Serum and Urinary Cell–free MiR-146a and MiR-155 in Patients with Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** Recent studies showed that micro-RNA play important roles in the pathogenesis of autoimmune diseases. We studied the levels of miR-146a and miR-155 in the serum and urinary supernatant of patients with systemic lupus erythematosus (SLE).

Methods. The serum and urinary supernatant levels of miR-146a and miR-155 were determined by real-time quantitative polymerase chain reaction in 40 patients with SLE and 30 healthy controls.

Results. Compared to controls, serum miR-146a and miR-155 levels were lower, and the urinary level of miR-146a was higher, in SLE. Estimated glomerular filtration rate (eGFR) correlated with both serum miR-146a ($r = 0.519$, $p = 0.001$) and miR-155 ($r = 0.384$, $p = 0.014$). Serum miR-146a inversely correlated with proteinuria ($r = –0.341$, $p = 0.031$) and the SLE Disease Activity Index ($r = –0.465$, $p = 0.003$). Serum miR-146a and miR-155 levels also correlated with red blood cell count, platelet count, and lymphocyte count. After treatment with calcitriol for 6 months, serum miR-146a level of SLE patients increased significantly ($p < 0.001$), and its change inversely correlated with the level of calcium-phosphate product ($r = –0.466$, $p = 0.003$).

Conclusion. The results suggested that serum miR-146a and miR-155 participate in the pathophysiology of SLE and might be used as biomarkers of SLE. (First Release Oct 15 2010; J Rheumatol 2010;37:2516–22; doi:10.3899/jrheum.100308)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS MiR-146a MiR-155 CALCITRIOL

Systemic lupus erythematosus (SLE) is a common and severe autoimmune disorder with complex but poorly understood etiology[1]. Clinical management of SLE remains a great challenge to physicians because it has heterogeneous manifestations and unpredictable course. Because the clinical course of SLE is often relapsing and remitting, it is particularly important to have reliable markers for disease monitoring and evaluation of treatment. Unfortunately, currently available tests, such as serum anti-double-stranded DNA, complement level, and the SLE Disease Activity Index (SLEDAI), are not entirely satisfactory[2,3]. Although many biomarkers have been investigated for SLE, few have been rigorously validated and widely accepted[4].

MicroRNA (miRNA) are small noncoding, single-stranded RNA molecules that regulate gene expression at the post-transcriptional level by degrading or blocking translation of messenger RNA (mRNA)[5]. Multiple miRNA, such as miR-146a, miR-155, miR-132, miR-16, and miR-101, are reported to be important regulators of the immune system[6,7,8]. In addition, studies have demonstrated that a number of miRNA species are differentially expressed between patients with SLE and normal controls, suggesting the involvement of miRNA in the pathogenesis of SLE[9,10]. Notably, a recent study showed that miR-146a was a negative regulator of the interferon (IFN) pathway, and underexpression of miR-146a in peripheral blood mononuclear cells (PBMC) contributed to the "interferon signature" of SLE[11].

It is notable that most previous studies focused on cellular miRNA. Recently, cell-free miRNA have also been found to exist in serum and urine supernatant and can be used as disease markers[12,13]. We examined serum-free and urinary-free miR-146a and miR-155 in patients with SLE.

MATERIALS AND METHODS

We recruited 40 patients with SLE who required maintenance immunosuppressive therapy between June 2008 and October 2008. All the patients were diagnosed according to the American College of Rheumatology diagnostic criteria[14]. All the patients were treated with calcitriol 0.25 μg/day for the prevention of osteoporosis. A whole-stream early morning urine specimen was collected and 5 ml of whole blood was drawn during clinic followup every 3 months. Clinical data including complete blood count, serum complement levels, serum creatinine, urea, proteinuria, serum high-sensitivity C-reactive protein (hsCRP), and SLEDAI were recorded.

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Glomerular filtration rate (GFR) was estimated by a standard equation[15]. We also studied 30 healthy subjects as controls. The study was approved by the Ethics Committee of the Chinese University of Hong Kong. All participating subjects gave written consent according to the Declaration of Helsinki.

Sample processing. Blood and urine samples were stored at 4°C and processed within 5 hours after collection. The whole blood and urine specimen was centrifuged at 3000 g for 30 min at 4°C. The serum and urine supernatant aliquots were then transferred in Eppendorf tubes and centrifuged at 12,000 g for 10 min at 4°C and stored at –80°C.

