

Expression Analysis of the Peripheral Blood Mononuclear Cells miR-21 and miR-155 in Systemic Lupus Erythematosus Patients and Healthy Controls

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ABSTRACT

Background: Systemic lupus erythematosus (SLE) is an autoimmune disease with variable clinical manifestations mostly affecting the skin, joints, hematopoietic system, kidney, and the central nervous system. The micro-RNAs (miR) are small, non-coding, endogenous RNAs, with key roles in many biological processes. The aim of this study was to investigate the cellular expression of miR-21 and miR-155 in SLE patients and healthy controls.

Methods: SLE patients (n=35) and matched healthy controls (n=35) were enrolled in this study. SLE activity was assessed with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score. The peripheral blood mononuclear cells (PBMC) were isolated and the RNA was extracted following cDNA synthesis. The expression of miR-21 and miR-155 was evaluated by

the real-time PCR technique.

Results: The results showed that the expression level of miR-21 was significantly reduced in patients (0.64 times and p-value=0.02) and significantly correlated with disease duration. However, no significant differences were observed between the patients and control subjects for miR-155 (p-value =0.42). Moreover, the expression of miR-21 was indirectly correlated with the disease duration (R=-0.24, p-value = 0.05), but miR-155 did not reveal any significant association with clinicopathological features of the patients.

Conclusion: The result of this study suggests a significant down-regulation of miR-21 in PBMC of SLE patient, mostly in individuals with a longer disease history.

Keywords: Systemic Lupus Erythematosus; miRNA-21; miRNA-155; PBMC; SLEDAI

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease associated with autoantibodies against nuclear self-antigens. It has variable clinical manifestations, and mostly affects the skin, joints, hematopoietic system, kidney, lung, and the central nervous system [1]. There is growing evidence that abnormal expression of micro-RNAs has a role in the pathogenesis of multiple autoimmune diseases such as SLE [2]. Micro RNAs (miRNA) are a class of small RNA molecules which play a

critical role in immune regulation and cellular processes such as differentiation, proliferation, apoptosis, and metabolism [3, 4].

Computational analysis of 72 genes predisposing to SLE has shown that the majority of these genes (71 of 72) have at least one miRNA target place for over 140 micro RNAs [5]. Despite the advances in the SLE diagnosis and treatment, the molecular pathogenesis of this autoimmune disease is not clear; however recent investigations support the important role of CD4+ T helper response along with the increased activity of B lymphocytes in the SLE progression

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and development. We selected two different miRNAs regulating each of the immunopathogenic features: the miR-21 as T helper regulator and the miR-155 as B-cell response modulator to cover both pathways.

Briefly, miR-21 is involved in most biological functions and diseases such as cancer, cardiovascular, and inflammatory diseases. It has a role in the regulation of Th1 and Th2 response to the antigen, T-cell maintenance in its effective phase [6], and also it has a role in the regulation of the cell proliferation, metastasis, and apoptosis [7]. Previous reports indicate miR-21 deregulation in different autoimmune conditions like SLE, rheumatoid arthritis (RA), multiple myeloma, vascular disease, and type 1 diabetes [8-12].

Another target miRNA, miR-155 is located within a gene known as *B-cell integration cluster (BIC)* [13] and regulates different genes in the immune system such as the production of antibody by B lymphocyte, and also the immunoglobulin class switching. Dysregulation of this micro-RNA is associated with inflammatory responses, and eventually some autoimmune diseases such as SLE [14], multiple sclerosis [15], and RA [16].

The present study was aimed to explore the expression patterns of miR-21, as a T helper response regulator and miR-155, which is associated with the B-cell function, in the peripheral blood mononuclear cells (PBMC) of SLE patients and healthy individuals.

METHODS

Subjects: The present study was performed as a matched case-control study in which 35 cases with SLE and 35 healthy controls were enrolled. The age and gender of controls were matched with patients. Cases were included in this study after the SLE diagnosis was confirmed by a clinical specialist and confirmed by antinuclear antibody (ANA), Anti-double stranded antibody (anti-dsDNA), Anti-Smith antibodies (Anti-Sm), Complement component 3 (C3), and Complement component 4 (C4) test. The individuals with cancer or cardiovascular disease were excluded from the study. We also abstracted the clinicopathological features of the two groups, including age, sex, ethnicity, family history, duration, and severity of the disease (Table 1).

Ethical Review: This study was approved by Ethical Committee of Golestan University of

Medical Science (approval code= 2518169310219).

RNA Isolation and cDNA Synthesis: Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA). The quantity and quality of the extracted RNA samples were assessed using the Nano Drop ND-100 spectrometer (Thermo Fisher Scientific, USA). RNase-free D NaseI (Thermo Fisher Scientific, USA) treatment of total RNA was performed according to the instruction to eliminate any potential contamination with genomic DNA. The cDNA synthesis was carried out on 200ng of total RNA, by using the reverse-transcriptase into the cDNA (Pars Genome Co), according to the manufacturer's protocol.

