Int J Clin Exp Pathol 2019;12(5):1578-1588
www.ijcep.com /ISSN:1936-2625/IJCEP0093022

*Original Article*

# MicroRNA-124 represents a novel diagnostic marker in human lupus nephritis and plays an inhibitory effect on the growth and inflammation of renal mesangial cells by targeting TRAF6

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Received February 25, 2019; Accepted March 27, 2019; Epub May 1, 2019; Published May 15, 2019

**Abstract:** microRNAs (miRs) are short non-coding RNAs that function as guide molecules in RNA silencing by inducing mRNA degradation or blocking protein translation. Increasing evidence has shown that miRNAs play an important role in regulating the pathological process of lupus nephritis (LN), but the precise role of miR-124 in LN is still unknown. Here, we found that miR-124 expression is significantly reduced in patients with active LN compared with those patients with non-active LN and the absence of LN. Additionally, the miR-124 level was negatively correlated with serum IL-1β, IL-6, TNF-α, and TRAF6 mRNA expressions in active LN patients. Receiver operating characteristic and logistic regression analyses revealed miR-124 is a significant diagnostic biomarker for active LN. Furthermore, transfection of the miR-124 mimic into human renal mesangial cells (HRMCs) resulted in significantly reduced cell proliferation, induced cell apoptosis, and decreased synthesis of inflammatory factors. Moreover, a dual luciferase assay showed that TRAF6 was a direct target of miR-124, and the expression of TRAF6 was suppressed by miR-124 through direct binding to the 3'-UTR of mRNA. Mechanistic studies demonstrated that the over-expression of TRAF6 could abrogate miR-124-related effects on cell proliferation, apoptosis and the synthesis of inflammatory factors in HRMCs. Taken together, these findings indicate that downregulated miR-124 represents a novel diagnostic marker in human LN and plays an inhibitory effect on the growth and inflammation of renal mesangial cells by targeting TRAF6.

**Keywords:** Lupus nephritis, miR-124, expression, mRNA, biomarker, TRAF6

## Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of a wide range of immune complexes in multiple organs [1]. Lupus nephritis (LN) is the most common manifestation of SLE and contributes greatly to the mortality and morbidity of patients with SLE [2]. LN is defined as progressive glomerulonephritis with a variety of pathological disorders, including glomerular damage, leukopenia, proteinuria, and hematuria [3]. In recent years, novel immunosuppressive drugs such as belimumab, mycophenolate mofetil, and anti-CD20 monoclonal antibody have improved the survival of patients with LN; however, approximately one-third of LN patients develop end-stage renal disease within 10-20 years [4, 5]. Therefore, finding new non-invasive biomarkers and identifying novel therapeutic targets are urgently needed for the treatment of LN.

MicroRNAs (miRs) are a group of highly conserved small non-coding RNA molecules composed of ~22 nucleotides, and they can regulate gene expression at the post-transcriptional level by binding to the 3'-untranslated region (UTR), resulting in the degrading or blocking of the translation of mRNA [6]. miRs have been implicated in playing important roles in the modulation of innate and adaptive immune responses [7]. Previous studies have shown that aberrant miR expression has been demonstrat-

ed in LN patients, such as in the blood serum, renal tissue, and urine, and is involved in the pathogenesis and progression of LN. For instance, Khoshmirsafa et al. reported that miR-21 and miR-155 are elevated in the peripheral blood of active LN patients, and the two miRs are significant risk factors for LN [8]. Tangtanatakul et al. showed that let-7a and miR-21 are significantly down-regulated in urine exosomes from LN patients and could be used to guide the clinical stages of LN patients [9]. Wang et al. found that miR-663a/miR-423-5p had a higher expression in renal biopsy tissues from LN patients as compared to the control group [10]. In addition, some studies also suggest that miRs play crucial roles in the pathogenesis of LN by interplaying with target genes and/or pathways involved in inflammation, proliferation, differentiation, and apoptosis [11-13]. Based on the above findings, discovering other miRs that mediate the onset and development of LN should provide novel avenues for developing treatments for LN.

While the precursors of miR-124 from different species are different, the sequences of mature miR-124 are completely identical. Interestingly, miR-124 is the most abundantly expressed miRNA in immune cells and organs, including the bone marrow, lymph nodes, the thymus, and peripheral blood mononuclear cells. Recent studies have reported that many inflammatory diseases and immune disorders show altered miR-124 expression, and miR-124 plays an important role in regulating innate and adaptive immune responses [14]. The study of Sun et al. implied that miR-124 is upregulated by cholinergic agonists in LPS-exposed cells and mice, and miR-124 modulates TLR4-induced cytokine production by targeting signal transducers and the activator of STAT3 to decrease IL-6 production and TNF-α's converting enzyme (TACE) to reduce TNF-α release [15]. TNF receptor-associated factor 6 (TRAF6) is one intermediate molecule between TLR4 and NF-κB. It is well-recognized that TRAF6 intensifies renal injury in the progression of diverse renal disorders [16].

According to the above information, we hypothesized that the miR-124/TRAF6 pathway is involved in the pathogenesis of LN. To verify the hypothesis, the expression profile and clinicopathological significance of miR-124 were first determined by examining blood samples from SLE patients. The relationship between miR-124 and inflammatory cytokines and TRAF6 expression was analyzed. Subsequently, a dual luciferase assay was applied to explore whether TRAF6 was a direct target of miR-124. Finally, the effects of miR-124's targeting of TRAF6 on cell proliferation, apoptosis, and the synthesis of inflammatory factors of human renal mesangial cells (HRMCs) were investigated.