Measurement of mRNA levels. The mirVana™ PARIS™ Kit (Ambion Inc., Austin, TX, USA) was used for the extraction of total RNA and TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used for reverse transcription, both according to the manufacturers' protocols. The resulting cDNA was stored at –80°C.

The expression of miR-146a and miR-155 was quantified by real-time quantitative polymerase chain reaction (RT-QPCR) using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). For RT-QPCR, 2.5 μl universal master mix, 0.25 μl primer, and probe set (all from Applied Biosystems), 0.33 μl cDNA and 1.92 μl H_2O were mixed to make a 5-μl reaction volume. Each sample was run in triplicate. RT-QPCR were performed at 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Data were analyzed with relative quantification software version 2.2.2 (Applied Biosystems). Because this study examined the miRNA level in cell-free body fluids (serum and urine), which are not known to have constant levels of a particular RNA species, normalization of expression level by an endogenous control or housekeeping gene was not possible. As a result, the same volume of serum and urine supernatant was used for miR-146a and miR-155 expression for each subject, and the same baseline and threshold cycle were set for each target, so that the level of expression could be compared between samples, and the levels of miR-146a and miR-155 are expressed as 50-CT.

Statistical analysis. Statistical analysis was performed by SPSS for Windows software version 13.0 (SPSS Inc., Chicago, IL, USA). All the results were presented in mean ± SD. Baseline data were compared by Student's t test or Fisher's exact test as appropriate. Since the miRNA expression and SLEDAI data are highly skewed, the Mann-Whitney U test was used to compare gene expression levels between groups, and Spearman's rank correlation to test association between gene expression levels and clinical measurements. A p value < 0.05 was considered statistically significant. All probabilities were 2-tailed.

RESULTS

The baseline and followup clinical data of the study subjects were summarized in Table 1 and Table 2. Renal function of patients with SLE at 3 and 6 months was lower than at baseline. All patients with SLE received maintenance prednisolone therapy, with a median dosage of 5 mg/day (range 2.5 to 10 mg/day); 10 also received azathioprine, and 2 received mycophenolate mofetil. There was no correlation between the dosage of prednisolone and the SLEDAI score.

Serum and urinary miRNA levels. The serum and urine supernatant levels of miR-146a and miR-155 in patients with SLE and controls are shown in Figures 1 and 2. Compared to controls, patients with SLE had lower serum levels of miR-146a (22.43 ± 0.87 in patients vs 27.50 ± 2.34 in controls; p < 0.001) and miR-155 (17.36 ± 1.08 vs 20.58 ± 1.90; p < 0.001; Figure 1). In contrast, the urine supernatant level of miR-146a was significantly higher in patients with SLE than controls (15.39 ± 2.79 in patients vs 13.61 ± 1.54 in controls; p = 0.007), while the urine supernatant level of miR-155 was similar (11.15 ± 4.45 in patients vs 11.79 ± 2.67 in controls; p = 0.683; Figure 2). Compared to urinary supernatant, serum had a higher level of miR-146a (23.81 ± 2.66 in serum vs 14.80 ± 2.58 in urine; p < 0.001) and miR-155 (18.24 ± 1.97 in serum vs 11.23 ± 3.96 in urine; p < 0.001). There were also significant internal correlations between serum level of miR-146a and miR-155 (r = 0.595, p < 0.001), as well as between urinary level of miR-146a and miR-155 (r = 0.443, p = 0.001).

Correlation with clinical measurements. There was no significant correlation between patients' ages and serum miR-146a or miR-155 level (details not shown). There was also no significant difference in serum miR-146a or miR-155 levels between men and women in the control group. Serum miR-146a level, however, correlated positively with GFR (r = 0.519, p = 0.001) and hsCRP (r = 0.350, p = 0.027), and correlated inversely with proteinuria (r = –0.341, p = 0.031) and SLEDAI (r = –0.465, p = 0.003; Figure 3). Serum miR-155 level correlated positively with GFR (r = 0.384, p = 0.014) and hsCRP (r = 0.412, p = 0.008), but did not correlate significantly with proteinuria (r = –0.286, p = 0.074) or SLEDAI (r = –0.129, p = 0.427; Figure 4). There was also a statistically significant but modest correlation between serum miR-146a and miR-155 with the dosage of prednisolone (Figures 3 and 4). Further, serum miR-146a level correlated positively with red blood cell count, lymphocyte count, and platelet count (Figure 3). Similarly, serum miR-155 level correlated positively with red blood cell count, lymphocyte count, and platelet count (Figure 4). In contrast, urine supernatant miR-146a and miR-155 levels did not have significant correlation with any clinical measurement (Table 3). In the control group, there was no significant correlation between serum or urine supernatant miRNA levels with renal function, blood count, or other baseline clinical measurements (details not shown).

