European Review for Medical and Pharmacological Sciences 2017; 21: 1041-1048

The role of miR-146a in modulating TRAF6-induced inflammation during lupus nephritis

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Abstract. – OBJECTIVE: Lupus nephritis (LN) is a major complication of systemic lupus erythematosus (SLE). A previous study showed decreased expression level of microRNA (miR)-146a in LN patients, indicating its possible role in LN pathogenesis.

PATIENTS AND METHODS: A total of 98 LN patients were recruited, for the collection of renal tissue samples during biopsy or surgery. Another cohort of 15 patients who had renal tumor resection was recruited as the control group, for the further comparison of expression levels of miR-146a, TRAF6 and p-p65 in tissues. Human glomerular mesangial cells were treated with miR-146a mimics, si-TRAF6 or both, followed by the evaluation of p65, p-p65, IL-1β, IL-6, IL-8 and TNF-α. Transwell assay was performed to detect the effect of mesangial cells on chemotaxis of macrophage.

RESULTS: MiR-146a expression was significantly depressed in renal tissues of LN patients, while TRAF6 expression, macrophage infiltration and p-p65 expression were all elevated as the activity of LN was induced. The up-regulation of miR-146a and/or down-regulation of TRAF6 can significantly inhibit NF-κB transcriptional activity of glomerular mesangial cells, while the gene expressions of IL-1β, IL-6, IL-8 and TNF-α were suppressed.

CONCLUSIONS: The expression of miR-146a in renal tissues of LN patients was significantly depressed, while the transcriptional activity of TRAF6 and NF-κB was enhanced. MiR-146 thus inhibited NF-κB transcriptional activity and inflammatory factor synthesis, and alleviated chemotactic effect towards macrophage via the inhibition of TRAF6 activity.

Key Words:
Lupus nephritis, MicroRNA-146a, TRAF6, NF-ÐB, Inflammatory factor, Macrophage.

Introduction

Systemic lupus erythematosus (SLE) is one autoimmune disease featured with multi-organ injury with unknown reasons. The kidney is the major affected organ of SLE, frequently causing lupus nephritis (LN)[1]. About 30-50% of SLE patients show a renal injury[2]. LN belongs to immune damage, which presents as various pathological types of SLE, in coupled with significant kidney damage such as hematuria, proteinuria or kidney dysfunction. The occurrence of LN is correlated with the immune complex formation, or the abnormal immunity of immune cells or cytokines[3-6]. The golden standard of LN diagnosis is the renal biopsy, but it has certain limitations due to the invasive nature. The exploration of LN-related biomarkers is, thus, of critical importance. MicroRNA (miR) is one non-coding small RNA molecule with about 22 to 25 nt in eukaryotes. It has highly conserved sequence across species. It can bind with 3'-untranslated region (3'-UTR) of target gene mRNA and regulate specific gene expression via the degradation of mRNA or inhibition of translation. Therefore, MiR is involved in the epigenetic modulation of multiple biological processes including immune inflammation[7-9].

miR-146a is the member of miR-146 family. Located on chromosome 5, miR-146a plays a critical role in modulating activation of NK-κB signal pathway and inflammatory factor release that were induced by toll-like receptor (TLR)-4. By inhibiting TNF receptor-associated factor 6 (TRAF6), which is one intermediate molecule between TLR4 and NF-κB, miR-146a can inhibit the transcriptional activity of NF-κB. The downstream inflammatory factors of which including IL-1β, IL-6, IL-8 and TNF-α were further downregulated, thus exerting negative regulation of immune inflammation to prevent over-reaction[10-13]. The dysregulation of miR-146a expression is also correlated with the occurrence of multiple autoimmune diseases including SLE[14], rheumatoid arthritis[15] and autoimmune thyroid disorder[16]. Some studies[17,18] showed decreased of

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miR-146a expression in LN patients, indicating a possible role of miR-146a in immune dysregulation and LN pathogenesis, although its detailed mechanism is still unclear.

