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Original Article

Clinical correlation of plasma miR-21, miR-126 and miR-148a in patients with lupus nephritis

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Abstract: Recent studies have shown that microRNAs play an important role in autoimmune diseases. In this study, we studied the plasma expressive levels of miR-21, miR-126 and miR-148a in 44 patients with lupus nephritis (LN) and 24 healthy controls, and their clinical relationships were further analyzed. Compared with the healthy controls, plasma miR-21, miR-126 and miR-148a levels were significantly higher in LN patients. Receiver operating characteristic curve analysis of plasma miR-21, miR-126a and miR-148a showed that the areas under curve were 0.807 ± 0.051 ($P < 0.0001$), 0.788 ± 0.053 ($P < 0.0001$) and 0.776 ± 0.055 ($P = 0.0002$), respectively, when differentiating LN patients from healthy controls. Plasma miR-21 levels were significantly negatively correlated with complement factors C3 ($r = -0.420$, $P = 0.005$), C4 ($r = -0.362$, $P = 0.016$), white blood cell ($r = -0.395$, $P = 0.008$) and red blood cell counts ($r = -0.357$, $P = 0.017$) and positively correlated with the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in patients with LN ($r = 0.472$, $P = 0.001$). In contrast, plasma miR-126 and miR-148a levels were not significantly correlated with any clinical data in our patient cohort. Therefore, our findings suggest that significantly increased plasma levels of miR-21, miR-148a, and miR-126 occur in LN patients, and plasma miR-21 levels are associated with C3 and C4 levels and the SLEDAI. The potential of circulating miR-21 as a non-invasive biomarker in LN patients warrants further study.

Keywords: miR-21, miR126, miR-148a, lupus nephritis, plasma biomarker

Introduction

Lupus nephritis (LN) is a common complication of patients with systemic lupus erythematosus (SLE). Although glucocorticoid and immunosuppressive therapy have shown some success, 10% to 40% of patients will still progress to end-stage renal disease by 15 years from the time of diagnosis [1]. LN is defined as autoimmune glomerulonephritis. Patients with LN exhibit a variety of clinical and pathological changes, from asymptomatic proteinuria or hematuria to rapidly progressive glomerulonephritis, and from slight hyperplastic to severe sclerosing glomerular pathological changes. Corresponding with this, the therapeutic response and prognosis often differ [2]. Several predictors have been recommended as biomarkers of disease activity or prognosis in patients with LN, including age at LN onset, increased serum creatinine and proteinuria, presence of anti-Ro antibodies, serum complement C3 and C4 levels, and an adverse renal pathology type [3-5]. However, a satisfactory and reliable biomarker remains to be found.

MicroRNAs (miRNAs) are a class of endogenous single-stranded non-coding RNAs containing 19-25 nucleotides [6]. They regulate more than 50% of the protein-coding genes by suppression of translated expression or degradation of target gene mRNAs after combining with the complementary sequences of 3'untranslated regions of these mRNAs [7, 8]. A number of studies have shown that miRNAs are involved in the pathogenesis and progression of SLE and LN, such as miR-21, miR-126 and miR-148a. Upregulation of these miRNAs in $CD4^+$ T cells from patients with SLE directly or indirectly inhibits expression of DNA methyltransferase 1 (DNMT1), in turn promoting activation of autoimmune-associated methylation-sensitive

Table 1. Clinical characteristics of LN patients and healthy controls

Parameter	LN, n = 44	Healthy Controls, n = 24
Age (year, median, range)	35, 18-54	36, 23-68
Gender (female/male)	42/2	21/3
24-h urine protein (g/d)	3.92 ± 3.26	N/A
Serum creatinine (μmol/l)	135.19 ± 123.84	63.87 ± 12.78*
eGFR (ml/min per 1.73 m^2)	76.24 ± 42.96	103.8 ± 18.35*
BUN (μmol/l)	9.8 ± 7.47	4.362 ± 1.495**
Uric acid (μmol/l)	377.82 ± 141.76	251.3 ± 79.68**
C3 (g/l)	0.61 ± 0.32	1.14 ± 0.23**
C4 (g/l)	0.15 ± 0.11	0.29 ± 0.08**
Red cell count (× 10^{12}/l)	3.41 ± 0.88	4.26 ± 0.60**
White cell count (× 10^9/l)	7.41 ± 3.2	6.12 ± 1.36
Lymphocyte count (× 10^9/l)	1.26 ± 0.67	1.96 ± 0.48**
Platelet count (× 10^9/l)	169.54 ± 69.35	210.10 ± 47.39*
SLEDAI	14.33 ± 6.27	0**