Response to calcitriol treatment. After treatment with calcitriol, both serum miR-146a (Friedman test, p < 0.001) and miR-155 (Friedman test, p = 0.004) levels changed significantly. Specifically, serum miR-146a rose from 22.43 ± 0.87 to 22.94 ± 0.90 after 3 months (Wilcoxon signed-rank test, p < 0.001) and further elevated to 23.64 ± 1.44 at the sixth month (p < 0.001). At the same time, the serum miR-155 level rose from 17.36 ± 1.08 to 17.90 ± 0.63 after 3 months (p = 0.005), yet dropped to 17.43 ± 1.00 at the sixth month (p = 0.687; Figure 5).

The change in serum miR-146a and miR-155 levels did not correlate with the change in renal function, proteinuria, or SLEDAI. However, there was a trend to an increase in calcium-phosphate product (Ca × P) following calcitriol treatment (2.85 ± 0.49 to 2.99 ± 0.60 $mmol^2/l^2$; paired t-test, p = 0.051). The change in Ca × P product correlated inversely with the change in serum miR-146a level (r = –0.466, p = 0.003), but not with miR-155 (r = 0.135, p = 0.413; Figure 6).

Table 1. Baseline clinical data of the subjects. Values are mean ± SD; data are compared by Student t test or Fisher exact test.

Characteristics	SLE, n = 40	Healthy Controls, n = 30	p
Male: female	0:40	14:16	< 0.0001
Age, yrs	48.4 ± 12.6	34.3 ± 6.7	< 0.0001
Proteinuria, g/day	0.38 ± 0.77	—	
Serum creatinine, μmol/l	82.1 ± 37.1	80.5 ± 20.7	0.9
GFR, ml/min/1.73 m^2	81.5 ± 27.9	79.3 ± 20.4	0.8
C3, g/l	0.88 ± 0.23	1.13 ± 0.41	0.014
C4, g/l	0.20 ± 0.10	0.35 ± 0.12	< 0.0001
Calcium, mmol/l	2.26 ± 0.11	2.37 ± 0.10	0.0003
Phosphate, mmol/l	1.24 ± 0.20	1.02 ± 0.18	0.0002
Ca × P product, $mmol^2/l^2$	2.85 ± 0.49	2.42 ± 0.44	0.004
ALP, IU/l	62.4 ± 20.4	59.4 ± 16.2	0.6
Red cell count, × 10^{12}/l	4.03 ± 0.68	3.98 ± 0.49	0.8
Lymphocyte count, × 10^9/l	1.35 ± 0.64	2.94 ± 0.83	< 0.0001
Platelet count, × 10^9/l	232.0 ± 64.9	257.6 ± 70.9	0.17
C-reactive protein	2.88 ± 4.56	0.77 ± 0.57	0.044
SLEDAI	1.7 ± 2.9	—	

GFR: glomerular filtration rate; ALP: alkaline phosphatase; SLEDAI: SLE Disease Activity Index.

Table 2. Followup clinical data of the patients with SLE. Values are mean ± SD.

Measures	0 Month	3 Months	6 Months
Proteinuria, g/day	0.38 ± 0.77	0.37 ± 0.79	0.27 ± 0.45
Serum creatinine, mmol/l	82.05 ± 37.13	92.75 ± 49.29*	94.33 ± 68.41*
GFR, ml/min/1.73m^3	81.52 ± 27.86	74.24 ± 28.43*	77.61 ± 29.95*
C3, g/l	0.88 ± 0.23	0.90 ± 0.23	0.90 ± 0.28
C4, g/l	0.20 ± 0.10	0.22 ± 0.14	0.21 ± 0.12
SLEDAI	1.68 ± 2.90	2.28 ± 2.92	1.95 ± 2.09
Calcium, mmol/l	2.26 ± 0.11	2.33 ± 0.13	2.35 ± 0.15
Phosphate, mmol/l	1.24 ± 0.20	1.25 ± 0.23	1.24 ± 0.20
Ca × P product, $mmol^2/l^2$	2.85 ± 0.49	2.99 ± 0.64	2.99 ± 0.60
ALP, IU/l	62.4 ± 20.4	54.2 ± 18.2	54.6 ± 22.5

GFR: glomerular filtration rate; ALP: alkaline phosphatase; SLEDAI: SLE Disease Activity Index. * $p < 0.01$, compared with 0 month.