Patients and Methods

Clinical Information

A total of 98 LN patients who received diagnosis and treatment in the Department of Nephrology in Traditional Chinese Hospital of Lu'An from August 2014 to January 2016 were recruited in this study. There were 12 males and 86 females in this cohort, aging at 38.2 ± 13.6 years. No immune suppressant, immune modulator or steroid hormone has been used within 3 months. No one was complicated with malignant tumor, acute/chronic infection or other autoimmune diseases. SLE disease activity index (SLEDAI) was employed based on clinical manifestation and lab tests. Those patients with equal or less than 4 points were classified as the static stage (N = 40, average score = 2.8 ± 0.9), while those with higher than 5 points were regarded as in the active phase (N=58, average score = 10.4 ± 3.7). Pathological samples were collected from renal tissues of LN patients. Another 15 normal renal tissue samples were collected from age/sex-matched patients who received renal tumor resection, but without primary glomerulonephritis, diabetes, and hypertension. Renal tissue samples were prepared for frozen section, and for protein/RNA extraction.

This study has been pre-approved by the Ethical Committee of Traditional Chinese Hospital of Lu'An. All patients have signed the consent forms before recruitment in this study.

Major Reagent and Materials

Human glomerular mesangial cell (HMC) was purchased from ATCC (ATCC, Manassas, VA, USA). Roswell Park Memorial Institute (RPMI)-1640, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (Rockville, MD, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Reverse transcription kit ReverTra Ace sPCR RT Kit was purchased from Toyobo (Toyobo, Osaka, Japan). SYBR Green Real-time PCR master mix was purchased from Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). siRNA interference sequence, microRNA mimic and PCR primer were synthesized by Gimma (Gimma, Shanghai, China). Rabbit anti-human NF-κB, p65, p-p65 and CD68 antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mouse anti-human histone H3.1, and rabbit anti-human TRAF6 antibodies were purchased from Abcam (Cambridge, MA, USA). Total tissue protein extraction kit was purchased from Phygene (Phygene, Fuzhou, Fujian, China).

Cell Culture and Grouping

HMC cells were cultured in 1640 medium containing 10% FBS, 10 mg/L insulin, 5.5 mg/L transferrin, and 6.7 μg/L sodium selenite, and were divided into 5 groups: mimic-NC, miR-146a mimic, si-NC, si-TRAF6 and miR-146a mimic+si-TRAF6. Nucleotide acid sequences were: si-TRAF6-sense: 5'- CCAGG AAUUU GACUC CCAAT T-3'; si-TRAF6-anti-sense: 5'-UUGGG AGUCA AAUUC CUGGC -3'; si-NC-sense: 5'-UUCUC CGAAC GUGUC ACGUT T-3'; si-NC-anti-sense: 5'-ACGUG ACACG UUCGG AGAAT T-3'.

qRT-PCR for Gene Expression

Trizol was used to extract total RNA from cells. ReverTra Ace aPCR kit was used to synthesize cDNA via reverse transcription. In a total of 20 μL system, there were 2 μg total RNA, 4 μL RT buffer (5×), 1 μL oligo dT+Random primer Mix, 1 μL RT enzyme mix, 1 μL RNase inhibitor and ddH_2O. The reverse transcription was performed under the follow conditions: 37°C 15 min, followed by 98°C 5 min. Using cDNA as the template, PCR amplification was performed with TaqDNA polymerase. Primer sequences were: miR-146a1P_F: 5'-TGAGA ACTGA ATTCC ATGGG TT-3'; miR-146a1P_R: 5'-TCACC CGTAG AACCG ACCTT-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; TRAF6P_F: 5'-ATGCG GCCAT AGGTT CTGC-3'; TRAF6P_R: 5'-TCCTC AAGAT GTCTC AGTTC CAT-3'; IL-1βP_F: 5'-ATGAT GGCTT ATTAC AGTGG CAA-3'; IL-1βP_R: 5'-GTCGG GATTC TAGCT GA-3'; IL-6P_F: 5'- ACTCA CCTCT TCAGA ACGAA TTG-3'; IL-6P_R: 5'-CCATC TTTGG AAGGT TCAGG TTG-3'; IL-8P_F: 5'-TTTTG CCAAG GAGTG CTAAA GA-3'; IL-8P_R: 5'-AACCC TCTGC ACCCA GTTTT C-3'; TNFαP_F: 5'-CCTCT CTCTA ATCAG CCCTC TG-3'; TNFαP_R: 5'-GAGGA CCTGG GAGTA GATGA G-3'; β-actinP_F: 5'-GAACC CTAAG GCCAA C; β-actinP_R: 5'-TGTCA CGCAC GATTT CC-3'. In a PCR system with 10 μL total volume, we added 4.5 μL 2 × SYBR Green Mixture, 0.5 μL of for-

ward/reverse primer (at 2.5 μm/L), 1 μL cDNA, and 3.5 μL ddH_2O. PCR conditions were: 95°C for 15 s, 60°C for 30 s and 74°C for 30 s. The reaction was performed on ABI ViiA7 fluorescent quantitative PCR cycler (Applied Biosystems, Foster City, CA, USA) for 40 cycles. Each sample was tested in triplicates.