Values are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, compared with LN patients. LN, lupus nephritis; N/A, not available; eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

genes and development of the disease [9-11]. In addition, miRNAs may be perfect biomarker candidates based on the characteristics of their stability and measurability in body fluids such as plasma and urine [8, 12]. However, the expression levels of circulating miR-21, miR-126 and miR-148a and their potential as non-invasive biomarkers in LN patients remain unclear.

In this study, we measured the plasma miR-21, miR-126 and miR-148a concentrations in patients with LN and further analyzed their clinical relevance and suitability as useful biomarkers.

Materials and methods

Patients

Forty-four patients with LN were recruited from December 2013 to June 2014 at the Department of Nephrology, Tongji Hospital, Huazhong University of Science and Technology. All of the patients met the American College of Rheumatology diagnostic criteria, with accompanying abnormal urinalysis (persistent proteinuria ≥ 0.5 g/d or greater than 3+ by dipstick, and/or cellular casts including red cell, hemoglobin, granular, tubular or mixed) [13]. Twenty-four age- and sex-matched healthy controls were also recruited. This study accords with the Declaration of Helsinki Principles, supported by the Ethics Com-mittee of Tongji Hospital, Huazhong University of Science and Technology. Written informed consent was obtained from all the participants.

Blood sample collection

Blood samples were collected from patients on the day of admission to hospital and from normal controls. After samples were obtained, they were centrifuged immediately at 2000× *g* for 10 min. The upper plasma was stored in aliquots at -80°C until RNA extraction.

RNA isolation

Plasma total RNA was extracted with TRIzol LS Reagent (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's protocol.

miRNA qRT-PCR

One microgram of total RNA was used to synthesize the cDNA of the target miRNA with the first-strand cDNA synthesis kit (Fermentas Inc., Vilnius, Lithuania) according to the manufacturer's protocol. Bulge-loop miRNA primes (Ribobio Co., Guangzhou, China) were used and are listed in Supplementary Table 1. In brief, a 20 μl reaction system was incubated at 42°C for 60 min, followed by 95°C for 5 min. Then, quantitative RT-PCR was performed according to the protocol of the SYBR Green/Fluorescein qPCR Master Mix kit (Fermentas Inc., Vilnius, Lithuania). The reaction conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec. The expression levels of target miRNAs were normalized to small RNA U6 and calculated as the $2^{-\Delta Ct}$ ($\Delta C_t = C_{t\ target\ miRNA} - C_{t\ U6}$) or $2^{-\Delta\Delta Ct}$ ($\Delta\Delta C_t = \Delta C_{t\ LN} - \Delta C_{t\ control}$) equation.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6 software (Graph software, San Diego, CA). The t-test was used to assess the differences in plasma miRNA expression between LN patients and healthy controls. Receiver operating characteristic