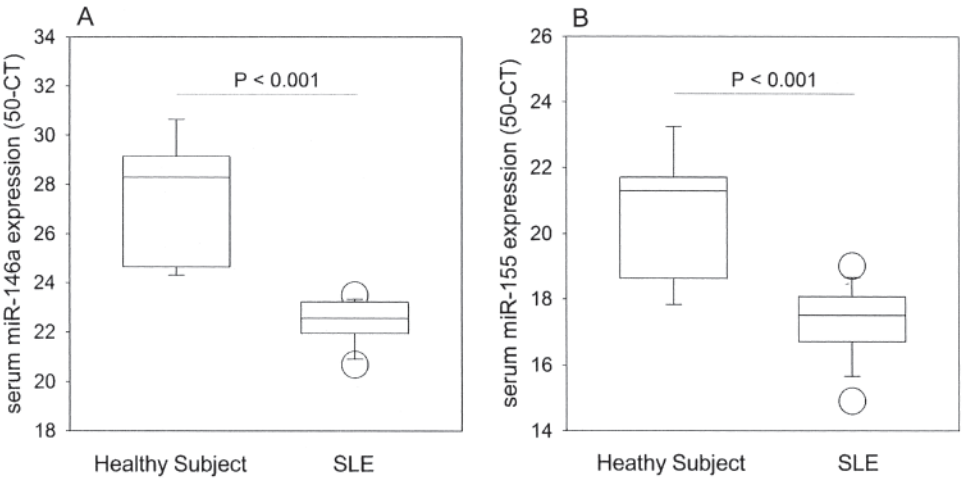

Figure 1. Comparison of (A) serum miR-146a and (B) serum miR-155 levels between patients with systemic lupus erythematosus (SLE) and healthy subjects. Data are compared by Mann-Whitney U test.

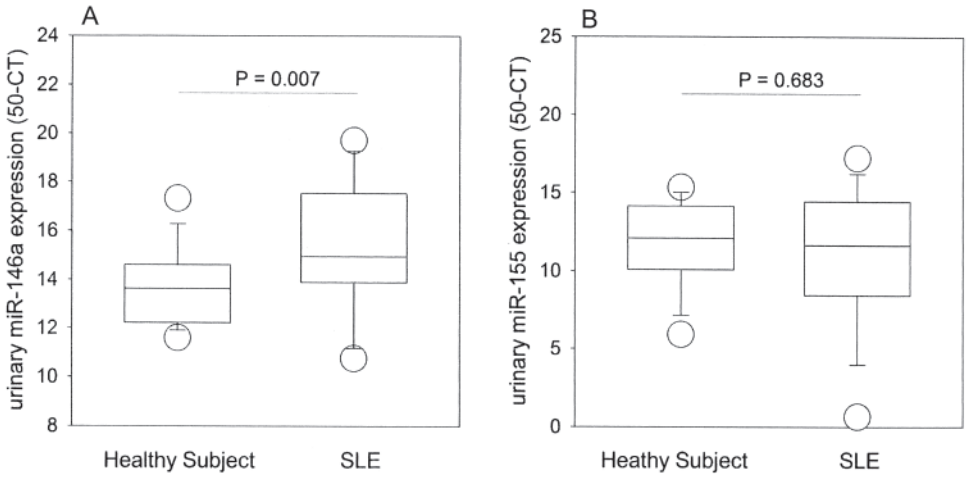

Figure 2. Comparison of (A) urinary miR-146a and (B) urinary miR-155 levels between patients with systemic lupus erythematosus (SLE) and healthy subjects. Data are compared by Mann-Whitney U test.

