microRNA146a Expression in Lupus Patients With and Without Renal Complications

Hashad DI,^{1*} Abdelmagid MH,² and Elsherif SH³

¹Clinical Pathology Department, Alexandria, Egypt

²Rheumatology Department, Alexandria, Egypt

³Physical Medicine, Rheumatology and Rehabilitation Department Faculty of Medicine, Alexandria University, Alexandria, Egypt

microRNA146a (miRNA) expression profiles are likely to become important diagnostic and prognostic tools in many disease aspects. This work aimed to study the expression of miRNA146a in lupus patients with and without renal complications and to assess its association with disease activity. Patients enrolled in the study included 52 females affected by systemic lupus erythematosus (SLE) and another 60 females with lupus nephritis (LN). Forty-eight age-matched healthy females were enrolled as a control group. miRNA146a expression was assessed using real-time PCR. In SLE patients, miRNA146a was underexpressed as compared to healthy controls and lower levels were detected among lupus patients with renal affection. In addition, miRNA146a expression was low and serum Interferon-

α (IFN- α) level was high in patients with active lupus as compared to those with inactive disease. miRNA146a expression was inversely correlated to serum IFN- α level and to anti ds-DNA titer in the three studied groups. In conclusion, miRNA146a might be implicated in lupus pathogenesis. Moreover, miRNA146a expression correlates negatively to lupus activity and LN, whereas serum IFN- α has a direct correlation to both disease activity and nephritis; hence, both miRNA146a expression and serum IFN- α could be potentially important diagnostic biomarkers and potential novel strategies for therapeutic interventions, which may possibly be implied to enhance the sensitivity and specificity for the prediction of flares and prognosis in SLE patients. *J. Clin. Lab. Anal.* 26:35–40, 2012. © 2012 Wiley Periodicals, Inc.

Key words: miRNA; SLE; nephritis-activity-marker

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease which is characterized by immune-mediated organ damage caused by immune complex deposition in various organs. Renal involvement in lupus patients is a common and serious complication that may terminate in end stage renal failure. Therefore, lupus nephritis (LN) is considered as one of the major causes of morbidity and mortality in SLE (1).

The unfavorable prognosis of LN together with the unsatisfactory laboratory markers lacking sensitivity and specificity had led to the continuous search for novel biomarkers that are able to detect the severity of kidney affection, monitor treatment response, and disease progression (2).

miRNAs are small noncoding RNAs (21–24 base duplexes) that are usually incompletely base paired and form

partial duplexes within the 3' un-translated region (UTR) of targeted transcripts via an association with RNA induced silencing complex. Via this association, one strand of the miRNA duplex binds to the target mRNA sequence in the 3' UTR, finally leading to either the translational repression or degradation of the target mRNA, and thus they are considered as important gene regulators (3). In the human genome, the miRNA-146 family consists of

*Correspondence to: Doaa Hashad, Lecturer of Clinical Pathology, Department of Clinical Pathology, Faculty of Medicine, Alexandria University Azarita, Alexandria Egypt. E-mail: doaa_hashad2003@yahoo.com

Received 8 July 2011; Accepted 16 September 2011

DOI 10.1002/jcla.20501

Published online in Wiley Online Library (wileyonlinelibrary.com).

two genes: miRNA-146a on chromosome 5 and miRNA-146b on chromosome 10 (4).

The role of miRNA in the regulation of the immune response and immune cell development is becoming evident. Their role as diagnostic markers and factors involved in the pathogenesis of SLE has also been suggested (5). One study has identified 16 miRNAs differentially expressed in SLE (6). Changes in the level of a single miRNA may have a significant impact on the level of hundreds or even thousands of proteins (5). This was shown by Tang et al. who found that miRNA-146a regulates the level of several factors all of which are important for the IFN pathway (7).