Real-Time PCR: Target gene amplification was carried out in the ABI 7300. Real-time PCR using the specific primers Pars Genome's miRNA amplification kit (pars Genome co. Iran) and the commercial SYBR Green kit developed by Kit (parsGenome co. Iran) according to the manufacturer's protocol. The expression levels of miR-21 and miR-155 in each sample were normalized to U6 snRNA, as the housekeeping gene control. The normalized gene expression was calculated by the 2-dct formula.

The thermal cycling condition was as follows: initial denaturation in 95°C for 10 minutes, 40 cycles of denaturation in 95°C for 5 seconds, following annealing in 63°C for 20 seconds and extension in 72°C for 30 seconds. To confirm the specific amplification, the melt curve analysis was carried out. The amplification plot and melting curve of one sample are shown in Figure 1 as an example.

Statistical Analysis: All the experiments in this study were performed at least in duplicates and the SPSS 22 software was used for statistical analysis. The p-value ≤ 0.05 was considered as the significance level. To compare the mean of normalized gene expression level between normal and SLE patients, the non-parametric Mann Whitney-U-test was used. To compare the mean of gene expression in different groups according to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) or disease duration categories, the Kruskal-Wallis test was performed. For more investigation, the Pearson's correlation test was used to investigate the gene expression changes along the disease severity.

Gene expression changes of miR-21 and miR-

155 in PBMC of SLE patients and healthy controls: The qRT-PCR results indicated that the mean of normalized miR-21 expression level in PBMC of patient group (0.16 ± 0.03) is significantly less than the healthy individual group (0.25 ± 0.04) (fold change = 0.64 times, p -value = 0.02). The normalized gene expression values were plotted in different categories of disease duration history (year, Figure 2B) or disease severity (SLEDAI score, Figure 2C). The graphs showed that the expression of miR-21 mainly declines in SLE patients with SLEDAI score 1-3 or in patients with longer disease duration. For miR-155 gene expression, however, we found some variation between the SLE patients and the healthy controls, but the average of normalized miR-155 gene expression in patients (0.1 ± 0.02) was not significantly (p -value = 0.42) different from controls (0.1 ± 0.015) (Figure 2A).

When categorizing the SLE patients according to clinicopathological criteria; interestingly, the expression of miR-155 is related to the disease duration, mostly downregulated in patients with 3-5 years of SLE history.

Association analysis of miR-21 and miR-155 expression with patient's clinicopathological characteristics: The expression level of miR-21 and miR-155 was re-analyzed in different clinicopathological categories of each individual. Present study findings revealed no significant differences between the mean of normalized miR-21 expression and duration of disease from the diagnosis (Table 2). Interestingly, the Pearson's correlation test showed that the mean of normalized miR-21 expression is indirectly related to the disease duration ($R = -0.24$, p -value = 0.05) and the SLEDAI score ($R = -0.2$, p -value = 0.1); however, the expression level of the miR-155 was not correlated with disease duration or the severity ($R \sim 0$). As was expected, the results showed a significant direct correlation between the disease duration and the SLEDAI score ($R = 0.63$, p -value = 0.0001).

Evaluation of the miR-21 down-regulation as a potential diagnostic marker: The receiver operating characteristic (ROC) analysis was performed to investigate the diagnostic value of miR-21 in discrimination of SLE patients from the healthy controls. ROC analysis revealed that miR-21 could differentiate SLE patients from healthy controls, the area under the ROC curve (AUC) was equal to 0.67 with P -value = 0.014

(Figure 3). This finding suggests that miR-21 expression can be potentially helpful in SLE diagnosis with sensitivity = 71% and specificity = 63%.