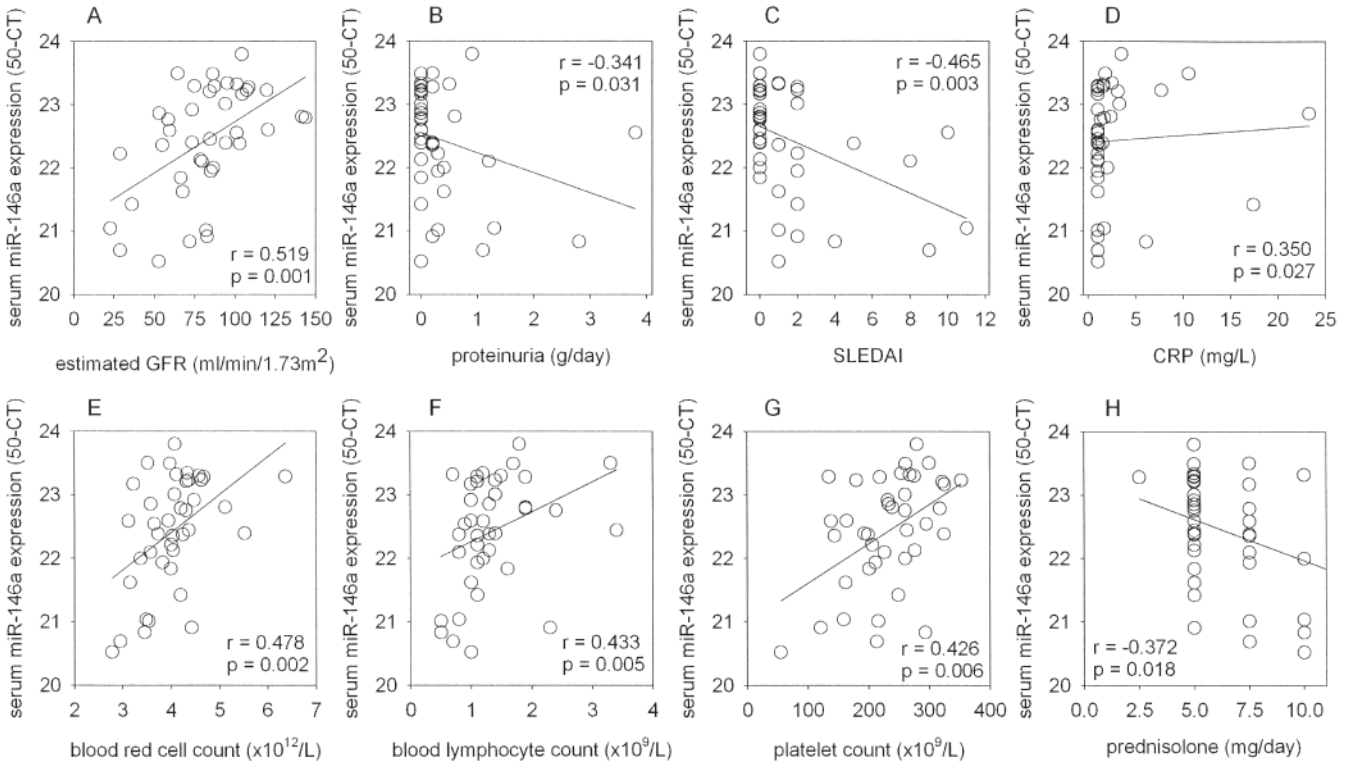

Figure 3. Correlations between serum miR-146a and (A) glomerular filtration rate (GFR); (B) proteinuria; (C) SLE Disease Activity Index (SLEDAI); (D) high-sensitivity C-reactive protein (hsCRP); (E) red blood cell count; (F) lymphocyte count; (G) platelet count; and (H) dosage of maintenance prednisolone. Data are compared by Spearman's rank correlation coefficient.

