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The role of miR-146b-5p in TLR4 pathway of glomerular mesangial cells with lupus nephritis

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) are now recognized as important regulators of gene expression. The aim of the study was to investigate the role of miR-146b-5p in the TLR4 pathway and provide the basis for the treatment of lupus nephritis.

MATERIALS AND METHODS: The glomerular mesangial cells were cultured *in vitro* and divided into 3 groups: control group, a group of miR-146b-5p mimic added, and a group of miR-146b-5p inhibitor added. The levels of IL-6 and IL-8 in the cell culture supernatant of the three groups were detected by ELISA. The cell proliferation was detected by MTT. The expressions of MiR-146b-5p and TLR4 pathway-associated factor TRAF6 were detected by RT- PCR. The expression of TRAF6 and IRAK1 protein was detected by Western blot.

RESULTS: The overexpression of miR-146b-5p could reduce the level of IL6 and IL8 in cell culture and inhibit glomerular mesangial cell proliferation in some degree. Also, the overexpression of miR-146b-5p could inhibit the expressions of TLR4 pathway-associated factor TRAF6 and IRAK1mRNA, and the expressions of TRAF6 and IRAK1 protein.

CONCLUSIONS: MiR-146b-5p attenuated the inflammatory response of glomerular mesangial cells by inhibiting the expressions levels of TRAF6 and IRAK1 in lupus nephritis.

Key Words:
miR-146b-5p, Lupus nephritis, TLR4 pathway.

Abbreviations

SLE = Systemic lupus erythematosus; miRNA = MicroRNA; UTR = Untranslated region; TLR = Toll-like receptor; TRAF6 = Tumor necrosis factor receptor-associated factor 6; IRAK1 = Interleukin-1 receptor associated kinase 1; IL-1 = Interleukin-1; DMEM = Dulbecco's Modified Eagle Medium; ELISA = Enzyme-linked immunosorbent assay; EB = Ethidium bromide; LPS = Lipopolysaccharide; SD = Standard deviation.

Introduction

Systemic lupus erythematous (SLE) is a chronic, multisystem autoimmune disease. Lupus nephritis is an inflammation of the kidney that is caused by SLE as well. The evidence indicates that lupus nephritis is more prevalent in Asians, Hispanics, Native Americans, and Blacks, especially in females of child-bearing age[1]. Although SLE typically has relapsing and remitting course, patients often eventually develop end-stage kidney disorder. Having a number of autoantibodies forming immune complexes in the kidneys contributes greatly to the pathogenesis of lupus nephritis[2]. Previous studies[3,4] pointed that various populations were in poor health, suffering from SLE. Recently, there are growing interests for studying the pathogenesis of lupus nephritis and exploring the new treatment options[5].

It was also shown that epigenetic defects one of the core contributors in lupus nephritis pathogenesis[6-8]. Altering the phenotype with stable and heritable changes, without affecting the underlying DNA sequence, it is called epigenetics, which also includes microRNA (miRNA) regulation[9]. MiRNAs are short, non-coding RNA molecules that govern gene expression by incomplete binding to the 3′ untranslated region (UTR) of target mRNA[10-13]. It was recognized that miRNAs not only regulate gene expression, but also affect innate and adaptive immune response. By regulating innate immune cell responses, proinflammatory mediator production, and toll-like receptor (TLR) and NF- κB signaling pathways, miRNAs are the key participants in the pathogenesis of lupus nephritis[14-18]. For example, miR21, miR146a, and miR155 implicated in the immunopathogenesis of lupus nephritis[19-24]. Tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin-1

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receptor associated kinase 1 (IRAK1) are the two signal transducers in the NF-κB activation pathway whose 3′UTRs contain multiple miR-146a target sequences. The production of TRAF6 and IRAK1 is directly reduced by miR-146a when lipopolysaccharide (LPS)-stimulated induction[25]. MiR-146a/b was forecasted to base-pair with sequences in the 3′UTRs of the TNF receptor-associated factor 6 and interleukin-1 (IL-1) f receptor-associated kinase 1 genes, and we found that these UTRs inhibit expression of an associated reporter gene[26]. However, it is unknown whether the expression of one miRNA alone (mi146b-5p) regulates the key immune complexes deposition and inflammatory reaction by regulation of inflammatory mediator production and the expressions of mRNA and protein of TLR4 pathway-associated factor TRAF6 and IRAK1.

