from Beijing Perlong New Technology Co., Ltd. (Beijing, China), the incubator from Chengdu Gaisen Electronic Equipment Co., Ltd. (Chengdu, China), the inverted fluorescence microscope from Nikon (Tokyo, Japan), the microtome from Leica (Munich, Germany), the ultra-low temperature refrigerator from Haier (Qingdao, China), and ultraviolet spectrophotometer from Shanghai Metash Instruments Co., Ltd. (Shanghai, China)

Establishment of Rat Model of Sepsis-Induced AKI

Sepsis-induced AKI in rats was established through cecal ligation puncture. Rats in each group fasted from food and water for 12 h before modeling, and then anesthetized *via* intraperitoneal injection of 10% chloral hydrate. After the rats were fixed in a supine position, the abdomen hair was shaved off. Rat abdomen was cut open along the midline, and the mesentery and cecum were separated. Then, the ileocecal valve was ligated, and the end of cecum was punctured twice using the needle. The feces were squeezed out, and finally the cecum was placed back into the abdominal cavity. In the control group, the mesentery and cecum were only separated after anesthesia, and the skin was sutured. All rats were resuscitated *via* injection of normal saline after the operation, and the serum levels of serine (Ser) and blood urea nitrogen (BUN) were detected using the biochemical method. Pathological morphology of kidney tissues was detected *via* HE staining.

Detection of IL-1β and TNF-α Levels in Kidney Tissues Via ELISA

The kidney tissues were taken, washed with pre-cooled Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA), ground and centri-

Table I. Changes in renal function of rats.

Group	Scr (μmol/L)	BUN (μmol/L)
Control	34.67±4.61	5.85±0.93
Model	149.35±10.28**	47.19±3.83**
LncRNA HOTAIR mimic	78.36±6.12#	19.45±5.12##

Note: **$p<0.01$: Model group *vs*. Control group, ##$p<0.01$, #$p<0.05$: LncRNA HOTAIR mimic group *vs*. Model group.

Table II. MiRNAs with potential binding sites to Bcl-2 mRNA 3'UTR.

miRNA	Position in the UTR	Context++ score
rno-miR-34c-5p	194-200	-0.35
rno-miR-34b-5p	194-200	-0.35
rno-miR-34a-5p	194-200	-0.29
rno-miR-449c-5p	194-200	-0.27
rno-miR-204-5p	200-207	-0.25
rno-miR-449a-5p	194-200	-0.24
rno-miR-211-5p	200-207	-0.24
rno-miR-30b-5p	2418-2424	-0.22
rno-miR-30c-5p	2418-2424	-0.22
rno-miR-384-5p	2418-2424	-0.21

fuged at 5000×g for 10 min, and the supernatant was retained. The standard working solution was diluted to be prepared into the standard working solution at a concentration of 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0 pg/mL. The samples to be detected and the standard working solution were added into a 96-well plate (100 μL/well) and covered with the film, followed by incubation at 37°C for 90 min. After the solution was discarded, 100 μL of biotinylated antibody working solution was added into each well, and the cover film was laid, followed by incubation at 37°C for 60 min. After the solution was discarded, each well was washed with 350 μL of washing liquid 3 times (2 min/time). Then 100 μL of enzyme-conjugated working solution was added into each well, and the cover film was laid, followed by incubation at 37°C for 30 min. After the solution was discarded, 90 μL of substrate solution was added, followed by incubation for 15 min. Finally, 50 μL of termination buffer was added into each well to terminate the reaction, and the absorbance was measured at a wavelength of 450 nm using the microplate reader.

Detection of Apoptosis of Kidney Tissues via TUNEL Staining

The kidney tissues were paraffin embedded, sliced, soaked in xylene for 30 min, deparaffinized and gradiently hydrated. After washing with PBS 3 times (5 min/time), the sections were fixed with 4% paraformaldehyde and permeabilized with 0.1% permeabilization solution. Then 100 μL of TUNEL staining solution was added for incubation at room temperature for 1 h, the sections were washed with PBS for 5 min, and the staining was observed under the fluorescence microscope. Finally, the apoptosis rate was calculated: the number of red-staining cells/the number of blue-staining cells × 100%.

Searching of Target Gene of Bcl-2 in Database

MiRNAs with potential binding sites to Bcl-2 were searched using the miRNA online database (TargetScan database, http://www.targetscan.org). Conserved 8mer and 7mer sites matched to each miRNA seed region, and BCL2 were used as the keywords.