Interferon- α (IFN- α) is an important cytokine that is not only involved in lupus etiology, but may also play a pivotal role in lupus pathogenesis and may affect its clinical manifestations. Many SLE patients have elevated serum levels of IFN- α , and these increased levels correlate with disease activity and severity. (7).

miRNA expression profiles are likely to become important diagnostic and prognostic tools. Hence, miRNA therapies could involve administration of a specific miRNA to downregulate specific target genes or the blocking of certain miRNA to increase expression of target genes (8,9).

As miRNAs play a key role in inflammatory and immunological response, this work was carried out to study the expression of miRNA146a in peripheral blood mononuclear cells of lupus patients with and without renal complications and to assess its association with disease activity.

MATERIALS AND METHODS

The study was approved by the ethics committee of the Faculty of Medicine, Alexandria University. All patients provided signed and written informed consent to participate in this study.

All 112 SLE female patients enrolled in the study fulfilled at least 4 out of the 11 revised criteria of the American College of Rheumatology (ACR), and 60 of these patients met the ACR criteria for LN (10). A group of 48 age matched and healthy females served as a control group. Subjects with other inflammatory diseases, chronic metabolic conditions, malignancies, or acute illness were excluded from the study.

A standard form was designed for registration of patients' important data. Personal history included inquiry about variable constitutional, musculoskeletal, articular, and dermatological symptoms. A detailed drug history (including corticosteroids, variable immune suppressants, and cytotoxic therapy) was also inquired about. Clinical examination focused on diagnostic ACR classifications for SLE as well as signs of complications, such as oedema and hypertension, in those who developed nephrotic syn-

drome. Assessment of disease activity was done using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (11). Patients were categorized as having active disease (score > 4) or inactive disease (scores ≤ 4) based on the SLEDAI results.

For all those participating in the study, urinalysis was performed focusing on detection of RBCs, urinary casts, and proteinuria which was quantitatively assayed in 24 hours collected urine samples. Complete blood count, renal functions, liver functions, and erythrocyte sedimentation rate were assayed. C-reactive protein was assessed using BNProSpec[®] Nephelometer (Siemens, Washington, DC). Serum IFN- α was assayed using Human Interferon alpha ELISA Kit (DRG International Inc, Mountainside, NJ).

Immunological studies included: antinuclear antibody assay using indirect immunofluorescence technique kit (ANAFLUOR[™], DiaSorin, Italy) and anti-double stranded DNA (anti-dsDNA) using anti-dsDNA enzyme immunoassay kit (ETI-dsDNA, DiaSorin, Italy).

EDTA blood was collected for miRNA isolation from peripheral blood mononuclear cells (PBMCs).

Peripheral Blood Mononuclear Cells Separation

Blood was processed within 2 hours of phlebotomy at room temperature and isolated by Ficoll density-gradient centrifugation according to the procedure of Lacelle et al. (12). Briefly, blood was centrifuged at $500 \times g$ for 10 minutes and the upper layer of plasma removed without disturbing the surface buffy coat. The remaining blood cells were immediately diluted two-fold with phosphate-buffered saline (PBS) and layered on Ficoll-Paque Plus (Amersham Biosciences, Canada) in 15 mL conical tubes. After centrifugation ($400 \times g$, 4°C , 30 minutes), the interphase layer containing PBMCs was carefully removed, washed in PBS and followed by centrifugation ($500 \times g$, 10 minutes).

miRNA Extraction and cDNA Synthesis

miRNA was extracted from the PBMCs using the *mirVana*[™] miRNA Isolation Kit (Applied Biosystems, Ambion, CA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the TaqMan[®] MicroRNA Reverse Transcription Kit (Applied Biosystems, Ambion, CA). Thermal profile was: 16°C (30 minutes), 42°C (30 minutes), and followed by 85°C for 5 minutes and a final hold at 4°C .

miRNA146a Expression Assay

Real-time PCR was performed on the Mx3000P[™] Real-Time PCR System (Stratagene, CA) using

TaqMan® MicroRNA Assay-miRNA146a reagents purchased from Applied Biosystems, CA. (Assay ID 002163). RNU6B Control miRNA Assay Reagents were used as an internal control (Inventory Assay ID 001093). Thermal cycling conditions included an initial hold at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec (denaturation step), and 60°C for 1 min (annealing/extension).