## Materials and methods

### *Cell culture and cell transfection*

Human renal mesangial cells (HRMCs) were purchased from Jennio Biological Technology Co., Ltd. (Guangzhou, China). The HRMCs were cultured in an RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Carlsbad, CA, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, MO, USA) at 37°C in a 5% $CO_2$-humidified atmosphere. The miR-124 mimic and its negative control, and the TRAF6 overexpressing vector and the control vector were ordered from GenePharma Co., Ltd (Shanghai, China). HRMCs in the logarithmic phase were placed into 6-well plates at 37°C for 24 h before cell transfection. The HRMCs were placed into the following groups: the miR-124 mimic group, the negative control group, the miR-124 mimic and TRAF6 overexpressing vector group, and the miR-124 mimic and control vector group. Briefly, the HRMCs were transfected by 100 pmol miR-124 mimic/negative control or 100 pmol miR-124 mimic with 2 μg TRAF6 overexpressing vector/control vector when their confluence reached 70% according to the instructions provided by Lipofectamine 2000 (Thermo Fisher Scientific Inc, MA, USA). The transfected cells were collected for total RNA isolation at 48 h post-transfection.

### *Clinical laboratory testing*

Serum samples were acquired from peripheral blood by centrifugation at 3000 g for 10 min at room temperature. Body mass index (BMI) was presented as weight divided by height squared ($kg/m^2$). White blood cells, hemoglobin, creatinine, blood urea nitrogen (BUN), proteinuria, and the erythrocyte sedimentation rate (ESR) were quantified using a Fully Automated Chemistry Analyzer (AU400, Olympus, Orlando, FL, USA). The concentrations of serum complement component 3 (C3) and complement com-

**Table 1.** Comparison of the clinical characteristics in systemic lupus erythematosus (SLE) patients and healthy controls

| Characteristics | SLE patients (n = 31) | Healthy controls (n = 31) | *P* |
|---|---|---|---|
| Age (years) | 39.42 ± 8.55 | 41.20 ± 6.96 | 0.16 |
| BMI (kg/m$^2$) | 24.30 ± 2.17 | 25.11 ± 3.02 | 0.74 |
| White blood cell (× 10$^9$/L) | 7.24 ± 1.05 | 7.10 ± 1.26 | 0.96 |
| Hemoglobin (g/dL) | 9.60 ± 2.46 | 13.01 ± 1.58 | < 0.01 |
| Creatinine (μmol/L) | 90.22 ± 18.47 | 75.49 ± 11.50 | < 0.01 |
| BUN (mmol/L) | 5.31 ± 1.20 | 4.70 ± 0.96 | 0.08 |
| Proteinuria (g/d) | 1.50 ± 0.44 | - | < 0.01 |
| C3 (g/L) | 0.54 ± 0.26 | 1.25 ± 0.30 | < 0.01 |
| C4 (g/L) | 0.17 ± 0.11 | 0.39 ± 0.09 | < 0.01 |
| ESR (mm/h) | 37.94 ± 7.88 | 10.39 ± 3.41 | < 0.01 |
| ANA | 1/640 | < 1/80 | < 0.01 |
| Anti-dsDNA | 10.33 ± 2.40 | - | < 0.01 |
| Anti-Sm | 4.10 ± 0.95 | - | < 0.01 |
| Anti-SSA | 9.22 ± 1.80 | - | < 0.01 |
| Anti-SSB | 3.78 ± 1.35 | - | < 0.01 |

BUN, blood urea nitrogen; C3, complement component 3; C4, complement component 4; ESR, erythrocyte sedimentation rate; Anti-dsDNA, anti-double-stranded DNA; ANA, antinuclear antibody; Anti-Sm, Anti-Smith; Anti-SSA, anti-Sjögren's syndrome (SS)-A; Anti-SSB, anti-Sjögren's syndrome (SS)-B.

ponent 4 (C4) were detected using the nephelometry method. Anti-double-stranded DNA (dsDNA), anti-Sjögren's syndrome (SS)-A, -SSB, and -Smith (Sm) were assayed using an enzyme linked immunosorbent assay (ELISA). Antinuclear antibody (ANA) was assayed using a chemiluminescence microparticle immunoassay (CMIA).

*Clinical information*

Thirty-one female patients with SLE (mean age 39.42 ± 8.55 years, range 16-67 years) and 31 healthy female volunteers (mean age 41.20 ± 6.96 years, range 18-60 years) were enrolled in the Department of Nephropathy, Tianjin Nankai Hospital (Tianjin, China) from February 2013 to April 2016. Six milliliters of peripheral blood from each subject was collected in K3EDTA tubes. SLE was diagnosed according to the American College of Rheumatology diagnostic criteria [17]. The diagnosis of LN was performed as per the criteria of the International Society of Nephrology/Renal Pathology Society (ISN/RPS) [18]. No patients had received immunosuppressive treatment or immune modulators within three months. Patients with malignant tumors, acute or chronic infections, or other autoimmune diseases were excluded. Based on kidney involvement, the SLE patients were divided into three subgroups, including active LN (n = 9), inactive LN (n = 16) and the absence of LN (n = 6). The clinical information of the SLE patients and healthy volunteers is summarized in **Table 1**. The present study was approved by the Ethics Committee of Tianjin Nankai Hospital and was performed in compliance with the Declaration of Helsinki. Written informed consent was obtained from each participant.