Detection of MiR-34a and Bcl-2 mRNA Levels in Kidney Tissues via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The kidney tissues were lysed with 1 mL TRIzol lysis buffer. The lysate was retained, extracted with chloroform and centrifuged. Then, it was added with isopropanol and centrifuged again. The supernatant was discarded, and the precipitate was resuspended with 75% ethanol and centrifuged. After being air-dried, the total RNA was extracted using the RNA extracting solution, and complementary deoxyribose nucleic acid (cDNA) was synthesized using the First Strand cDNA Synthesis Kit according to the instructions, followed by Polymerase Chain Reaction (PCR) for 35 cycles. After that, the agarose gel electrophoresis was performed, followed by photography under a gel imager. The optical density of the band was analyzed using the Image J software (Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Measurement data in the normal analysis were expressed as mean ± standard deviation. One-way analysis of variance was adopted for the intergroup comparison of data with homogeneous variance, followed by Post-hoc test (Least Significant Differ-

ence). $p<0.05$ suggested that the difference was statistically significant.

Results

Successful Establishment of Sepsis-Induced AKI Model in Rats

The serum levels of Ser and BUN in each group of rats were detected. The results revealed that the serum levels of Ser and BUN significantly increased in the model group compared with those in the control group ($^{**}p<0.01$, $^{**}p<0.01$), which were markedly declined in lncRNA HOTAIR mimic group compared with those in the model group ($^{\#\#}p<0.01$, $^{\#}p<0.05$) (Table I). It is indicated that lncRNA HOTAIR mimics could remarkably improve the renal function of sepsis rats. HE staining revealed normal kidney structure in the control group. In the model group, cell necrosis, shedding and hyperemia could be observed. The renal function was improved significantly in lncRNA HOTAIR mimic group (Figure 1). The above results suggested the successful establishment of sepsis-induced AKI model in rats, and lncRNA HOTAIR mimics can also improve the renal function and cell pathological morphology.

LncRNA HOTAIR Mimics Could Reduce IL-1β and TNF-α Contents

To explore the effect of lncRNA HOTAIR mimics on the inflammatory response in AKI in sepsis rats, contents of IL-1β and TNF-α in kidney tissues were detected *via* ELISA. The results showed that IL-1β and TNF-α increased in the model group compared with those in the control group ($^{**}p<0.01$, $^{*}p<0.05$, respectively). In lncRNA HOTAIR mimic group, IL-1β and TNF-α levels declined in kidney tissues ($^{\#\#}p<0.01$, $^{\#}p<0.05$, respectively; Figure 2), indicating that lncRNA HOTAIR mimics can inhibit the inflammatory response in AKI in sepsis rats.

LncRNA HOTAIR Mimics Could Inhibit the Apoptosis of Kidney Cells

The effect of lncRNA HOTAIR mimics on the apoptosis of kidney cells in sepsis rats with AKI was detected *via* TUNEL (Figure 3A). The apoptotic rate of kidney tissues was markedly enhanced in the model group compared with that in the control group ($^{**}p<0.01$), while it was significantly reduced in lncRNA HOTAIR mimic group compared with that in the model group ($^{\#}p<0.05$) (Figure 3B). It is indicated that lncRNA HOTAIR mimics can inhibit the apoptosis rate of kidney tissues in sepsis rats with AKI.

MiR-34a Could Regulate Bcl-2 Predicted by MiRNA Database

MiRNAs that may regulate the Bcl-2 gene were screened using the TargetScan database, and the top ten miRNAs ranking from high to low were shown in Table II. It can be seen that the miR-34 family had the highest score, and some studies[11] have proved that miR-34a is correlated with apoptosis. Therefore, miR-34a was selected as the object in the present work. The screening results of potential binding sites between miR-34a and Bcl-2 were shown in Figure 3. The results manifested that miR-34a had binding sites at 194-200 of the 3'UTR of Bcl-2 mRNA, and there was high homology in rats, mice, squirrels, rabbits and humans.