Western Blotting for Protein Expression

Cells were collected and extracted for total proteins. After quantification, 50 μg samples were loaded for separation by SDS-PAGE for 3 h. Proteins were then transferred to PVDF membrane (1.5 h), which was then blocked by 5% defatted milk powder for 1h, followed by primary antibody (TRAF6 at 1:2000, p65 at 1:2000, p-p65 at 1:1000, Histone H3.1 at 1:5000 or β-actin at 1:10000) incubation at 4°C overnight. PBST (phosphate buffered saline and Tween 20) was used to wash the membrane for three times to remove excess antibody. A secondary antibody (goat anti-mouse IgG at 1:5000 or goat anti-rabbit at 1:5000) was then added for 1h room temperature incubation, followed by PBST washing. ECL reagent was then added for development, followed by exposure and scanning the film.

Immunofluorescence for Macrophage Infiltration

Renal tissue sections were dried at room temperature for 20 min, and were rinsed three times in PBS to remove OCT. The tissue was blocked in 10% goat serum for 60 min at room temperature, and was washed with PBS three times. Mouse anti-human CD68 monoclonal antibody (1:200 dilution) was added for 4°C overnight incubation. After rinsing in PBS for three times, Alexa Fluor 594 labeled secondary antibody (1:400) was added for 60 min incubation, followed by PBS washing. After DAPI staining and mounting, images were captured with a fluorescent microscope.

Transwell Assay for Migration of Macrophage

Type IV collagen was paved on the upper surface of Transwell chamber (pore size: 8 μm), which was filled with 200 μL macrophage cell suspension (2×10^6/mL). The lower chamber was inoculated with HMC from all five groups. After 48 h incubation, the liquid in the upper chamber was discarded. Un-penetrated cells were removed by sterilized cotton swabs, followed by PBS washing twice and methanol fixation for 30 min. The membrane was stained with 0.1% crystal violet for 20 min. Under the microscope, five randomly selected fields were counted for the number of cells.

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean±standard deviation (SD), while enumeration data were presented as percentage. Student *t*-test or analysis of variance (ANOVA) was used to compare measurement data among groups. Statistical significance was defined when $p < 0.05$.

Results

MiR-146a was Downregulated in Renal Tissues of LN Patients

qRT-PCR results showed that, compared to normal kidney tissues, miR-146a expression in renal cortical tissues in LN patients was significantly depressed. MiR-146a level was further higher in non-active LN patients compared to those in active LN cases (Figure 1A). TRAF6 gene expression level was significantly higher in LN patient renal cortical tissues compared to normal ones (Figure 1B). The immune fluorescent assay showed more macrophage infiltration in renal tissues of active LN patients compared to non-active ones, which further led to the higher level of macrophage infiltration compared to the normal tissues (Figure 1C). Western blotting results exhibited higher TRAF6 and p-p65 protein levels in renal cortical tissues of active LN patients compared to those in non-active LN patients and normal tissues (Figure 1D).

Elevation of miR-146a Inhibited NF-κB Transcriptional Activity of HMC

The abnormal function of glomerular mesangial cells is critical in the pathogenesis of LN. This study found significantly lower miR-146a and TRAF6 expression in renal tissues of LN patients, which was correlated with disease activity of LN. Due to the known effect of miR-146a in modulating TRAF6 and NF-κB transcriptional activity, this study investigated the effect of miR-146a in HMC. Compared to NC group, the transfection of miR-146a mimic elevated miR-146a expression (Figure 2A), and significantly depressed the expression of TRAF6