DISCUSSION

We found that the serum level of miR-146a and miR-155 in patients with SLE was significantly lower than that of healthy controls, and the level correlated positively with renal function. We further found that the serum level of miR-146a correlated negatively with SLEDAI and proteinuria in patients with SLE. Our result is consistent with a report by Tang, *et al*[11], which showed that the expression of miR-146a in the PBMC of patients with SLE is downregulated and is inversely related to SLEDAI, and the expression of miR-146a in patients with proteinuria is significantly lower than in those without. We also found both serum miR-146a and miR-155

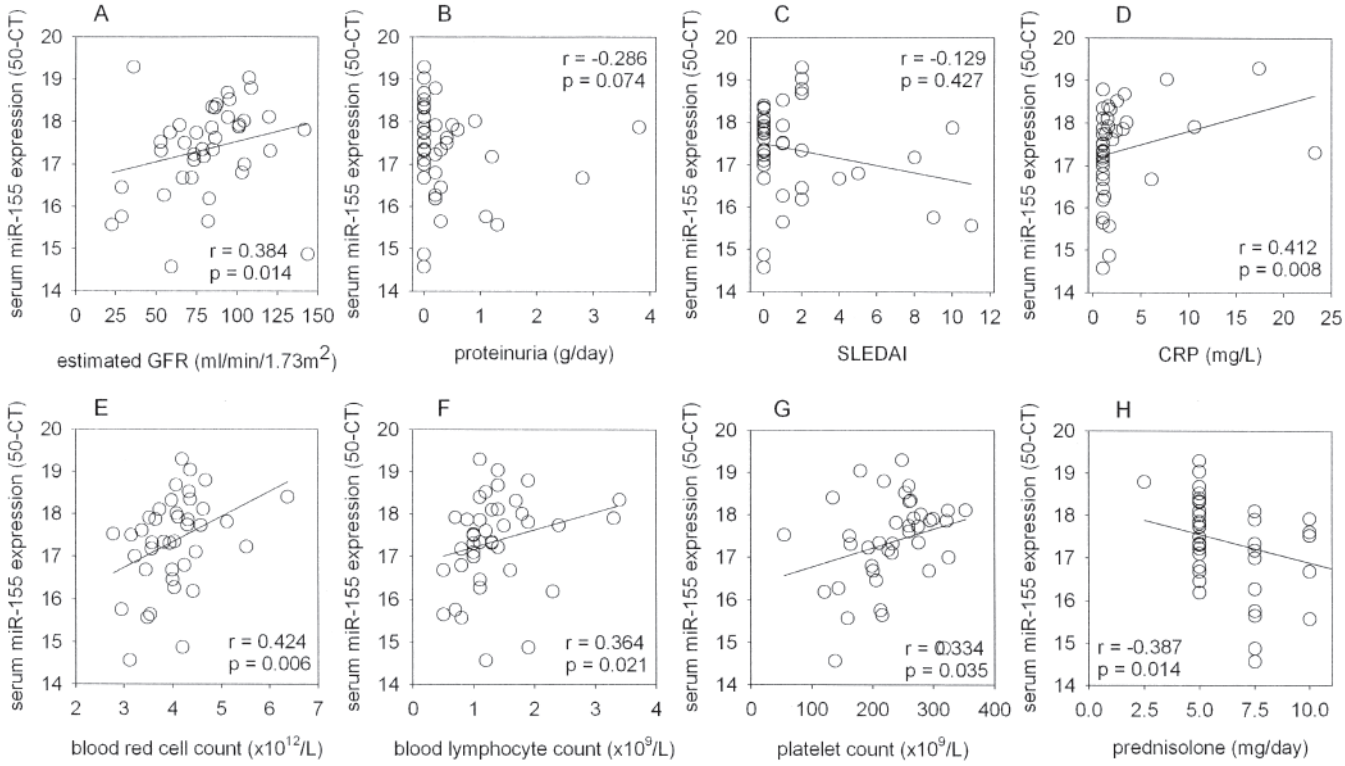

Figure 4. Correlations between serum miR-155 and (A) glomerular filtration rate (GFR); (B) proteinuria; (C) SLE Disease Activity Index (SLEDAI); (D) high-sensitivity C-reactive protein (hsCRP); (E) red blood cell count; (F) lymphocyte count; (G) platelet count; and (H) dosage of maintenance prednisolone. Data are compared by Spearman's rank correlation coefficient.

Table 3. Correlations between miRNA in urine supernatant and clinical measurements.