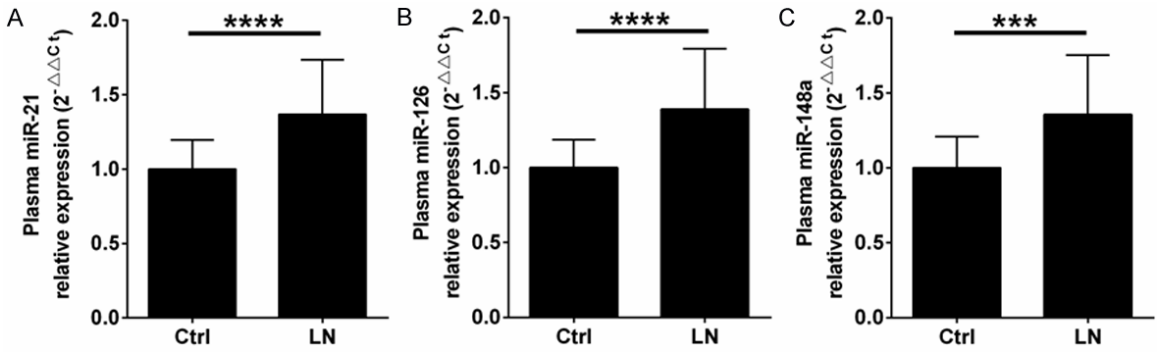

Figure 1. Relative expression of plasma miR-21, miR-126 and miR-148a in LN patients and healthy controls. The levels of plasma miR-21 (A), miR-126 (B) and miR-148a (C) were significantly higher in patients with LN compared with healthy controls. Ctrl, control; LN, lupus nephritis. $^{****}P < 0.0001$, $^{***}P < 0.001$.

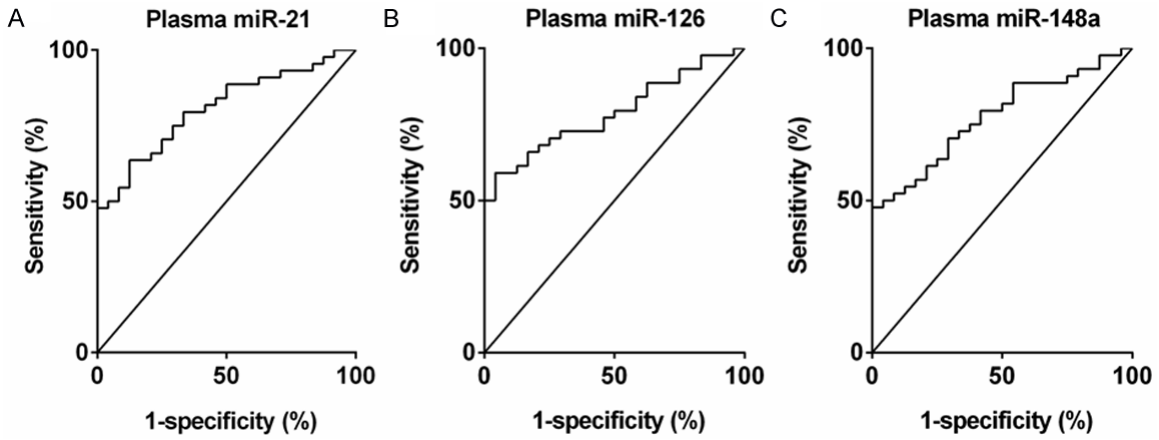

Figure 2. Receiver operating characteristic (ROC) analysis of the plasma miR-21, miR-126 and miR-148a levels for differentiating LN patients from healthy controls. The area under the ROC curve (AUC) for plasma miR-21 (A), miR-126 (B) and miR-148a (C) was 0.807 ± 0.051 (95% CI: 0.706-0.907, $P < 0.0001$), 0.788 ± 0.053 (95% CI: 0.683-0.893, $P < 0.0001$) and 0.776 ± 0.055 (95% CI: 0.668-0.884, $P = 0.0002$), respectively.

(ROC) curve analysis was performed to analyze the diagnostic value of these plasma miRNAs. The Pearson's correlation coefficient or the nonparametric Spearman's correlation test was performed to analyze the correlations between plasma miRNA levels and clinical parameters. All statistical analyses were two tailed and $P < 0.05$ was considered to be statistically significant.

Results

Characteristics of LN patients

The clinical characteristics of 44 patients and 24 healthy controls are shown in **Table 1**. In patient group, the median age was 35 years, ranging from 18 to 54 years. Of these, two were male, and 42 were female. Renal function-related indictors were recorded, such as 24-h urine protein, serum creatinine (Scr), estimated glomerular filtration rate (eGFR), blood urea nitrogen (BUN) and uric acid (UA). In addition, C3 and C4 levels and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) of the patients were recorded. Blood cell data, including red cell count, white cell count, lymphocyte count and platelet count, were also analyzed.