For quantification, miRNA146a expression was normalized to the expressed miRNA control; RNU6B using Stratagene Mx3000P™ Software (MX.PRO software) which determines miRNA146a expression using the $2^{-\Delta\Delta C_t}$ method (7, 13, 14).

STATISTICAL ANALYSIS

Data were analyzed using SPSS software package version 18.0 (SPSS, Chicago, IL). Qualitative data were analyzed using Chi-square and Fisher's exact test. Normally distributed quantitative data were analyzed using F-test (ANOVA). Nonparametric tests were used. Mann-Whitney *U* test and Kruskal-Wallis test. $P < 0.05$ was considered statistically significant. To assess the relationship between miRNA serum level and disease activity, Pearson's coefficient was used to analyze the correlation between miRNA146a expression and activity parameters as serum IFN- α level and anti-dsDNA titer.

RESULTS

There was no statistically significant difference between the control and the patients' group as regards age ($P = 0.569$). A statistically significant difference was observed between lupus patients and the control females as regards hemoglobin ($P < 0.001$), WBCs ($P = 0.006$) and platelets' count ($P = 0.002$).

All inflammatory markers were significantly higher in lupus patients as compared to the healthy control group, including serum IFN- α .

To illustrate the effect of drug intake on miRNA146a expression, 50 lupus patients with active disease and receiving high doses of corticosteroids were compared as regards their miRNA146a expression demonstrating no association between miRNA146a expression level and intake of higher doses (more than 40 mg/day) of corticosteroids ($P = 0.8$). The same was applied on a group of patients treated with anti-rheumatics (chloroquine, cyclophosphamide, methotrexate, and azathioprine) ($n = 45$) and still no association was detected ($P = 0.34$).

As for miRNA146a expression, it was significantly lower in lupus patients' group as compared to the control group ($P = 0.001$).

For more illustration, SLE patients were divided into two groups. The first group included SLE patients who had no renal affection (SLE patients group), whereas the second one included lupus patients who had renal involvement (LN patients group).

As regards disease duration, LN patients suffered longer disease duration as compared to the SLE group ($P = 0.038$).

Table 1 illustrates biochemical features, serum IFN- α and miRNA146a expression of all lupus patients and the control group. Table 2 shows the clinical features and immunological investigations of all lupus patients.

Relationship Between miRNA146a Expression, Serum IFN- α and LN

miRNA146a was significantly overexpressed in healthy controls ($P < 0.001$) as compared to the SLE group and significantly lower values were detected among LN group as compared to the SLE group ($P = 0.009$) (Fig. 1).

TABLE 1. Biochemical Features and miRNA146a Expression of All Patients and Controls

	SLE patients (<i>n</i> = 52)	LN patients (<i>n</i> = 60)	Control (<i>n</i> = 48)	<i>P</i>
Erythrocyte sedimentation rate: first hour (mm)	86 (50–150)	68.5 (30–150)	5 (5–9)	0.002*
C-reactive protein (mg/l) (Up to 3 mg/dL)	9.5 (1–43)	8.5 (3.9–34)	1 (0.2–3)	<0.001*
Serum creatinine (mg/dL)	0.9 (0.7–1)	2.3 (1–5.6)	0.95 (0.7–1.2)	0.002*
Urea (mg/dL)	24 (15–40)	70 (35–135)	22.5 (15–35)	0.002*
Urine protein (mg/24 hr)	58.50 (30–120)	1875 (550–3,900)	75 (6–98)	0.002*
Aspartate transaminase (AST)(U/L)	43 (15–45)	45 (23–54)	44 (23–54)	0.126
Alanine transaminase (ALT)(U/L)	28 (12–34)	52 (20–32)	30 (15–32)	0.169
Hemoglobin (g/dl)	11 (8.9–12.7)	11 (8–12)	10.90–13	<0.001*
WBCs (white blood cells) $\times 10^3/\mu\text{L}$	4,980 (2,300–8,000)	4,400 (3,000–9,800)	6,000 (4,000–8,900)	0.004*
Platelets $\times 10^3/\mu\text{L}$	158 (120–450)	160 (80–340)	220 (160–400)	0.002*
IFN- α (pg/mL)	74 (0–155)	175 (10–245)	22.5 (0–45)	<0.001*
miRNA146a expression	8.5 (1–29)	3.1 (1–17)	54.5 (40–62)	0.001*