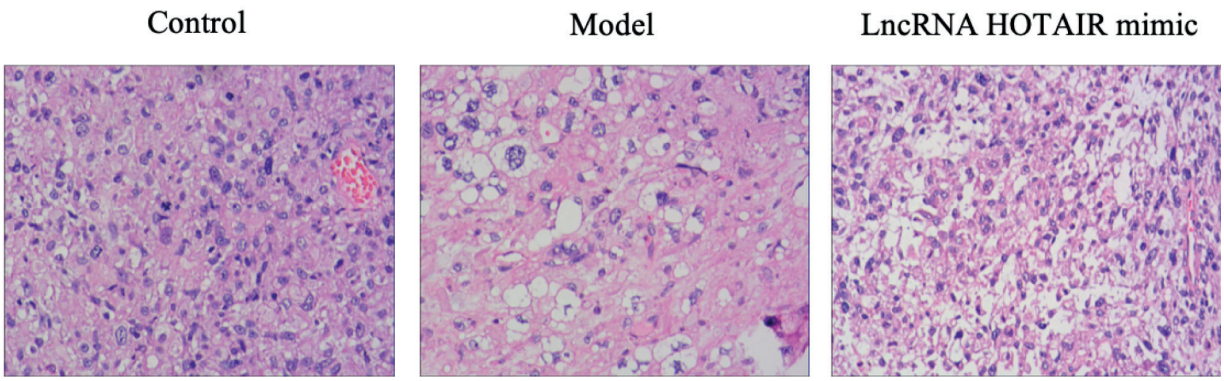

Figure 1. HE staining of rat kidney (20×).

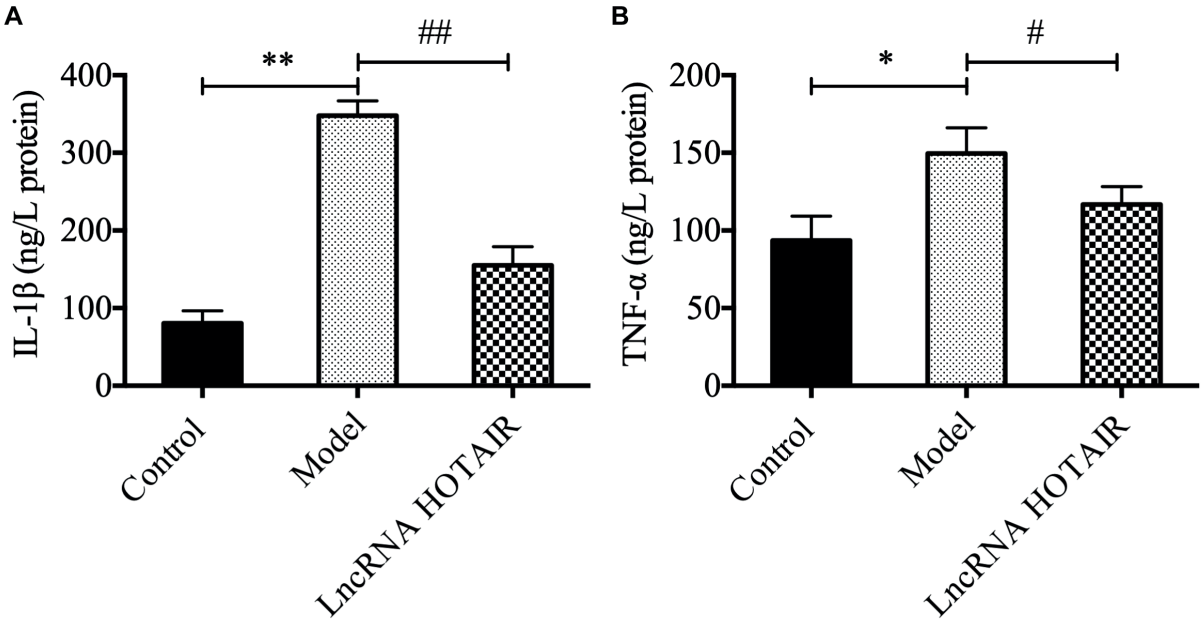

Figure 2. Content of IL-1β and TNF-α in rat kidney. ***A,*** IL-1β content; ***B,*** TNF-α content ($^{**}p<0.01$, $^{*}p<0.05$, $^{\#\#}p<0.01$, $^{\#}p<0.05$).

LncRNA HOTAIR Mimics Could Down-Regulate Bcl-2 and Inhibit MiR-34a

To further study the mechanism of anti-apoptosis effect of lncRNA HOTAIR mimics on kidney tissues, mRNA levels of miR-34a and Bcl-2 were detected *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The band diagram was shown in Figure 5A. It was found that the miR-34a level increased ($^{**}p<0.01$) and the Bcl-2 mRNA level decreased ($^{**}p<0.01$) in the model group compared with those in the control group. Compared with those in the model group, the miR-34a level declined ($^{\#\#}p<0.01$) and the Bcl-2 level increased ($^{\#}p<0.05$) in lncRNA HOTAIR mimic group (Figure 5B).