Plasma miRNA levels in patients and healthy controls

Next, we detected the plasma concentrations of miR-21, miR-126, and miR-148a in LN patients and healthy controls. Compared with that of the healthy controls, the plasma values of miR-21 in LN patients were increased by 1.369

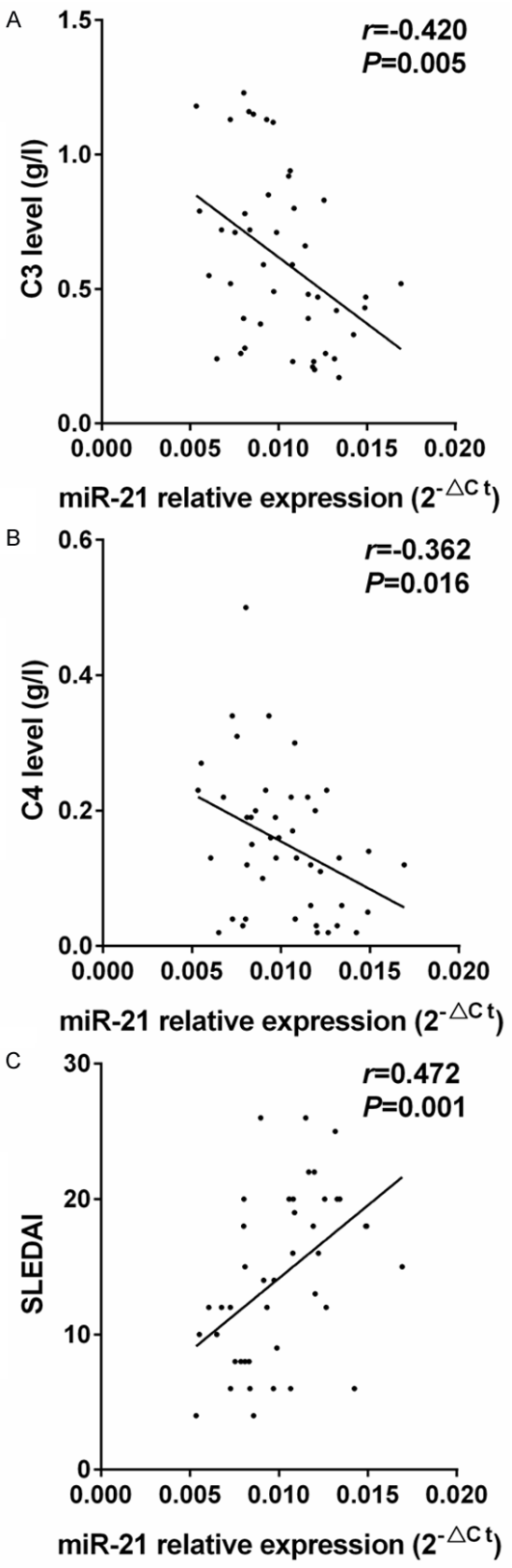

Figure 3. Correlations between plasma miR-21 levels and C3, C4 and SLEDAI in LN patients. A. Plasma miR-21 concentrations of LN patients were significantly correlated with their serum C3 values. B. Plasma miR-21 values were also correlated with serum C4 levels in LN patients. C. A significant association was observed between plasma miR-21 levels and SLEDAI in LN patients.

(± 0.366)-fold. Similarly, the plasma miR-126 levels in the patient cohort showed a 1.388 (± 0.403)-fold increase. In addition, the plasma miR-148a concentration in patients with LN exhibited a 1.356 (± 0.396)-fold increase. These details are shown in **Figure 1**.