Measures	miR-146a	miR-155
GFR, ml/min/1.73m^2	r = –0.158, p = 0.343	r = 0.046, p = 0.776
Proteinuria, g/day	r = 0.057, p = 0.732	r = 0.059, p = 0.718
SLEDAI	r = 0.110, p = 0.512	r = 0.150, p = 0.354
hsCRP	r = –0.031, p = 0.856	r = –0.132, p = 0.418
Red blood cell count	r = 0.104, p = 0.535	r = 0.146, p = 0.368
Lymphocyte count	r = 0.047, p = 0.781	r = –0.044, p = 0.786
Platelet count	r = –0.065, p = 0.697	r = –0.017, p = 0.919

miRNA: micro RNA; GFR: glomerular filtration rate; SLEDAI: SLE Disease Activity Index; hsCRP: high-sensitivity C-reactive protein.

were significantly correlated with hsCRP. Given the controversial role of CRP in SLE[16,17], further studies are required to clarify the implication of these relationships.

Underexpression of miR-146a in PBMC has been proved to be involved in the pathogenesis of SLE by negatively regulating key molecules of the type I IFN pathway[11]. At present, there is no study of the clinical implication of cell-free miR-146a and miR-155. Recent studies, however, suggested that cell-free miRNA could be transferred between cells with biological function[18,19,20]. Therefore, it is at least theoretically possible that serum miR-146a and miR-155 participate in the pathogenesis of SLE.

Cellular expression of miR-146a and miR-155 has been proved to be altered by calcitriol treatment[21]. We found that the levels of serum miR-146a and miR-155 increased after calcitriol treatment. Since the degree of change of serum miR-146a correlated with the change in calcium-phosphate product, it is likely to be a genuine alteration. The clinical relevance of this observation, however, is uncertain. Nonetheless, vitamin D does not only control calcium and bone metabolism; it exerts exquisite immunoregulatory properties[22,23], which might contribute to the change in miRNA. Further, vitamin D has been recently found to suppress the expression of IFN signature in SLE[24]. It is possible that miR-146a contributes to the calcitriol-induced suppression of IFN. This hypothesis requires further studies to confirm.

We also studied cell-free miR-146a and miR-155 in urine. The urinary cell-free miR-146a and miR-155 probably originate from the kidney *per se* rather than plasma filtrate because its level is different from the corresponding serum level, and there was no correlation between them. We further found a significant difference between urinary level of miR-146a between patients with SLE and normal controls. However, neither urinary cell–free miR-146a nor miR-155 significantly correlated with clinical measurements. The significance of urinary cell–free miR-146a and miR-155 remains doubtful.

We found that the serum miR-146a level correlates with

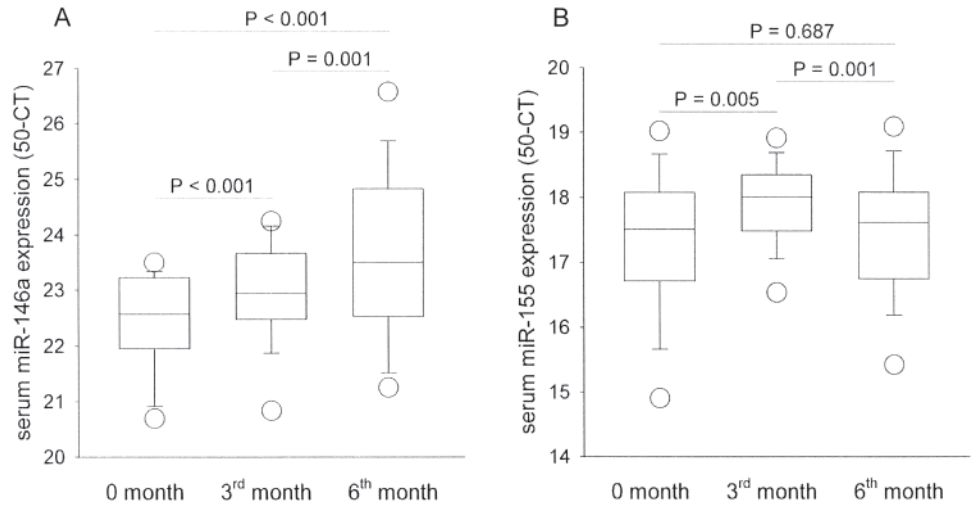

Figure 5. Comparison of (A) serum miR-146a and (B) serum miR-155 levels at 0 month, third month, and sixth month of calcitriol treatment. P values indicate posthoc comparison by Wilcoxon signed-rank test.