Data are presented as median (min/max) and were analyzed by Mann-Whitney test, $P < 0.05$ is statistically significant. *Statistically significant.

TABLE 2. Clinical Features and Immunological Investigations of All Lupus Patients

	SLE patients (n = 52)	LN patients (n = 60)
Age (years)	32 (23–45)	32 (25–45)
Duration of disease (years)	5 (1–11)	7.75 (4–10)
Skin manifestations	32 (61.5%)	40 (66.6%)
Oral ulcers	10 (19.2%)	6 (10%)
Arthritis	36 (69.2%)	32 (53.3%)
Cardiac and pulmonary involvement	4 (7.6%)	6 (10%)
Hematological disorders	18 (34.6%)	17 (28.3%)
Neurologic affection	9 (17.3%)	11 (18.3%)
Drugs		
Corticosteroids	100%	100%
Others	53.80%	66.60%
ANA		
Negative	0	0
Positive	52 (100%)	60 (100%)
anti-dsDNA (U/ml)	50 (10–150)	43.5 (12–220)

Data are presented as number (%); ANA: negative and positive, anti-dsDNA: median (minimum/maximum). Hematological disorders: leucopenia, lymphopenia Hemolytic anemia, or thrombocytopenia. Drugs (others): chloroquine, cyclophosphamide, methotrexate, and azathioprine.

Fig. 1. Scatter plot showing miRNA146a expression in the studied groups.

As regards serum IFN- α , a statistically significant difference was observed between the three studied groups ($P < 0.001$) being highest in LN group, less in SLE group, and least in the control group.

Relationship Between miRNA146a Expression and Measures of Disease Activity

To assess the association between SLE disease activity and miRNA146a expression levels, lupus patients were classified into inactive and active groups based on SLEDAI scores.

According to SLEDAI, 24 patients (46.2%) of SLE group had active disease while the rest (28 patients) (53.8%) had inactive lupus. On the other hand, 44 females (73.3%) had active disease among the LN group and the remaining 16 patients (26.7%) had inactive disease.

miRNA146a expression was lower ($P < 0.001$) in those with active SLE as compared to the expression in inactive lupus cases ($P < 0.001$), as shown in Figure 2 and Table 3.

Fig. 2. Scatter plot showing miRNA146a expression in the group of active and inactive lupus patients.**TABLE 3. Relationship Between miRNA146a and Disease Activity Status Among SLE Group and LN Group**

		SLEDAI		<i>P</i>
		Active (n = 24 + 44)	Inactive (n = 28 + 16)	
microRNA 146a expression	SLE group	January 1–5	August 8–29	<0.001*
		3.25 ± 1.36	15.0 ± 5.48	
	LN group	1–6.5	October 10–17	<0.001*
		2.76 ± 1.50	12.31 ± 2.71	

Data are presented as median (min/max) and as mean ± SD.

Data were analyzed by Mann-Whitney test, $P < 0.05$ is statistically significant (*).

miRNA146a expression level was correlated with anti-dsDNA titer among the LN group and a significant negative correlation was found ($P = 0.007$, $r = -0.048$).

Serum IFN- α level was associated in all patients with lupus activity ($P = 0.03$ and 0.04 , respectively). In addition, miRNA146a expression was inversely correlated to serum IFN- α level in lupus patients ($P < 0.001$, $r = -0.44$).