The diagnostic value of these plasma miRNAs: differentiating LN patients from healthy controls

Then, we performed ROC curve analysis on LN patients and healthy controls to analyze the predictive value of these plasma miRNAs. For plasma miR-21, the optimal cutoff value of relative expression normalized to U6 was 0.009. The sensitivity and specificity were 0.636 and 0.875. The area under the ROC curve (AUC) was 0.807 ± 0.051 (95% CI: 0.706-0.907, $P < 0.0001$) (**Figure 2A**). The ROC curves analysis of plasma miR-126 and miR-148a showed that the AUC value was 0.788 ± 0.053 (95% CI: 0.683-0.893, $P < 0.0001$) and 0.776 ± 0.055 (95% CI: 0.668-0.884, $P = 0.0002$), respectively. When the relative expression was 0.044 for plasma miR-126, the corresponding sensitivity and specificity were 0.591 and 0.958. When plasma miR-148a levels reached 0.028, the sensitivity and specificity were 0.477 and 1, respectively (**Figure 2B** and **2C**).

Associations between plasma miRNA levels and clinical data of LN patients

To further evaluate the potential of plasma miR-21, miR-126 and miR-148 as biomarkers, we then analyzed the relationships between the plasma expression levels of these miRNAs and their clinical parameters. We found that plasma miR-21 levels in the LN patients were significantly negatively correlated with C3 and C4 values ($r = -0.420$, $P = 0.005$ and $r = -0.362$, $P = 0.016$, respectively) (**Figure 3A** and **3B**). Moreover, the plasma miR-21 concentrations were also positively correlated with the SLEDAI in our LN patient cohort ($r = 0.472$, $P = 0.001$) (**Figure 3C**). For the associations between plasma miR-21 levels and blood cell parameters, significant relevance was observed between plasma miR-

Table 2. Correlations between plasma miR-126 and miR-148a levels and clinical parameters in LN patients

Parameter	miR-126	miR-148a
24-h urine protein (g/d)	r = 0.089, P = 0.562	r = 0.015, P = 0.926
Serum creatinine (μmol/l)	r = -0.041, P = 0.792	r = 0.075, P = 0.627
eGFR (ml/min per 1.73 m^2)	r = 0.011, P = 0.942	r = -0.003, P = 0.983
C3 (g/l)	r = 0.023, P = 0.883	r = -0.041, P = 0.792
C4 (g/l)	r = -0.018, P = 0.905	r = 0.202, P = 0.189
SLEDAI	r = -0.129, P = 0.411	r = -0.015, P = 0.923
Red cell count (× 10^{12}/l)	r = -0.134, P = 0.385	r = -0.146, P = 0.344
White cell count (× 10^9/l)	r = 0.181, P = 0.240	r = -0.067, P = 0.665
Lymphocyte count (× 10^9/l)	r = 0.150, P = 0.331	r = -0.097, P = 0.531
Platelet count (× 10^9/l)	r = 0.061, P = 0.692	r = -0.035, P = 0.821

eGFR, estimated glomerular filtration rate; BUN, blood urea nitrogen.

21 values and white blood cell counts (r = -0.395, P = 0.008) and red blood cell counts (r = -0.357, P = 0.017); no significant correlation was found with lymphocyte counts (P = 0.167) and platelet counts (P = 0.122). In contrast, plasma miR-21 levels did not have significant relevance with data related to renal function, such as 24-h urine protein (P = 0.455), Scr (P = 0.092) and eGFR (P = 0.148). In addition, we did not find that plasma miR-126 and miR148a levels had significant correlations with any clinical parameter, as shown in **Table 2**.

Discussion

Approximately 50-80% of patients with SLE will develop renal involvement, termed LN, which is the most important cause of patient death [1, 5]. However, there is still no perfect biological marker that can reflect the degree of the disease activity, or the prognosis and treatment response of patients with LN. In this study, we detected the levels of plasma miR-21, miR-126 and miR-148a, all of which regulate the process of SLE emergence and development, and further assessed their potential as novel biomarkers in patients with LN.