*Reverse transcription quantitative PCR (RT-qPCR)*

Total RNA from HRMCs and peripheral blood was extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and RNA concentration was determined using NanoDrop 2000 (Thermo Fisher Scientific Inc, MA, USA). cDNA was achieved with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real time PCR was performed in 96-well plates on an ABI 7500 system using the Taqman Gene Expression assay with a reaction system of 20 μl consisting of 1 μl template, 1 μl primers, 10 μl Taq PCR MasterMix, and 8 μl dd$H_2O$. Amplifications were performed under the following parameters: a denaturation step at 95°C for 20 min, 40 cycles of the amplification step at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The primers used were as follows: GAPDH, forward 5'-AAGAAGGTGGTGAAGCA-3' and reverse 5'-AGGTGGAGGAGTGGGT-3'; TRAF6, forward 5'-ATGCTGGTCATTCATC-3' and reverse 5'-GTAGGAGATTGGGAA-3'; miR-124, forward 5'-GCGGCCGTGTTCACAGCGGACC-3' and reverse 5'-GTGCAGGGTCCGAGGT-3'; U6, forward 5'-CGCTTCGGCAGCACATATACTA-3', and reverse 5'-CGCTTCACGAATTTGCGTGTCA-3'. The data were normalized based on the expression of the endogenous control, the relative expression of TRAF6 and miR-124 was calculated according to the formula of $2^{-\Delta\Delta ct}$.

*Dual luciferase assay*

The partial length TRAF6 3'-UTR was cloned into the pGL3 vector between the Xbal and

BamHI sites to establish pGL3-TRAF6 luciferase reporter vector. The mutant TRAF6 3'-UTR was generated as (GTGCCTT to ACATTCC) using a Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), and also inserted into the pGL3 vector between the Xbal and BamHI sites to build the pGL3-TRAF6 mutant luciferase reporter vector. The recombinant luciferase reporter vectors were confirmed by DNA sequencing. HRMCs were cultured in a 24-well plate the day before transfection. The cells were co-transfected with the recombinant luciferase reporter vectors, the pRL-TK vector, and a miR-124 mimic or negative control. At 48 h after transfection, cell lysates were collected and a luciferase assay was performed using a Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) under a chemiluminescence apparatus (NOX 4000, Aix-en-Provence, France). The pRL-TK vector served as an internal control. The luciferase activities were normalized to Renilla luciferase activity.

*Western blotting*

HRMCs were lysed using a RIPA lysis buffer with a protease inhibitor cocktail (Beyotime Biotechnology, Shanghai, China). Protein extracts were diluted in a 5 × SDS-PAGE loading buffer and heated at 102°C for 5 min. Subsequently, 30 μg proteins were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were incubated in a TBST buffer with 5% non-fat dry milk for 60 min at room temperature and incubated overnight at 4°C with TRAF6 (Catalog #8028) and a GAPDH (Catalog #51332) primary antibody (Cell Signaling Technology, USA) and then washed with a TBST buffer. After further incubation with a secondary antibody conjugated with horseradish peroxidase (HRP) for 60 min at room temperature, the membranes were visualized with the ECL Western Blotting Detection System (GE Healthcare Life Sciences, USA).

*Cell proliferation assay*

Cell proliferation was determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. HRMCs were plated into 96-well plates at 3000 cells per well and cultured in the growth medium overnight. Cells were then transfected with either 100 pmol miR-124 mimic/negative control or 100 pmol miR-124 mimic with 2 μg TRAF6 overexpressing vector/control in RPMI 1640 (without serum or antibiotics). 10 μL CCK-8 solution was added to each well, which were then incubated for 3 h in a cell incubator, and then we measured the absorbance at 450 nm using an ELISA reader. The number of viable cells was assessed at 0, 12, 24, 48, and 96 h after treatment.

*Enzyme-linked immunosorbent assay (ELISA)*

The levels of IL-1β, IL-6, and TNF-α were quantified using specific ELISA kits (Beyotime Biotechnology, Shanghai, China). Briefly, the HRMCs and the peripheral blood were lysed according to the manufacturer's protocols, and the supernatants were added into 96-well plates coated with primary antibodies for 60 min at room temperature. After washing the plates three times, biotinylated antibodies were added into each well for 30 min, and they were quantified using a HRP-conjugated streptavidin and chromogen reagent. The absorbance was immediately observed at 570 nm in an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

*Cell apoptosis assay*

Cell apoptosis was detected using a Caspase 3 ELISA assay and flow cytometry. After incubation for 48 h, a human Caspase 3 ELISA Kit (Catalog #EK1425, Boster Biological Technology, Wuhan, China) was used to detect the activity of Caspase 3 under an ELISA reader. In order to understand the accurate rate of apoptotic cells, flow cytometry was also performed. After transfection for 48 h, the HRMCs were collected and washed with a PBS buffer. Subsequently, 5 μl Annexin V-FITC (AV) and 2 μl Propidium Iodide (PI) were added to the cells, which were then incubated in darkness for 20 min. The rate of the apoptotic cells was measured using a Coulter Epics XL™ Flow Cytometer (Beckman, CA, USA).