In the present work, the glomerular mesangial cells were cultured in *vitro* and divided into 3 groups: control group, a group of miR-146b-5p mimic, and a group of miR-146b-5p inhibitor added to examine alterations miR-146b-5p expression that may contribute to the production of IL-6 and IL-8. To further investigate how epigenetics can affect inflammatory mediator production in lupus nephritis, and the role of miR146b-5p in the TLR4 pathway of glomerular mesangial cells in lupus nephritis, in *vitro* techniques were used. The data may be used for a potential promising treatment of lupus nephritis by investigating whether the overexpression of miR146-5p can reduce inflammatory mediator production with immune stimulation. Thus, miR-146a could be monitored as diagnostic biomarker and treatment response.

Materials and Methods

Glomerular Mesangial Cells of Lupus Nephritis Separation and Culture

All experiments were carried out according to the guidelines set forth by the Animal Ethics Committee. MRL/faslpr mice (28 weeks old, weight 45 g-55 g) were purchased from Nanjing University Model Animal Institute. The mice were sacrificed and exsanguinated, then the kidneys were removed and capsules were taken. Renal cortex and medulla were separated in the sterile conditions. Next, the renal cortex was chopped and sieved successively by 80 mesh, 100 mesh, and 150 mesh stainless steel sieves. The obtained composition under the last sieve was placed on the 200 mesh sieve net to collect glomeruli, which was digested with 0.1% collagenase and then inoculated in collagenase-treated culture bottle. Glomerular mesangial cells of lupus nephritis of mice were isolated in our laboratory and were maintained by Dulbecco's Modified Eagle Medium (DMEM)/F12 (3:1) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Hangzhou, China) for cell culture. Cells were cultured in an incubator maintained at 37°C with saturated humidity and 5% CO_2 until the cells grew up to 80% and were digestively subcultured. Cells were subcultured by using 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) digestion, observed to round under the microscope; then, they were added to the serum culture medium to suspend digestion, disperse the cells and packaging.

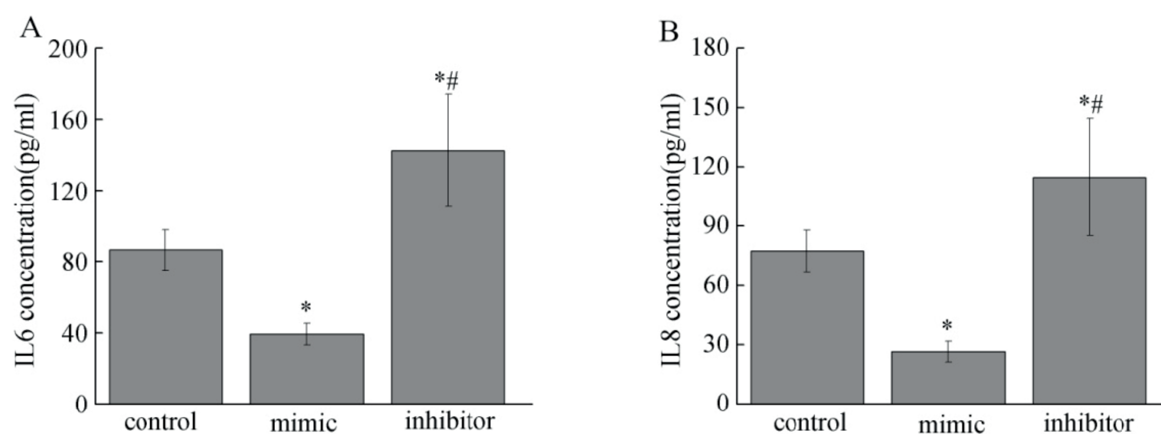

Figure 1. The IL-6 and IL-8 production. ***A,*** IL-6; ***B,*** IL-8. Asterisk was that compared with positive group. *$p < 0.01$; Pound sign was that compared with model group $^{\#}p < 0.01$.