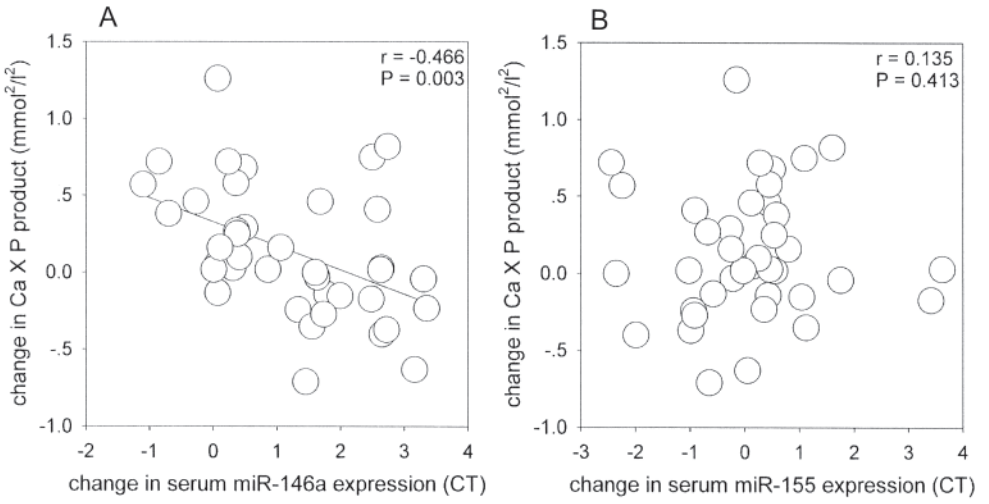

Figure 6. Correlation between change in calcium-phosphate (Ca × P) product after 6 months of calcitriol treatment and change in (A) serum miR-146a; and (B) serum miR-155. Data are compared by Spearman's rank correlation coefficient.

the GFR in patients with SLE. However, after 6 months of calcitriol treatment, serum miR-146a levels increased significantly, but contrary to the prediction, the GFR decreased. The reason for this paradoxical observation is not clear. The small number of patients with SLE in our study may have prevented uncovering a clear trend of changes in laboratory or clinical measurements; a larger and more diverse cohort of patients with SLE with a prolonged followup would be necessary to confirm our observation.

There are a few inadequacies of our study. First, most of our patients had quiescent lupus. Although we observed a significant correlation between serum miR-146a and SLEDAI score, implying that the serum level of this miRNA species is related to lupus activity, it would be ideal to have a separate group of patients with active lupus for comparison. Because of the small sample size and limited duration of followup, our study did not have sufficient statistical power to determine the relation between the change in serum miR-146a and miR-155 levels and the fluctuation of lupus disease activity with time. In addition, the effect of immunosuppressive therapy on serum miR-146a and miR-155 level also requires further study.

Second, we did not attempt to differentiate cell-free miRNA in microvesicles or truly "naked" miRNA, each of which has its own biological relevance[25,26]. In fact, the definite origin of free miRNA in the body fluid remains incompletely understood[27]. It is possible that circulating miRNA are derived from cells in the body fluid. For example, PBMC can be a source of serum miR-146a[11]. We believe that lymphocytes, rather than monocytes, might be the major source

of serum miR-146a and miR-155 in our patients, because serum miR-146a and miR-155 levels correlated with lymphocyte but not monocyte counts. The other formed elements, such as mature erythrocytes and platelets, might also contribute to the production of serum miRNA[28,29].

Third, the age and sex of controls did not perfectly match with patients in this study, making comparison of the 2 groups difficult. Although previous studies have shown similar levels of serum and urinary free miRNA between men and women[26,30], and we found no significant correlation between age and serum or urinary miR-146a level, the reference ranges of some measurements in this study (for example, red blood cell count, or GFR) are age-dependent or sex-dependent, which makes the relevance of our analysis difficult to interpret.

Finally, although it is likely that the studied miRNA are related to the pathogenesis of SLE, the use of serum miRNA levels as biomarkers of SLE is less certain. Further studies are necessary to include a panel of differentially regulated miRNA in patients with SLE and to examine their correlation with serum or urinary levels.

We found that serum miR-146a and miR-155 levels are lower in patients with SLE than in healthy controls; and that their levels correlate with renal function. Serum miR-146a levels also correlate with proteinuria and SLEDAI. In addition, serum miR-146a and miR-155 levels increase with calcitriol treatment. The results suggest that serum miR-146a and miR-155 may play important roles in the pathogenesis of SLE and have the potential for further development as biomarkers of SLE.

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