Discussion

Sepsis is characterized by high morbidity and mortality rates, and its main sources of infection include pulmonary infection, abdominal infection and urinary tract infection. Organ dysfunction is closely related to the mortality rate of sepsis. Even if sepsis patients are recovered after treatment, the mental impairment has not been improved, seriously affecting the quality of life of patients[12]. Studies[13] have demonstrated that more than 50% of sepsis patients are often accompanied by complications of AKI, causing a decline in renal function and AKI due to the excessive release of inflammatory mediators and factors.

Inflammatory response plays an important role in the process of AKI, and the excessive release of a variety of pro-inflammatory factors causes damage to renal tubular epithelial cells[14]. Studies[15] have revealed that cisplatin-induced AKI can be prevented, and renal epithelial cells can be protected from toxic damage in TNF-α knockout mice. The apoptosis caused by inflammatory responses in AKI is noteworthy. Bcl-2, as a key factor regulating apoptosis, also plays an important role in AKI[16]. Zhang et al[17], in the study on acute renal ischemia-reperfusion, found that the longer the duration of ischemia, the lower the Bcl-2 expression and the higher the Bax expression. It further stimulates the expressions of NF-κB and downstream COX2, and aggravates apoptosis. The above results indicated that drugs target Bcl-2 may provide new therapeutic strategies for sepsis-induced AKI.

The preliminary mechanism and function of lncRNA, a research hotspot in the field of medicine in recent years, have been mastered by researchers. Studies[18] have shown that lncRNAs play important roles in the complex pathogenesis of AKI. Ding et al[19] found that lncRNA MALAT1 may treat AKI by regulating the miR-146a/NF-κB