We found that LN patients had significantly higher levels of plasma miR-21, miR-126 and miR-148a compared with healthy controls, as previously reported in SLE patients [14]. ROC curve analysis of these miRNAs showed their ability to distinguish LN patients from healthy controls. We also found that plasma miR-21 levels were significantly correlated with C3, C4, SLEDAI, red blood count and white blood count in our LN patient cohort. No correlation was observed between plasma miR-126 and miR-148a levels and the clinical indicators in our patient cohort. The findings suggest that significantly increased plasma levels of miR-21, miR-148a, and miR-126 levels could be found in LN patients. Circulating miR-21 may be a novel potential candidate biomarker in LN patients.

The mechanism and source of higher expression of plasma miR-21, miR-126 and miR-148a and their roles in the pathogenesis of LN are currently unclear. Recently, several studies reported circulating miRNAs may mainly arise from cell apoptosis, necrotic cell death and active secretion [15]. The apoptotic and necrotic cells release cell-free miRNAs that then form stable complexes with specific RNA-binding proteins. The apoptotic process also produces apoptotic bodies containing miRNAs that may be common in SLE and LN patients based on defects in apoptotic cell clearance ability [16]. In addition, circulating miRNAs may be stored in various extracellular vesicles, termed exosomes, which have been shown to be involved in the progression of renal disease [17, 18]. Exosomes are secreted by activated cells and contain high amounts of miRNAs, proteins and mRNAs. The role of miR-21, miR-126 and miR-148a in the occurrence and progression of SLE is mainly to promote lymphocyte activation and hyperresponsiveness. Previous studies on patients with SLE and lupus mice reported that the expression of miR-21 was significantly increased in $CD4^+$ T cells and B cells. Increased miR-21 levels promoted $CD4^+$ T cell phenotype activation, B cell hyperresponsiveness and overexpression of autoimmune-associated methylation-sensitive genes through repression of DNMT1 [9], PDCD4 [19] or PTEN [20] expression. Inhibition of miR-21 in $CD4^+$ T cells from patients with SLE reversed the activation of T cells [19]. Silencing of miR-21 in lupus mice reduced splenomegaly, the ratio of splenic $CD4^+$ to $CD8^+$ T cells and splenic B cell activity [21]. Similarly, miR-148a expression was also upregulated in $CD4^+$ T cells from patients with SLE and lupus mice, and

miR-126 levels were increased only in $CD4^+$ T cells from SLE patients. Their overexpression resulted in DNA hypomethylation in $CD4^+$ T cells by directly inhibiting DNMT1 protein expression, thus inducing $CD4^+$ T cell activation and secretion of autoimmune-related proteins, such as CD70, CD11a and LFA-1 [9, 10]. Accordingly, we hypothesized that the significant increase in circulating miR-21, miR-126 and miR-148a in patients with LN may be because of the increase in circulating apoptotic bodies and exosomes containing higher levels of target miRNAs secreted by activated lymphocytes, especially activated $CD4^+$ T cells and B cells. In addition, target miRNAs contained in apoptotic bodies and exosomes could be delivered to target cells through microvesicle-mediated cell-cell communication [22]. Thus, for the functional role of these miRNAs, we conjectured that increased circulating miR-21, miR-126 and miR-148a in turn may also accelerate disease progression through the cell-cell communication process between these apoptotic bodies and exosomes and target cells, such as quiescent lymphocytes.