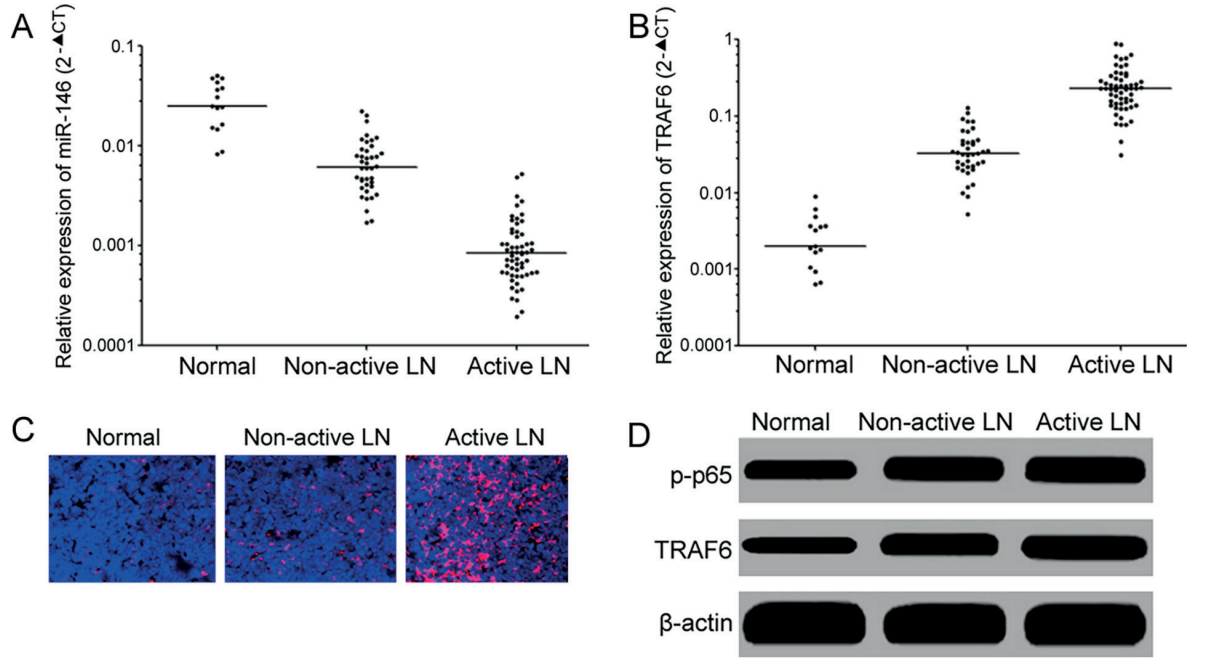

Figure 1. Decreased miR-146a expression in renal tissues of LN patients. ***(A)*** qRT-PCR for miR-146a expression; ***(B)*** qRT-PCR for TRAF6 mRNA expression; ***(C)*** Immunofluorescence for macrophage infiltration; ***(D)*** Western blotting for protein expression level.

mRNA and protein in HMC (Figure 2B and 2C). Meanwhile, phosphorylation level of NF-κB p65 protein and nuclear expression level of p65 were remarkably decreased. The silencing of TRAF6 also inhibited the transcriptional activity of NF-κB (Figure 2B and 2C).

Over-expression of miR-146a Inhibited Synthesis of Inflammatory Factors by HMC and Macrophage Migration

Based on the effect of miR-146a in the modulation of TRAF6-mediated NF-κB transcriptional activity and inflammatory response, we further measured the effect of miR-146a on the synthesis of inflammatory factor inside HMC. Results showed that the over-expression of miR-146a and/or silencing of TRAF6 significantly depressed the synthesis of inflammatory factors including IL-1β, IL-6, IL-8 and TNF-α mRNA, and decreased the chemotactic effect of HMC towards macrophage. The strategy, which combined over-expression of miR-146a with silencing of TRAF6, contributed to potent inhibitory effects on inflammatory factor and chemotaxis of macrophage (Figure 3A and 3B).