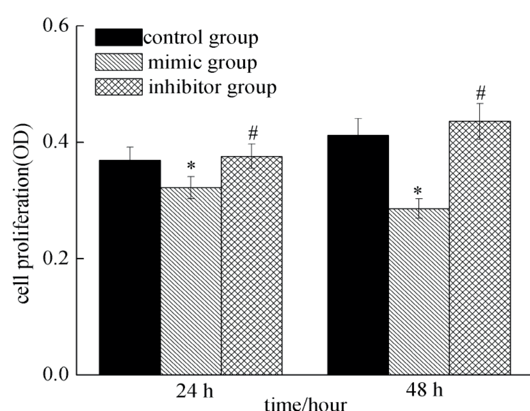

Figure 2. The levels of cell proliferation in different study group. Asterisk was that compared with positive group. $*p < 0.01$; Pound sign was that compared with model group $^{\#}p < 0.01$

Liposome-mediated mi RNA Transfection

The cells were divided into three groups: control group (liposomal transfection), mimic group (miR-146b-5p mimic (Gene Pharma, Shanghai, China) mixed with liposome transfection) and inhibitor group (miR-146b-5p inhibitor (Gene Pharma, Shanghai, China) mixed with liposomal transfection).

The fully digesting cells that were seeded in 96-well plates (Sigma-Aldrich, St. Louis, MO, USA) at the density of 10^5 cells/well until the cells were cultured about 80% density and then began the transfection. The miR-146b-5p mimic and the inhibitor were mixed with medium diluted Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) respectively, configured to miR-146b-5p mimic and inhibitor concentration of 50 nM solution at room temperature for 20 min, according to the instruction requirements. The cells were collected when the complex was added to each well and mixed gently and kept in an incubator maintained at 37°C and 5% CO_2 for 48 h.

ELISA

IL-6 and IL-8 were detected by enzyme-linked immunosorbent assay (ELISA) according to ELISA kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) instruction requirements.

MTT

The 5×10^3 cells in logarithmic growth phase were seeded in 96-well plates with each set five wells. 200 µL cell suspension were added into each well and cultured by adding 5 mg/mL MTT (Sigma-Aldrich Inc., St. Louis, MO, USA) solution 20 µL after 24 h and 48 h, respectively at 37°C for 4 h. The supernatant that was obtained from the extract was added 150 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to dissolve the resulting crystals. We measured OD values of each well on a microplate reader by 490 nm wavelength.

RT-PCR

Purity and integrity of total RNA were measured when total RNA was extracted by using TRIzol kit (Invitrogen, Carlsbad, CA, USA). Reverse transcription reaction was performed by reverse transcription kit (TaKaRa, Dalian, China). PCR reaction was carried out by primers utilized SYBR Green master kit to take 1 µL cDNA reaction system configuration. Horizontal electrophoresis was performed with agarose gel after the PCR reaction. RNA was stained by ethidium bromide (EB) solution and the band intensities were analyzed using Bio-Gel imagery apparatus (Bio-Rad, Hercules, CA, USA). ImageJ analysis software was used to calculate and analyze relative gray values between each band and internal control (β-actin), respectively. Relative expression levels of miR-146b-5p and TRAF6, IRAK-1mRNA of cells in each group were compared after normalization. Primers used in this study were as follows: TRAF6: 5'-CAGTCCCCTG-CACATT-3'(Forward) and 5'-TACG CTACG-GAGGAG-3'(Reverse); IRAK1: 5'-GAGAGTGT TCCTGGCCTCTC-3'(Forward) and 5'- GCTG-GGTTGATGATGATCTG-3'(Reverse); 5'-GTG-GGGATAATGAACTTGCAG-3'(Forward) and 5'-GGAACCCCTGGTAGAACAGT-3'(Reverse) for β-actin.