*Statistical analysis*

SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used to analyze all the experimental data. Each experiment was independently repeated 3 times, and the data were expressed as the mean ± standard deviation. Comparisons of the differences between 2 groups were performed using a *t* test, and data between multiple groups were compared with the use of a one-way analysis of variance followed by Tukey's post

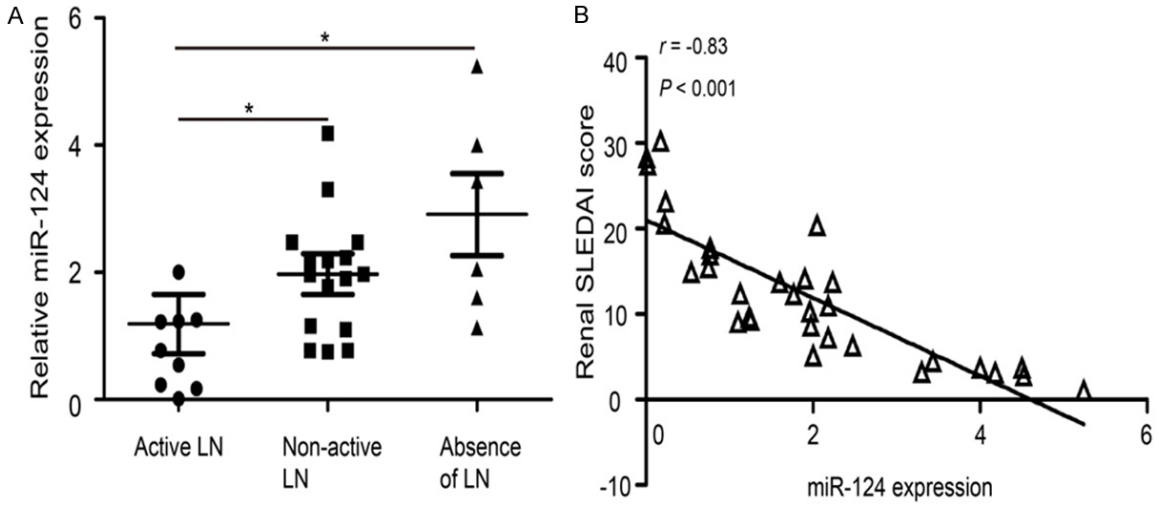


**Figure 1.** Serum miR-124 was downregulated in patients with active LN. A. RT-qPCR assay showed that lower levels of miR-124 were observed in active LN patients compared with those patients with non-active LN and the absence of LN. Data are presented as the mean ± standard deviation. B. A direct negative correlation was observed between miR-124 level and renal SLEDAI score. Three independent experiments were conducted. miR-124, microRNA-124; SLE, systemic lupus erythematosus; LN, lupus nephritis; RT-qPCR, reverse transcription quantitative PCR. *$P < 0.05$.

hoc test. Linear regression and Spearman's correlation coefficient were performed to assess the relationships between miR-124 level and IL-1β, IL-6, TNF-α, and TRAF6 expression. The diagnostic accuracy of miR-124 was evaluated using receiver operating characteristic (ROC) analysis and the areas under the ROC curve (AUC). Values of $P < 0.05$ were regarded as statistically significant.

## Results

*Comparison of clinical information in SLE patients and healthy volunteers*

As shown in **Table 1**, there were no significant differences in age, BMI, white blood cell, and BUN between the SLE patients and the healthy volunteers. Creatinine, proteinuria and ESR were higher in the SLE patients than in the healthy volunteers ($P < 0.01$). Hemoglobin, C3 and C4 were significantly lower in the SLE patients than in the healthy volunteers ($P < 0.01$). In addition, anti-dsDNA, -SSA, -SSB, -Sm, and ANA were significantly greater in the serum of the SLE patients than in the healthy volunteers ($P < 0.01$).

*Serum miR-124 was downregulated in patients with active LN*

To determine the expression status of miR-124 in human LN, the association of miR-124 expression and the kidney involvement of the SLE patients was analyzed. The results showed that miR-124 expression was lower in the patients with active LN compared with those with non-active LN and the absence of LN (**Figure 1A**, $P < 0.05$). Moreover, a direct negative correlation was observed between the miR-124 level and the renal SLEDAI score (**Figure 1B**, $r = -0.83$, $P < 0.001$).

*The miR-124 level was negatively correlated with serum IL-1β, IL-6, TNF-α, and TRAF6 mRNA expressions in patients with active LN*

A RT-qPCR assay revealed that, compared with the other SLE subgroups, TRAF6 mRNA expression was clearly upregulated in the serum of patients with active LN (**Figure 2A**, $P < 0.05$). Subsequently, the levels of serum IL-1β, IL-6, and TNF-α were detected using ELISA assay, and the data showed that these inflammatory factors were apparently higher in active LN patients than they were in the other SLE subgroups (**Figure 2B**, $P < 0.05$). Moreover, the correlation analysis shown in **Table 2** demonstrated that the miR-124 level was clearly negatively correlated with serum IL-1β ($r = -0.67$, $P < 0.01$), IL-6 ($r = -0.63$, $P < 0.01$), TNF-α ($r = -0.56$, $P = 0.03$), and TRAF6 mRNA expression ($r = -0.80$, $P < 0.001$) in patients with active LN.