In our study, we also found a positive association between plasma miR-21 concentrations and the SLEDAI, which may reflect the disease activity, as Pan et al. [9] recently reported that miR-21 and miR-148a levels in $CD4^+$ T cells were significantly correlated with SLEDAI in patients with SLE. However, a significant correlation was not observed between plasma miR-148a levels and the SLEDAI. This may be on account of the functional differences between miRNAs in blood circulation and lymphocyte cells. These results also show in part that the impact of miR-21 may be wider than miR-148a and miR-126. Plasma miR-126 levels were not correlated with the SLEDAI, as Zhao et al. [10] have shown that miR-126 expression levels in $CD4^+$ T cells were not of relevance to the SLEDAI in their SLE patient cohort. We also found that plasma miR-21 levels were significantly negatively correlated with C3 and C4 values. For the mechanism, we speculate that increased circulating miR-21 could increase the production of autoantibody and antigen–antibody complexes through promoting $CD4^+$ T cell and B cell activity. Then, enhanced antigen-antibody complexes can accelerate complement pathway activity and complement consumption, thus resulting in the reduction of C3 and C4 levels. No remarkable relationships were observed between plasma miR-126 and miR-148a levels and C3 and C4 concentrations in our LN patient cohort. In addition, plasma miR-21 levels, but not plasma miR-126 and miR-148a values, were negatively correlated with the red blood cell counts and white blood cell counts in our patients with LN. Since the assessments of low complement and leukopenia were included in the SLEDAI measurement, the correlation between miR-21 concentrations and complement levels and blood counts will account in part for its relationship with the SLEDAI. These results suggest that miR-21 may play a more important role in development of SLE and LN than miR-126 and miR-148a.

Although we found the plasma miR-21 levels correlated with the values of C3 and C4 and the SLEDAI, the association between plasma miR-21 and renal function indictors was not observed in our LN patient cohort. This may because of a limited sample size that included only a small number of new-onset LN patients and the influence of treatment of glucocorticoid or immunosuppressants recently administered to patients. Moreover, the lack of pathological information for most patients also limited further study. However, the potential of circulating miR-21 as a novel biomarker for renal injury has been shown in previous studies of different kidney diseases. Du et al. [23] reported that plasma and urine miR-21 levels were correlated with severe acute kidney injury (AKI) and poor outcomes following cardiac surgery. The predictive ability of plasma and urine miR-21 for AKI severity was comparable with other biomarkers such as urinary albumin to creatinine ratio, urinary IL-18, and urinary and plasma neutrophil gelatinase-associated lipocalin [24, 25]. Glowacki et al. [26] showed that the levels of circulating miR-21 were significantly positively correlated with the severe degree of renal interstitial fibrosis and the glomerular filtration rate in renal transplant patients. In type 1 diabetes patients, the upregulated plasma miR-21 levels were related with the occurrence of proteinuria and the increased risk of end-stage renal disease [27]. In our recent study, we also found the plasma miR-21 levels in LN patients were significantly higher than those in SLE patients with normal urinalysis, and IgAN patients had higher plasma miR-21 values compared with normal controls (data not shown). Accordingly,

we speculated that the association between circulating miR-21 and renal injury in LN patients warrant further study.

There are several limitations in this study. First, since the study examined a small sample size and single ethnic groups, a larger scale cohort study or wider racial multicenter study is required to verify the universality of these results obtained in our study. Second, the medication used by the majority of patients prior to hospital admission could have an effect on the patients' immune response, such as glucocorticoid, cyclophosphamide and mycophenolate mofetil, thus post-study should recruit more new-onset LN patients to further illustrate the roles of these miRNAs. Third, follow-up information was not incorporated into our study, and further follow-up research should be performed to determine the predictive ability and responsiveness of treatment of these plasma miRNAs. Finally, because only four patients in our study had information about renal pathological injury, we could not analyze the association between these circulating miRNAs and renal pathological damage. Furthermore, the expression of these miRNAs in urinary and intra-renal should also be detected to elucidate their roles in renal injury in patients with SLE or LN.

In conclusion, the results of this study indicate that significantly increased plasma levels of miR-21, miR-148a, and miR-126 could be found in LN patients, and plasma miR-21 levels were associated with C3 and C4 levels and the SLEDAI. The potential of circulating miR-21 as a non-invasive biomarker in LN patients requires further study.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Primers of miRNAs used for qRT-PCR

Primers	Sequences
MiR-21	
RT loop primer	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAAC
Forward primer	5'-TGCGCTAGCTTATCAGACTGAT
MiR-126	
RT loop primer	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCAT
Forward primer	5'-TGCGCTCGTACCGTGAGTAATA
MiR-148a	
RT loop primer	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAAA
Forward primer	5'-TGCGCTCAGTGCACTACAGAAC
Reverse primer	CCAGTGCAGGGTCCGAGGTATT