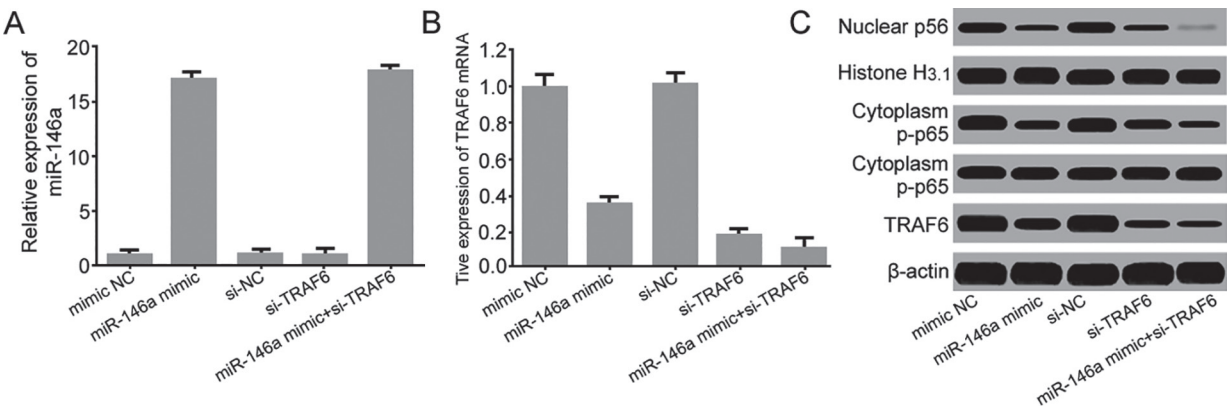

Figure 2. Inhibition of NF-κB transcriptional activity in HMC cells by miR-146a over-expression. ***(A)*** qRT-PCR for miR-146a expression; ***(B)*** qRT-PCR for TRAF6 mRNA expression; ***(C)*** Western blotting for protein expression level.