*The diagnostic value of serum miR-124 on LN*

The diagnostic accuracy of serum miR-124 on LN was evaluated using a receiver operating

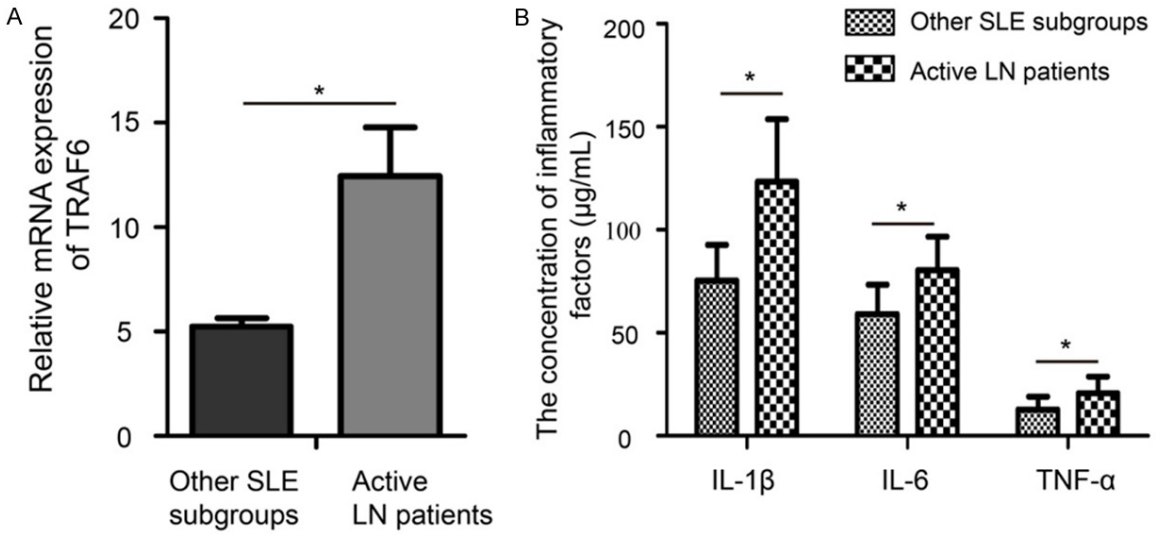


**Figure 2.** The serum IL-1β, IL-6, TNF-α, and TRAF6 mRNA expression was upregulated in patients with active LN. A. RT-qPCR assay revealed that TRAF6 mRNA expression was obviously upregulated in the serum of patients with active LN compared with the other SLE subgroups. B. An ELISA assay showed that serum IL-1β, IL-6, and TNF-α levels were apparently higher in active LN patients than they were in the other SLE subgroups. The experiments were repeated three times. IL-1β, interleukin 1 beta; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; TRAF6, TNF receptor associated factor 6; ELISA, enzyme-linked immunosorbent assay. *$P < 0.05$.

**Table 2.** Correlation analysis of miR-124 expression with serum IL-1β, IL-6, TNF-α and TRAF6 mRNA levels in SLE patients

| Gene | *r* | *P* |
|---|---|---|
| IL-1β | *r* = -0.67 | < 0.01 |
| IL-6 | *r* = -0.63 | < 0.01 |
| TNF-α | *r* = -0.56 | = 0.03 |
| TRAF6 mRNA | *r* = -0.80 | < 0.001 |

IL-1β, interleukin 1 beta; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; TRAF6, TNF receptor associated factor 6.

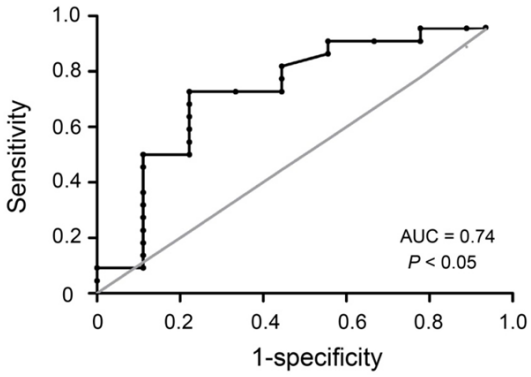


**Figure 3.** A ROC curve analysis of miR-124 on LN diagnosis. The sensitivity and specificity were 0.77 (53.43.8% and 94.55%) for miR-124 to differentiate active LN patients from the other SLE subgroups, the AUC of miR-124 on the diagnosis of active LN was 0.74. The experiments were independently repeated 3 times. ROC, receiver operating characteristic curve; AUC, area under the curve.

characteristic (ROC) analysis and the areas under the ROC curve (AUC). **Figure 3** revealed that the maximum Youden's J indexes (sensitivity and specificity) were 0.77 (53.43% and 94.55%) for miR-124 to differentiate the active LN patients from the other SLE subgroups. The area under the curve (AUC) of miR-124 on the diagnosis of active LN was 0.74.

*Verification of TRAF6 as a directly target gene of miR-124 in HRMCs*

To observe whether TRAF6 was regulated by miR-124, HRMCs were transiently transfected with an miR-124 mimic or a negative control. As presented in **Figure 4A**, RT-qPCR showed that the relative level of miR-124 was considerably higher in the miR-124 mimic group compared to the level in the negative control group ($P < 0.05$). The mRNA and protein levels of TRAF6 were significantly reduced in the HRMCs transfected with miR-124 mimic compared to the cells treated with the negative control (**Figure 4B** and **4C**, $P < 0.05$). miRs repress translations in the guidance of the RNA-induced silencing complex (RISC) in an Ago2-slicer-dependent