Western Blot

50 µg protein was extracted from the collected cells and added to 1/5 volume of 6 times sample buffer, boiled for 5 min and then loaded on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein sample with the gel was sustained with voltage at 90 V. When the protein sample came into the separated gel, the voltage was changed to 120 V until the protein pre-stained marker was sufficiently dispersed and the electrophoresis was stopped. The target band was placed in transfer buffer to balance for 5 min. Polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) was immersed in methanol for 5-10 min and washed by distilled water and put into electrical transfer buffer and into transfer cassette, which was put into the power tank. The membrane was trans-

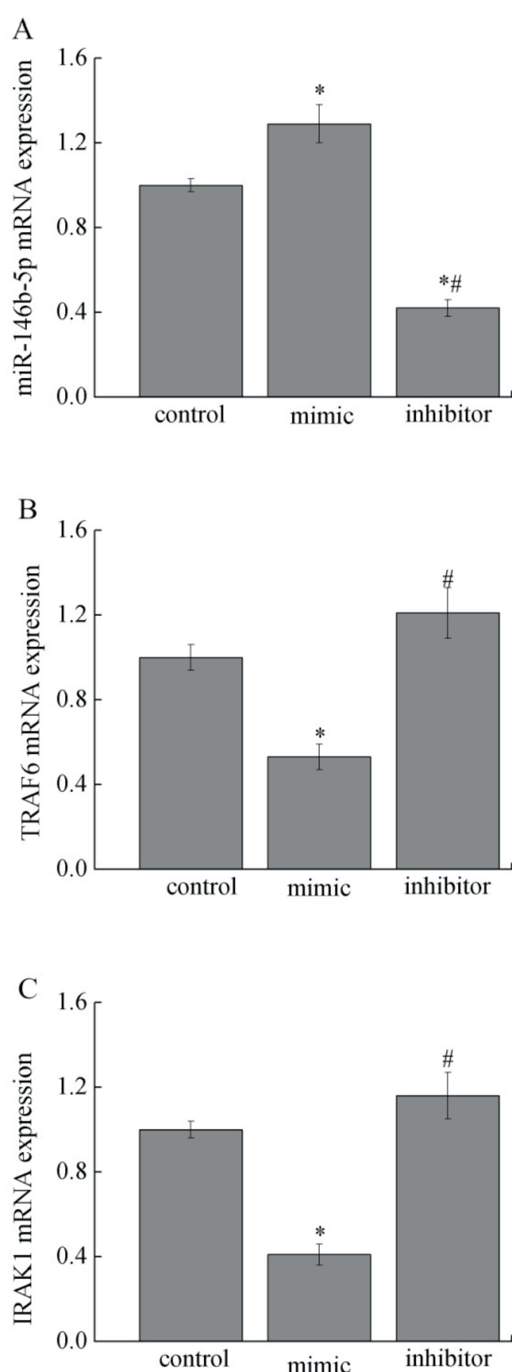

Figure 3. miRNA expression levels of each group. ***A,*** miR-146b-5p; ***B,*** TRAF6 mRNA; ***C,*** IRAK1 mRNA. Asterisk was that compared with positive group. *$p < 0.01$; Pound sign was that compared with model group $^{\#}p < 0.01$.

ferred at 90 V at a constant low temperature and followed by blocking with 5% not-fat dry milk. The monoclonal anti-rabbit antibody TRAF6 (Abcam, Cambridge, MA, USA) and anti-rabbit polyclonal antibody IRAK1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), were added to maintain a concentration of 1:1000 and incubated at 4°C overnight. The membrane was washed three times to incubate goat anti rabbit IgG with concentration dilution, according to the instructions at 4°C overnight and then was washed three times. Chemiluminescence was utilized to detect. Protein expression was standardized according to β-actin and gray scale scanning and quantitation were obtained by software.

Statistical Analysis

All data were performed for variance analysis by SPSS19.0 statistical software (SPSS Inc. Chicago, IL, USA). The analysis results were presented by mean ±standard deviation (SD). Data was analyzed by *t*-test. The $p<0.05$ indicated that there was statistical significance.

Results

Overexpression of mir-146b-5p Reduced the IL-6 and IL-8 Levels in the Cell Culture

Patients with active lupus nephritis showed elevated urinary IL-6 secretion[27] and the expression of IL-6 was increased along glomerular and tubular tissue in lupus nephritis kidneys *in situ*[18]. IL-6 and IL-8 were detected in the cell culture. As shown in Figure 1, the IL-6 and IL-8 in the miR-146b-5p mimic group were significantly lower than those in the control group ($p<0.01$); they were reduced 52.8% and 65.7%, respectively. Furthermore, the IL-6 and IL-8 in the miR-146b-5p inhibitor group were significantly higher than those in the control group ($p<0.01$), increased 65% and 48.5%, respectively. These data suggested that overexpression of miR-146b-5p may attenuate the inflammatory response by reducing IL-6 and IL-8, and inhibited the miR-146b-5p expression, can increase the cell inflammatory response.