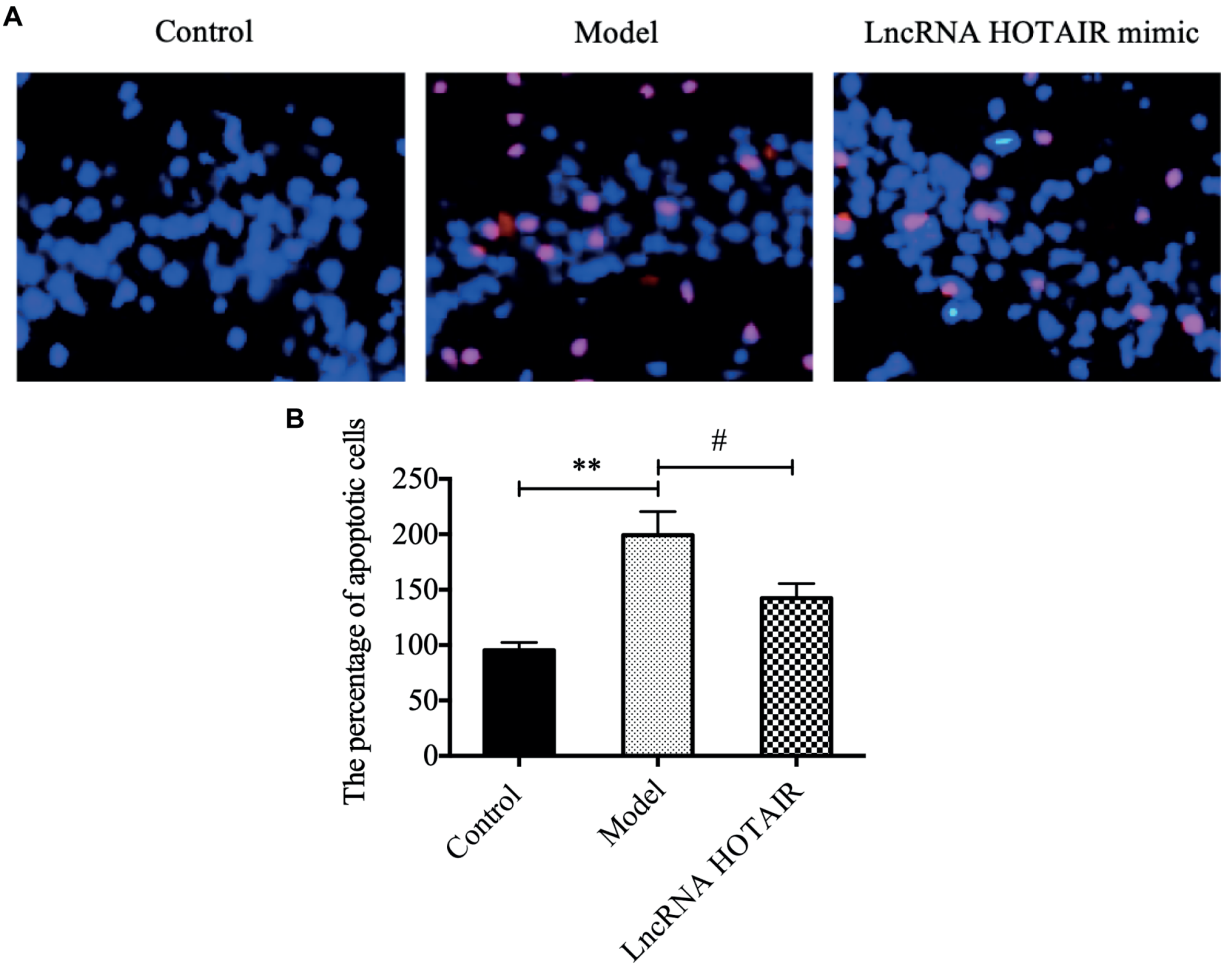

Figure 3. Apoptosis of kidney tissues detected *via* TUNEL (20×). ***A,*** TUNEL staining (red: apoptotic cells, blue: nuclei). ***B,*** Apoptosis rate ($^{**}p<0.01$, $^{\#}p<0.05$).

Figure 4. Prediction results in TargetScan database.

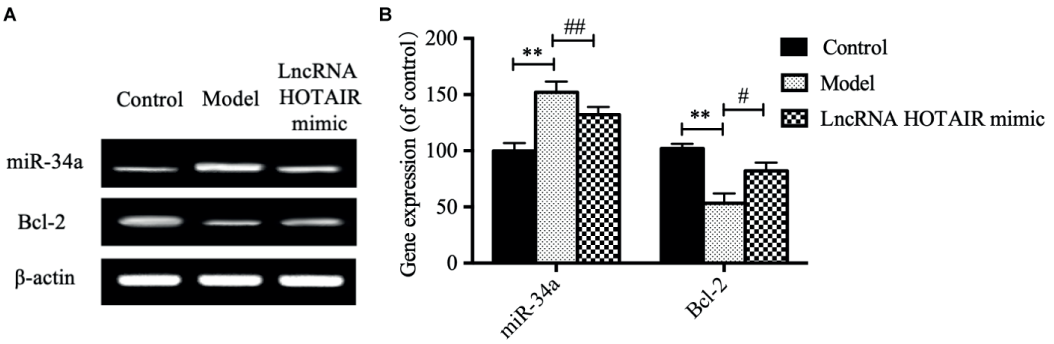

Figure 5. Expression of miR-34a and Bcl-2 mRNA in kidney tissues. ***A***, RT-PCR band; ***B***, statistical graph of band ($^{**}p<0.01$, $^{\#\#}p<0.01$, $^{\#}p<0.05$).

signaling pathway. In addition, Qiu et al[20] found that lncRNA ATB can exert a therapeutical effect on AKI through TNF-α, indicating that lncRNAs play important roles in the pathogenesis and treatment of AKI.

In this paper, the sepsis-induced AKI model in rats was established through cecal ligation puncture, and the successful modeling was confirmed *via* detection of renal function and HE staining. It was found that lncRNA HOTAIR mimics could improve both renal function and pathological injury. The ELISA results revealed that lncRNA HOTAIR mimics could significantly inhibit the release of inflammatory factors and apoptosis of kidney tissues. Then the screening results using miRNA database showed that miR-34a could regulate Bcl-2 in a targeted way. Moreover, the changes in miR-34a and Bcl-2 levels were detected *via* RT-PCR, and it was found that lncRNA HOTAIR mimics could down-regulate the miR-34a level and raise the Bcl-2 level. Therefore, lncRNA HOTAIR mimics can inhibit the apoptosis of kidney tissues of rats with sepsis-induced AKI by reducing the miR-34a level and increasing the Bcl-2 level.

Conclusions

We found that lncRNA HOTAIR mimics can alleviate AKI in sepsis rats by inhibiting the apoptosis of kidney tissues by down-regulating the miR-34a/Bcl-2 signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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