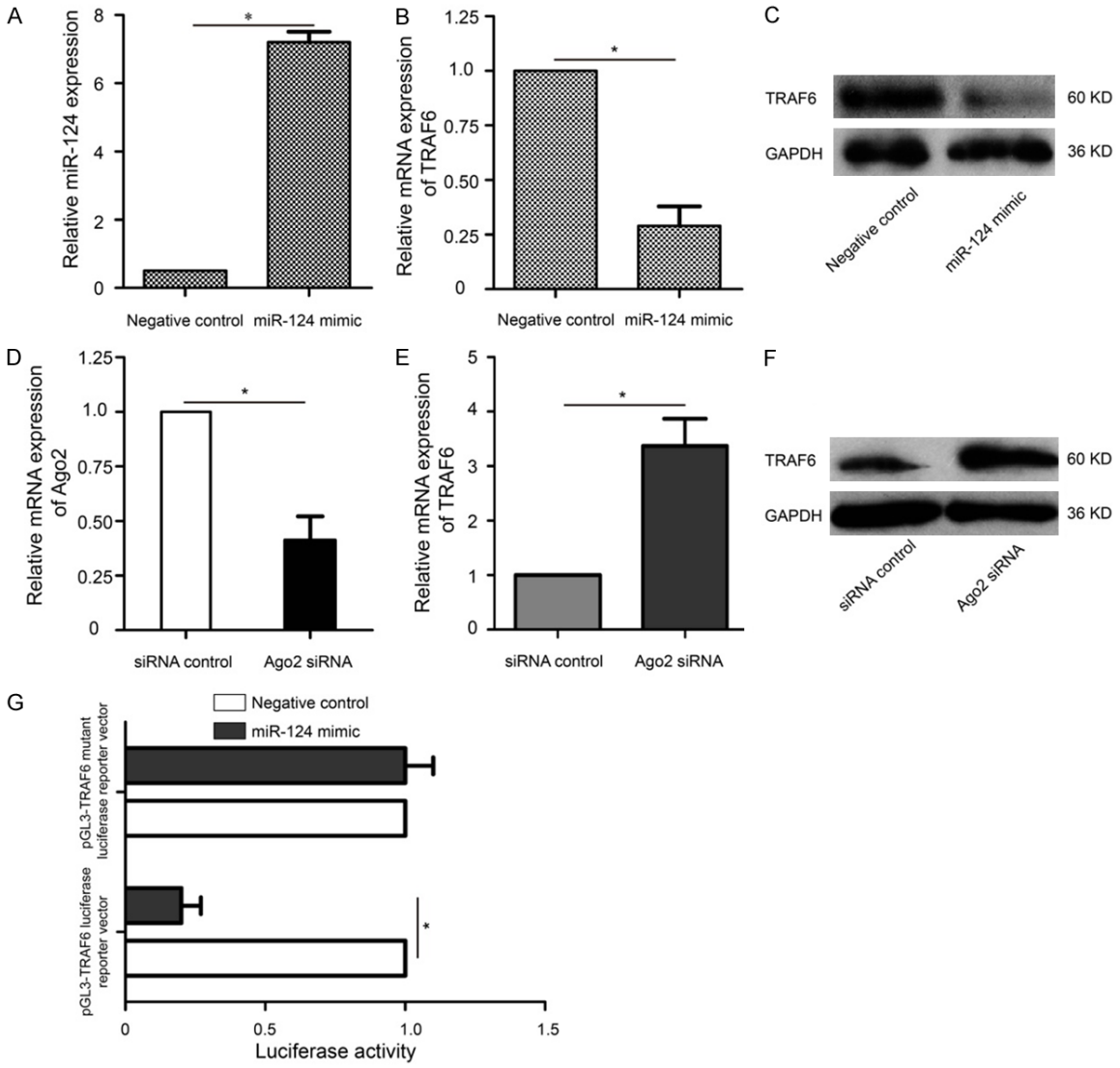


**Figure 4.** TRAF6 was a direct target gene of miR-124 in HRMCs. A. RT-qPCR assay showed that the relative level of miR-124 was considerably higher in the miR-124 mimic group as compared to the negative control group. B and C. In HRMCs, the over-expression of miR-124 by the miR-24 mimic significantly repressed the mRNA and protein expression of TRAF6. D. Ago2 siRNA was transfected into HRMCs to inhibit Ago2 expression, and Ago2 expression was significantly down-regulated. E and F. RT-qPCR and Western blotting assays were used to detect the mRNA and protein expressions of TRAF6. G. The overexpression of miR-124 could decrease the luciferase activity in the pGL3-TRAF6 luciferase reporter vector, but the activity of the pGL3-TRAF6 mutant luciferase reporter vector was not altered significantly by miR-124 overexpression. Three independent experiments were conducted. Ago2, argonaute RISC catalytic component 2. *$P < 0.05$.

manner in mammalians [19]. Simultaneously, Ago2 siRNA was used to inhibit Ago2 expression in HRMCs, and the data showed that the Ago2 expression was significantly down-regulated (**Figure 4D**, $P < 0.05$), but the TRAF6 expression was remarkably increased (**Figure 4E** and **4F**, $P < 0.05$). All of these results hinted that the expression of TRAF6 could be modulated by miR-124. To verify whether miR-124 could potentially bind to TRAF6 3'-UTR, a dual luciferase assay was performed in the HRMCs. As shown in **Figure 4G**, the overexpression of miR-124 could decrease the luciferase activity by 80% in the pGL3-TRAF6 luciferase reporter vector ($P < 0.05$), but the activity of pGL3-TRAF6 mutant luciferase reporter vector was not altered significantly by miR-124 overexpression. Thus, TRAF6 is a direct target gene of miR-124 in HRMCs.