Overexpression of MiR-146b-5p Inhibited Glomerular Mesangial Cell Proliferation

Next, we examined whether miR-146b-5p overexpression inhibits glomerular mesangial cell proliferation. As shown in Figure 2, compared to the cell proliferation at 24 h and 48 h in control group (0.369±0.023 and 0.412±0.029), those in miR-146b-5p mimic group (0.322±0.019 and 0.286±0.017) were significantly lower ($p < 0.01$). In contrast, there was no significant difference in the cell proliferation of miR-146b-5p inhibitor

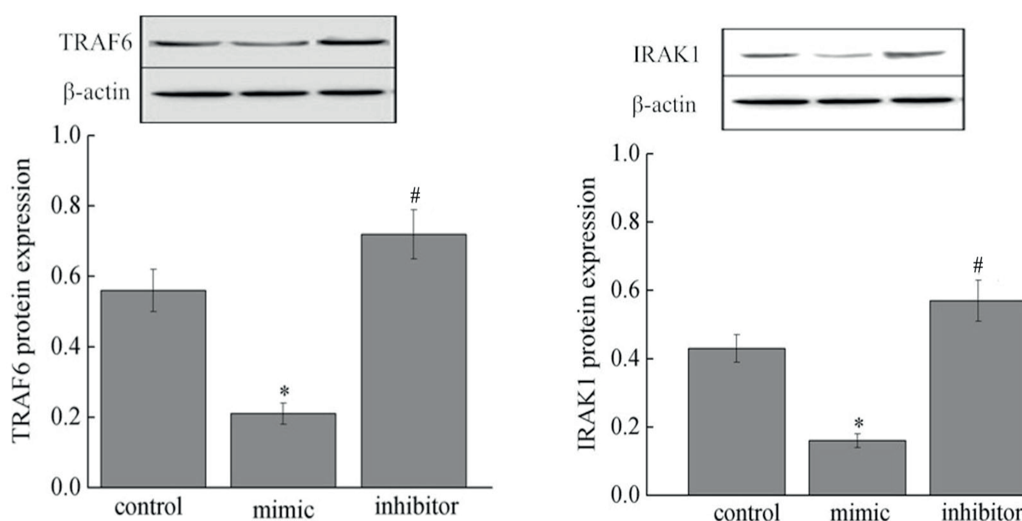

Figure 4. Protein expression levels. ***A,*** TRAF6; ***B,*** IRAK1. Asterisk was that compared with positive group. $^*p < 0.01$; Pound sign was that compared with model group $^{\#}p < 0.01$.

group (0.376±0.021 and 0.436±0.031) compared to the control group ($p > 0.05$), and there was a significative difference compared with the miR-146b-5p mimic group ($p < 0.01$). The result indicated that miR-146b-5p overexpression could inhibit glomerular mesangial cell proliferation in lupus nephritis to some degree.

Overexpression of miR-146b-5p Inhibited the Expression of TRAF6 and IRAK1 mRNA

To explore the mechanism of miR-146b-5p overexpression regulating the expressions of TRAF6 and IRAK1 mRNA, the expressions of TRAF6 and IRAK1 mRNA were measured by RT-PCR. Compared with the control group, the expression of TRAF6 and IRAK1 mRNA in miR-146b-5p mimic group were significantly decreased ($p < 0.01$), reduced 47% and 59%, respectively. Furthermore, the mRNA expression level of TRAF6 and IRAK1 in miR-146b-5p inhibitor group was significantly higher than those in control group ($p < 0.01$), increased 21% and 16%, respectively (Figure 3). These data suggested that miR-146b-5p overexpression could inhibit the expression of TRAF6 and IRAK1 mRNA.