DISCUSSION

SLE is a complex autoimmune disease characterized by chronic immune activation and multiple immunologic phenotypes. The pathogenesis of SLE is poorly understood and current therapies are based on nonspecific immunosuppression (7). The development of new biomarkers could help in avoidance of treatment adverse effects by screening asymptomatic patients, predicting the onset, activity, severity, and progression of disease, thus permitting earlier disease modifying intervention at potentially reversible stages (15, 16).

miRNAs are important regulators of gene expression, their biological functions and relevance to diseases are being thoroughly studied, aiming to elucidate their implication in various pathways that may prove valuable in detecting such autoimmune diseases or anticipating their complications (17).

A study conducted by Dai et al. (15) used miRNA microarray chip analysis to identify 66 miRNAs that were found to be differentially expressed (36 upregulated and 30 downregulated) in renal biopsies of patients with LN, suggesting a possible role for miRNAs as diagnostic markers and as factors involved in the pathogenesis of LN.

Tang et al. (7) used real-time polymerase chain reaction with SYBR green technique to elucidate the link between miRNA146a expression and IFN system as a possible pivotal point in lupus pathogenesis in a group of Chinese patients. This study (7) proved that miRNA146a expression regulates the level of many important key steps for the IFN pathway and reported that reduction (six-fold) of miRNA146a in SLE patients as compared to the control groups will likely affect the levels of these key factors significantly and contribute to overexpression of type I IFN, and thus supporting the association between miRNA146a expression and lupus pathogenesis.

The present study comes as a confirmation of the results of Tang et al. (7) with some differences in the methods of achievement. The present work used realtime polymerase chain reaction (Taqman technique) to study alterations of miRNA146a expression in peripheral blood mononuclear cells of a group of Egyptian females affected by SLE and assayed serum IFN- α level using ELISA technique, whereas Tang et al. (7) studied miRNA146a expression in peripheral blood samples of a group of Chinese lupus

patients using SYBR green technique. In addition, IFN system was investigated using IFN score.

In the present study, lupus patients complicated by nephritis suffered a significantly longer disease duration when compared with those patients without renal affection ($P = 0.038$). This might be the time needed for the pathological changes to affect the kidneys producing the full-blown clinical picture of LN.

Drug intake, either in the form of corticosteroids or anti-rheumatic treatment, did not affect the miRNA146a expression levels in lupus patients participating in the present study.

miRNA146a expression in the present work was downregulated in SLE patients as compared to the control group ($P < 0.001$). Lower values for miRNA146a expression were detected in LN group as compared to the SLE group ($P < 0.001$), confirming the importance of miRNA146a expression in the pathogenesis of lupus and LN in accordance with previous studies (7, 15).

On comparing serum IFN- α level in the three studied groups, it was highest in LN patients, followed by those with SLE, and lowest in the control group ($P < 0.001$).

In a trial to further investigate the relation between lupus activity and miRNA146a expression, SLEDAI score was used to identify lupus activity.

miRNA146a was downregulated in active cases from both patients' groups as compared with the inactive ones, suggesting a possible association with disease activity. This was further illustrated when a significant correlation was found between miRNA146a expression and anti-dsDNA titer which has been correlated with lupus activity in some studies (18).

In addition, serum IFN- α level, which is related to disease activity (19), was inversely correlated with miRNA146a expression level ($P < 0.001$) in all lupus patients, and it related well to lupus activity, so patients with active lupus had a combination of underexpressed miRNA146a and a high serum IFN- α level.

Evidence for the role of IFN as a key cytokine in lupus development is assured by the development of lupus in non-rheumatic disease patients following treatment with IFN (19). Also many studies (7, 20, 21) have shown that IFN- α is involved in multiple aspects of lupus pathogenesis. In addition, heightened levels of serum IFN- α and dysregulated expression of IFN response genes are common in lupus patients.