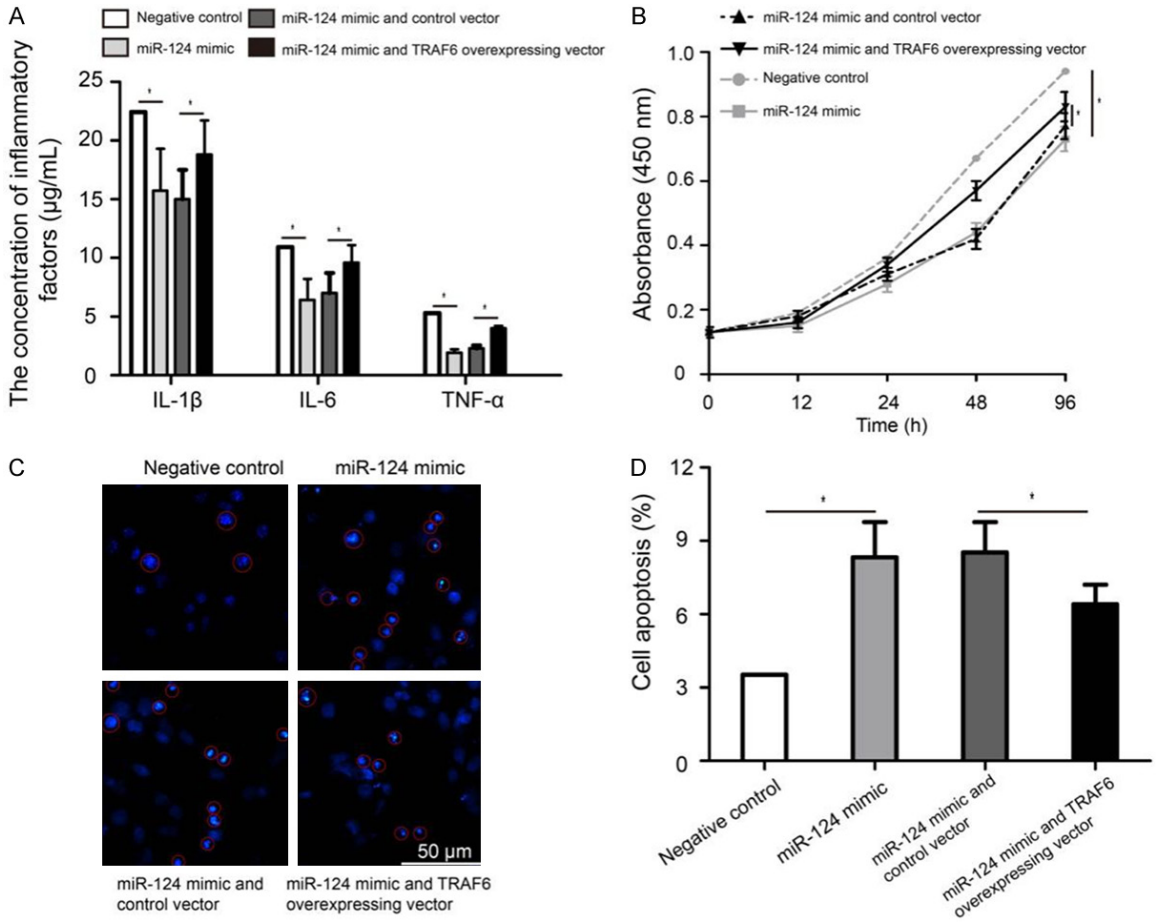


**Figure 5.** miR-124 over-expression inhibits the proliferation and induces the apoptosis and decreases the synthesis of inflammatory factors in HRMCs by targeting TRAF6. A. An ELISA assay showed that the over-expression of miR-124 significantly suppresses the synthesis of inflammatory factors in HRMCs, while increasing TRAF6 using the TRAF6 overexpression vector could abrogate the miR-124-related inhibition of the inflammatory factors expression. B. A CCK-8 assay showed that miR-124 mimic treatment could significantly decrease the cell proliferation of HRMCs in vitro. Transfection of the TRAF6 overexpression vector to increase TRAF6 could reverse the miR-124-related inhibition of HRMCs proliferation. C and D. Caspase 3 ELISA assay and flow cytometry analyses of the apoptosis of HRMCs transfected with miR-124 mimic with or without TRAF6 overexpression vector treatment. Experiments were independently repeated 3 times. *$P < 0.05$.

*The over-expression of miR-124 inhibits the synthesis of inflammatory factors by regulating TRAF6 in HRMCs*

Based on the negative association of miR-124 and inflammatory factors in the serum of SLE patients, we further measured the effect of miR-124 on the synthesis of the inflammatory factors in HRMCs including IL-1β, IL-6, and TNF-α. As illustrated in **Figure 5A**, the results showed that the over-expression of miR-124 significantly suppressed the synthesis of the inflammatory factors, but increasing of TRAF6 abrogated the miR-124-related inhibition of the inflammatory factors' expression ($P < 0.05$).

*Increasing miR-124 reduced the proliferation and induced the apoptosis of HRMCs by targeting TRAF6*

In order to determine the effects of miR-124 on the proliferation and apoptosis of the HRMCs, we transfected the miR-124 mimic into the HRMCs to increase miR-124 expression and used a CCK8 assay to examine the effect of increasing miR-124 on cell growth. We observed that the miR-124 mimic treatment could significantly decrease the cell proliferation of the HRMCs in vitro (**Figure 5B**, $P < 0.05$). Moreover, HRMCs treated with the miR-124 mimic were then transfected with or without the TRAF6

overexpressing vector, and cell proliferation was detected after 0, 12, 24, 48, and 96 h. The data showed that the transfection of the TRAF6 overexpression vector to increase TRAF6 could reverse the miR-124-related inhibition of the HRMCs proliferation (**Figure 5B**, $P < 0.05$). In addition, a Caspase 3 ELISA assay and flow cytometry revealed that the overexpression of miR-124 induced apoptosis of the HRMCs, but the co-transferred miR-124 mimic and the TRAF6 overexpression vector could reverse the effect of miR-124-induced cell apoptosis (**Figure 5C** and **5D**, $P < 0.05$).