Overexpression of miR-146b-5p Could Inhibit the Expression of TRAF6 and IRAK1 Protein

We detected the expression of TRAF6 and IRAK1 protein to investigate the effect of miR-146b-5p over expression on the expressions of TRAF6 and IRAK1 protein, and the proteins were measured by Western blot. As shown in Figure 4, the expression of TRAF6 and IRAK1 protein in the miR-146b-5p mimic group (0.21±0.03 and 0.16±0.02) were significantly lower than those in the control group (0.56±0.06 and 0.43±0.04) ($p<0.01$). Conversely, there was no significant difference in the miR-146b-5p inhibitor group (0.72±0.07 and 0.57±0.06) ($p<0.05$). The results demonstrated that over expression of miR-146b-5p could inhibit the expression of TRAF6 and IRAK1 protein and reduce the inflammatory response of lupus nephritis.

Discussion

In lupus nephritis, glomerular immune complexes deposition is known to be pathogenesis of renal disease. In previous studies, some miRNAs were recognized as the important regulators of renal disease[28,29]. For example, increased expression of let-7a may lead to enhanced expression of signaling molecules that induce or maintain IL-6 expression in immune-stimulated mesangial cells. Also, expression of miRNA let-7a increased significantly in pre-diseased and actively diseased NZB/W mice compared to controls[30]. However, the pathway of miR-146b-5p regulation immune complex is not known well yet. In the present work, we investigated the

role of miR-146b-5p in TLR4 pathway of glomerular mesangial cells in lupus nephritis. The glomerular mesangial cells were cultured in *vitro* and divided into 3 groups: control group, a group of miR-146b-5p mimic added and a group of miR-146b-5p inhibitor added. The levels of IL-6 and IL-8, the cell proliferation and TLR4 pathway-associated TRAF6, IRAK1 mRNA and protein, were detected by protein assays and analysis methods, respectively. We demonstrated that the overexpression of miR-146b-5p could reduce IL6 and IL8 in cell culture and could inhibit glomerular mesangial cell proliferation to certain degree. These data suggested that an overexpression of miR-146-5p may incline lupus mice to decrease inflammatory mediator production with immune stimulation.

While the overexpression of miR-146-5p could reduce cell proliferation in mesangial cells, the cell proliferation level in miR-146b-5p inhibitor group was not significantly different to those in controls. This suggests that increasing miR146b-5p is sufficient to reduce mesangial cell proliferation, but inhibiting the expression of miR146b-5p alone is not sufficient to increase mesangial cell proliferation. If there is an inherent difficultly in increasing mesangial cells proliferation through miR146b-5p alone, some other signaling pathway may play a role in the cells proliferation and inflammatory mediator production.

Then, in our investigation, we have shown that the overexpression of miR-146b-5p could inhibit the expressions mRNA and protein expressions of TLR4 pathway signaling TRAF6 and IRAK1. It was illustrated that a potential therapeutic approach to resolve TLR4 pathway related infiltrating inflammatory cells may affect overexpression of miR-146b-5p. The protein expression levels of TRAF6 and IRAK1 in the miR-146b-5p inhibitor group do not significantly increased compared to the controls. This suggests that miR-146b-5p alone is not sufficient factor to affect TLR4 pathway related infiltrating inflammatory reaction. There may be other factors that play a role in the process of immune complexes deposition. In the present study, miR-146a was found to be a NF-κB-dependent gene[31]. Upon LPS-stimulated induction, miR-146a directly decreased the TRAF6 and IRAK1 production. While acknowledging the complexity of the underlying mechanism, not only one miRNA regulates the expressions of mRNA and protein of TLR4 pathway-associated signaling TRAF6 and IRAK1. Our work may be utilized for the potential treatment of lupus nephritis to target miR-146b-5p to reduce IL-6 and IL-8 production and the expression levels of TRAF6 and IRAK1 in lupus nephritis. Since miR-146b-5p reduces IL-6 and IL-8, which may be a potential therapeutic target of other diseases as well.

Conclusions

These results demonstrated that the overexpression of miR-146b-5p could reduce IL6 and IL8 production in cell culture and could inhibit glomerular mesangial cell proliferation to some degree. Furthermore, the overexpression of miR-146b-5p could inhibit the expressions of mRNA and protein of TLR4 pathway-associated factor TRAF6 and IRAK1. Our data were significantly important for the potential novel gene therapy for lupus nephritis. We suggested overexpression of miR-146b-5p may result in an improvement in lupus nephritis outcome.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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