CONCLUSION

miRNA146a might be implicated in lupus pathogenesis. Moreover, miRNA146a expression correlates negatively to lupus activity and LN, whereas serum IFN- α has a direct correlation to both disease activity and nephritis; hence, both miRNA146a expression and serum

IFN- α could be potentially important diagnostic biomarkers and potential novel strategies for therapeutic interventions which may possibly be implicated to enhance the sensitivity and specificity for the prediction of flares and prognosis in SLE patients.

REFERENCES

- Hakim A, Frnrohr BG, Amann K, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *PNAS* 2010;107:9813–9818.
- Mok C. Biomarkers for lupus nephritis: a critical appraisal. *J Biomed Biotechnol* 2010;2010:1–11.
- Soifer H, Rossi J, Strom P. MicroRNAs in disease and potential therapeutic applications. *Mol Ther* 2007;12:2070–2079.
- Sonkoly E, Pivarcsi A. microRNAs in inflammation. *Int Rev Immunol* 2009;28:535–561.
- Chan EK, Satoh M, Pauley KM. Contrast in aberrant microRNA expression in systemic lupus erythematosus and rheumatoid arthritis: is microRNA-146 all we need? *Arthritis Rheum* 2009;60:912–915.
- Dai Y, Huang YS, Tang M, et al. Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. *Lupus* 2007;16:939–946.
- Tang Y, Luo X, Cui H, et al. MicroRNA-146a contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins. *Arthritis Rheum* 2009;60:1065–1075.
- Calin GA, Croce CM. microRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857–866.
- Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. *J Autoimmun* 2009;32:189–194.
- Hochberg MA. Updating the ACR revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997;40:172.
- Petri M. Disease activity assessment in SLE: do we have the right instruments? *Ann Rheum Dis* 2007;66:iii61–iii64.
- Lacelle C, Riol H, Xu S, et al. Blood sample processing for the study of age-dependent gene expression in peripheral blood mononuclear cells. *J Gerontol A Biol Sci Med Sci* 2002;57:B285–B287.
- Nakasa T, Miyaki S, Okubo A, et al. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis Rheum* 2008;58:1284–1292.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. *Diagn Mol Pathol* 2001;25:402–408.
- Dai Y, Sui W, Lan H, et al. Comprehensive analysis of microRNA expression patterns in renal biopsies of lupus nephritis patients. *Rheumatol Int* 2009;29:749–754.
- Te JL, Dozmorov IM, Guthridge JM, et al. Identification of unique microRNA signature associated with lupus nephritis. *PLoS ONE* 2010;5:1–7.
- Sonkoly E, Pivarcsi A. microRNAs in inflammation. *Int Rev Immunol* 2009;28:535–561.
- Lpez-Hoyos M, Cabeza R, Martnez-Taboada VM. Clinical disease activity and titers of anti-dsDNA antibodies measured by an automated immunofluorescence assay in patients with systemic lupus erythematosus. *Lupus* 2005;14:505–509.
- Niewol T, Swedler W. Systemic lupus erythematosus arising during interferon-alpha therapy for cryoglobulinemic vasculitis associated with hepatitis C. *Clin Rheumatol* 2005;24:178–181.
- Niewold T, Clark D, Salloum R, Poole B. Interferon alpha in systemic lupus erythematosus. *J Biomed Biotechnol* 2010;2010:1–8.
- Bagavanta H, Fua S. Pathogenesis of kidney disease in systemic lupus erythematosus. *Curr Opin Rheumatol* 2009;21:489–494.
- Dall’Era MC, Cardarelli PM, Preston BT, Witte A, Davis Jr JC. Type I interferon correlates with serological and clinical manifestations of SLE. *Ann Rheum Dis* 2005;64:1692–1697.
- Bengtsson A. Activation of type I interferon system in systemic lupus erythematosus correlates with disease activity but not with antiretroviral antibodies. *Lupus* 2000;9:664–671.