## Discussion

SLE is an autoimmune disease affecting multiple organs and having a recurrent progression. As a major and severe complication of SLE, LN easily develops into end-stage renal disease, which is associated with higher morbidity and mortality [20]. Currently, there is lack of robust and sensitive biomarkers for the early and accurate diagnosis of LN. Thus, an in-depth understanding of the pathogenesis of LN and a search for novel diagnostic tools have important practical significance to judging LN activity, evaluating its prognosis, and applying appropriate treatments to decrease the burden of SLE. In recent years, studies have demonstrated that miRs play an important role in modulating immune cell function and inflammatory factors and participate in the pathogenesis of LN [21]. The results of the current study have demonstrated that serum miR-124 represents a novel diagnostic marker in human lupus nephritis and plays an inhibitory effect on the growth and inflammation of renal mesangial cells by targeting TRAF6.

miR-124 is a critical modulator in the immune system and immune disorders which could regulate innate and adaptive immune responses [14]. miR-124 is a critical mediator for the differentiation of naive $CD4^+$ T cells into T helper type 1 and T(H)17 cells in vitro and in vivo [22]. More and more studies indicate that miR-124 has the ability to restrain the activation of inflammatory signaling in RAW264.7 cells and macrophages [23]. Previous studies have shown that the expression of miR-124 is decreased in ulcerative colitis and rheumatoid arthritis due to promoter hypermethylation [24, 25]. Up to date, no study has focused on the role of miR-124 in the pathogenesis of LN. In the current study, the expression levels of miR-124 in serum from SLE patients were detected with a RT-qPCR assay. Our experiments revealed that the average level of serum miR-124 was significantly reduced in active LN compared with the other SLE subgroups. Moreover, a direct negative correlation was observed between miR-124 levels and the renal SLEDAI score. The most interesting finding obtained from the ROC analysis was the diagnostic value of miR-124 on active LN. It was shown that miR-124 presents relatively high diagnostic values for LN activity.

Inflammatory factors play a fundamental role in the pathogenesis of LN [26]. Immune complex deposition may induce a large number of inflammatory cytokines resulting in lupus glomerulonephritis. Yung et al. reported that anti-DNA antibodies may induce glomerular mesangial and renal tubular epithelial cells via secreting IL-1β, IL-6, and TNF-α [27]. Cash et al. found that the knockout of IL-6 significantly alleviates the symptoms of proteinuria, hematuria, and lymphocyte infiltration in a mouse model of LN [28]. The present study showed that IL-1β, IL-6, and TNF-α expressions were apparently higher in active LN patients than they were in the other SLE subgroups, which was similar to the findings of the study of Tsai et al. [29]. The miR-124 level was negatively correlated with serum IL-1β, IL-6, and TNF-α in SLE patients. Increasing miR-124 in the HRMCs blocked the synthesis of the inflammatory factors. This suggests that miR-124 may participate in inhibiting inflammation, and thus takes part in LN pathogenesis. Aberrant proliferation and the apoptosis of mesangial cells are frequently observed in LN [30]. Here, by using cell functional experiments, we found that miR-124 over-expression significantly reduced cell proliferation and induced cell apoptosis. The data was consistent with previous studies to some extent. For example, miR-124 inhibits growth and enhances radiation-induced apoptosis in non-small cell lung cancer [31]. miR-124 regulates STAT3-mediated cell proliferation, migration and apoptosis in bladder cancer [32]. These results demonstrated that the overexpression of miR-124 could reduce cell proliferation, induce cell apoptosis, and decrease synthesis of inflammatory factors in HRMCs.

TRAF6 has been reported to be functionally involved in the pathogenesis of LN. miRs can

inhibit gene expression by binding to the 3'-UTRs of target mRNAs. It has also been shown that TRAF6 is a target of miR-146a [33-35]. In this study, we found that miR-124 was able to bind to the 3'-UTR of TRAF6 mRNA, indicating that TRAF6 might be a potential target for miR-124 in LN. To confirm whether miR-124 had a direct regulation of TRAF6, we transiently transfected it with a miR-124 mimic into HRMCs. RT-qPCR showed that the mRNA and protein levels of TRAF6 was significantly reduced in the cells transfected with miR-124 mimic. Subsequently, Ago2 siRNA was used to inhibit Ago2 expression in HRMCs, and our data showed that the Ago2 expression was significantly down-regulated, but the TRAF6 expression was remarkably increased. Moreover, a dual luciferase assay confirmed that TRAF6 was a direct target gene of miR-124 in HRMCs. In order to determine the importance of TRAF6 as a functional target of miR-124, we hypothesized that the forced expression of TRAF6 could circumvent the effects of miR-124 on cell proliferation and apoptosis. HRMCs treated with the miR-124 mimic were then transfected with or without the TRAF6 overexpressing vector, and the cell proliferation and apoptosis were then quantified. Mechanistic studies showed that the transfection of the TRAF6 overexpression vector to increase TRAF6 could reverse the miR-124-related inhibition of HRMCs proliferation and the induction of apoptosis.

In conclusion, the present study is the first to demonstrate that downregulated miR-124 is a significant diagnostic biomarker for human LN. miR-124 over-expression inhibits cell proliferation and induces apoptosis and decreases the synthesis of inflammatory factors in HRMCs by downregulating the target gene of TRAF6.

## Acknowledgements

This work was supported by the Project of Science and Technology Plan of Tianjin City (no. 16ZXMJSY00190).

## Disclosure of conflict of interest

None.

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