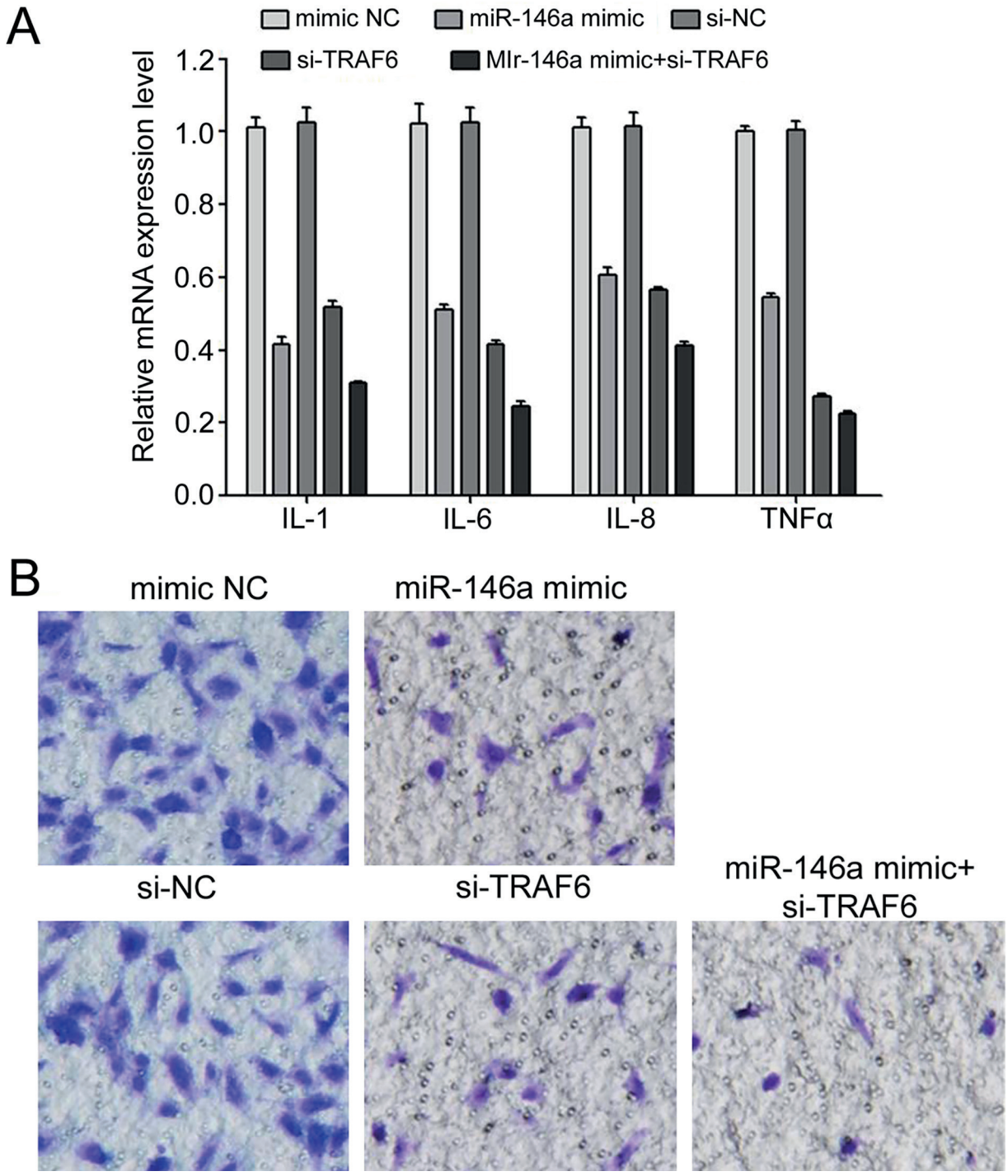

Figure 3. MiR-146a over-expression and the related effects. ***(A)*** qRT-PCR for mRNA level of inflammatory factors; ***(B)*** Transwell assay for macrophage migration.

Discussion

SLE is an autoimmune disorder affecting multiple organs and having recurrent progress. Major features of SLE are the over-production of auto-antibody and immune complex, both of which deposit in host tissues/organs, and induce immune inflammatory response, injury or damage[19]. The kidney is the major affecting organ of SLE, as about 30-50% SLE patients developed renal injury, causing LN[20,21]. As a major and severe complication of SLE, LN patients may

develop into end-stage renal disease, which is associated with higher mortality and unfavorable prognosis[22]. During the whole process of LN, recurrence and remission may alter repeatedly. Those patients at active stage frequently develop progressive renal damage and kidney dysfunction, both of which are major reasons for SLE-related mortality[23]. Therefore, the knowledge of activeness of LN, and the observation of disease condition, are of critical importance for the treatment and prognosis. Renal glomerular mesangial cells play an important function for immune modulation in the focal region of glomerulus. Under pathological conditions, it can be activated to release various inflammatory factors, and induce infiltration of various inflammatory cells including macrophage, neutrophils, B cells, T cells and dendritic cells, further exaggerating inflammatory cascade reaction and worsening renal damage[24,25]. MicroRNAs play an important role in modulating immune cell function and inflammatory mediator, and participate in LN pathogenesis[26,27]. Kato et al[28] found that miR-216a and miR-217 could facilitate the synthesis and secretion of inflammatory factors by renal glomerular mesangial cells via targeting PTEN/AKT signal molecules. Chafin et al[29] found that elevated let-7a in renal glomerular mesangial cells from LN patients might facilitate the synthesis and secretion of inflammatory factor IL-6, which leads to the high reactivity of mesangial cells towards inflammation. Recent studies[17,18] also reported down-regulation of miR-146a in LN patients, indicating that miR-146a might play a role in immune reaction dysfunction and LN pathogenesis, although detailed mechanism is still unclear.

This study showed that, compared to normal renal tissues, renal cortex of LN patients had significantly depressed miR-146a expression. Those LN patients at the active stage even showed the lowest level. TLR4-induced signal transduction plays an important role in the initiation of the inflammatory response. miR-146a targets TRAF6, which is one of the downstream factors of TLR4, modulates the transcriptional activity of NK-κB, which is critical for inflammatory response, exerting negative control on immune functions, and protecting the immune system from over-activation[30-33]. This study showed remarkably higher TRAF6 expression in renal tissues of LN patients, as opposite to that of miR-146a, suggesting that down-regulation of miR-146a and up-regulation of TRAF6 might play a role in LN pathogenesis. Moreover, LN patients had more macrophage infiltration in renal tissues, especially in the active stage, indicating that the transcriptional activity of NK-κB was also related with the active stage of LN.

Renal glomerular mesangial cells play an important role in the mediation of inflammatory factor release, inflammatory cell infiltration, and inducing LN pathogenesis[24,25]. In Toll/TRAF6/NK-κB induced inflammatory response, the activation of NK-κB transcription up-regulates the synthesis and secretion of various inflammatory factors including IL-1β[34], IL-6[35], IL-8[36] and TNF-α[37]. This study showed that the up-regulation of miR-146a and/or down-regulation of TRAF6 significantly inhibited the transcriptional activity of NK-κB, and simultaneously inhibited synthesis of IL-1β, IL-6, IL-8 and TNF-α, thus weakening their chemotactic effects on macrophage.

Conclusions

MiR-146a relieved inflammatory response via the inhibition of TRAF6 and may present as a promising drug in the future renal therapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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