DISCUSSION

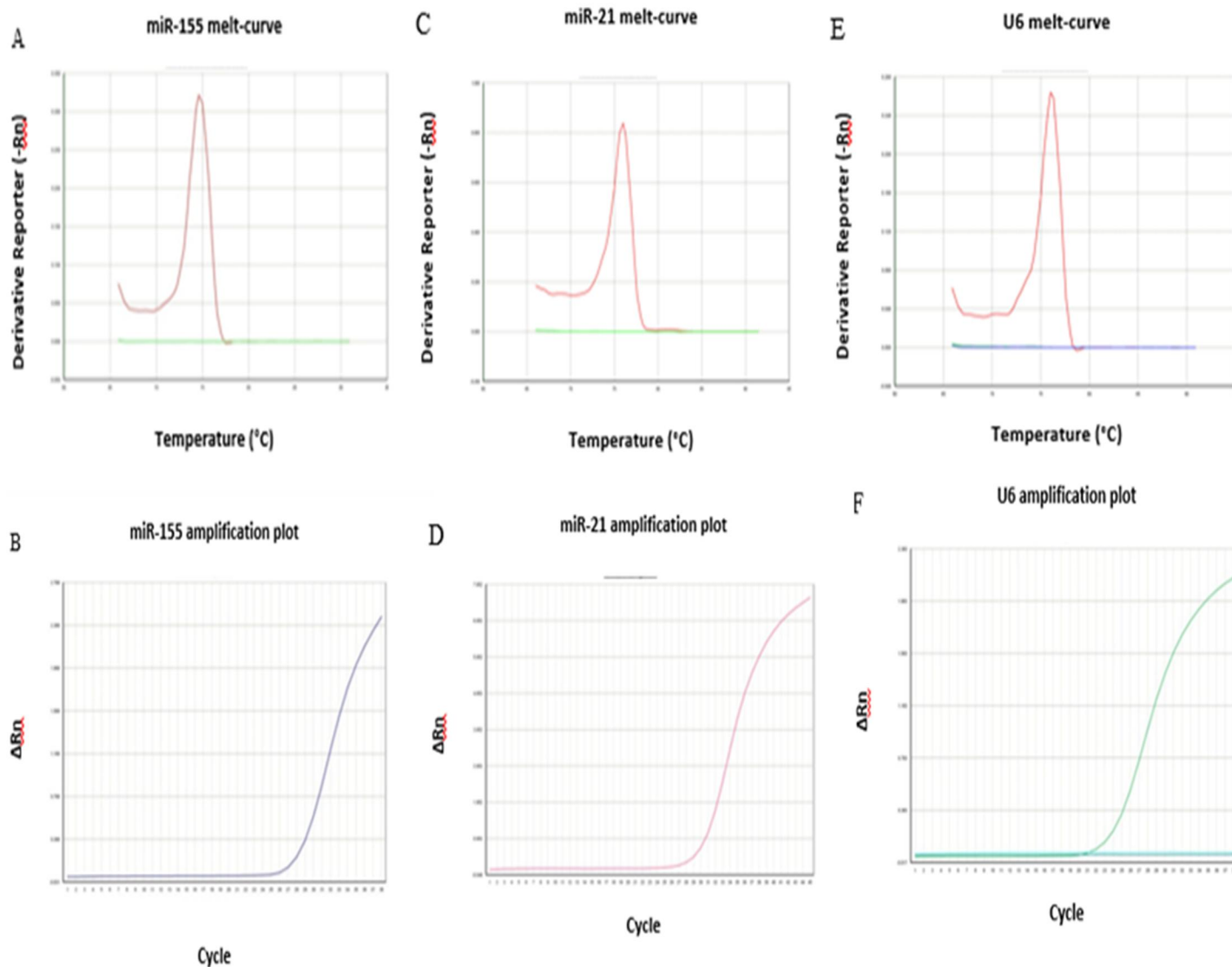
The autoimmunity observed in SLE indicates the combination of many factors, including increased production of the self-antigens, defective clearance of apoptotic cells, and the subsequent tolerance failure. The cellular and humoral immunity systems are associated with the SLE progression and development and it is difficult to identify one of them as the causal factor [17].

Micro-RNAs as the emerging regulators of the immune system have been reported to be associated with the SLE pathogenesis. Of interest, miR-21 which is reported to be deregulated in SLE patients [18] is reported to regulate the Toll-like receptors (TLR), Nuclear factor κ B (NF κ B), and signal transducers and activators of transcription (STAT) signaling pathways [19, 20]. The results revealed that expression of miR-21 is downregulated in PBMC of the SLE patient and indirectly correlated with the disease duration. Similarly, Rasmussen et. al. in 2015 reported that miR-21 expression is lower in the CD4+ T cells of SLE patients than controls; leading to high levels of its target, tumor suppressor programmed cell death 4 (PDCD4) with pro-inflammatory function. They concluded that less miR-21 could in part explain the increased programmed cell death in T cells from SLE patients [21].

Moreover, Dong et. al. in 2014 showed that miR-21 expression is lowered in the PBMC and CD4+ T cells of the rheumatoid arthritis patients; which is negatively correlated with the Th17/T reg cell ratio in the blood of the RAs [22]. However, there is evidence supporting that miR-21 expression is upregulated in PBMC of SLE patients leading to PDCD4 expression inhibition and aberrant T-cell responses [23]. Considering miR-21 as candidate circulatory biomarker in the plasma of SLE patients [24], some studies have investigated miR-21 expression in the plasma samples instead of blood cells; for example, Tang et. al. studied 44 untreated SLE patients' plasma samples and found that miR-21 expression in SLE is higher than control and positively correlated with SLEDAI score [25].

In contrast in the present study, new cases and treated SLE patients were both included for miR-21 expression and miR-21 had a disease duration

Figure 1: Representative amplification and melt-curve plots (A, B), the amplification and melting curves of one case are represented as a sample for miR-155 (A, B), miR-21 (C, D) and U6 snRNA (E, F). The melting curve analysis was done to confirm the specificity of the amplification



dependent decline in the miR-21 expression level with almost no significant decrease in the new patients. It seems that time factor and the anti-inflammatory therapy intervention might affect the miR-21 expression changes.

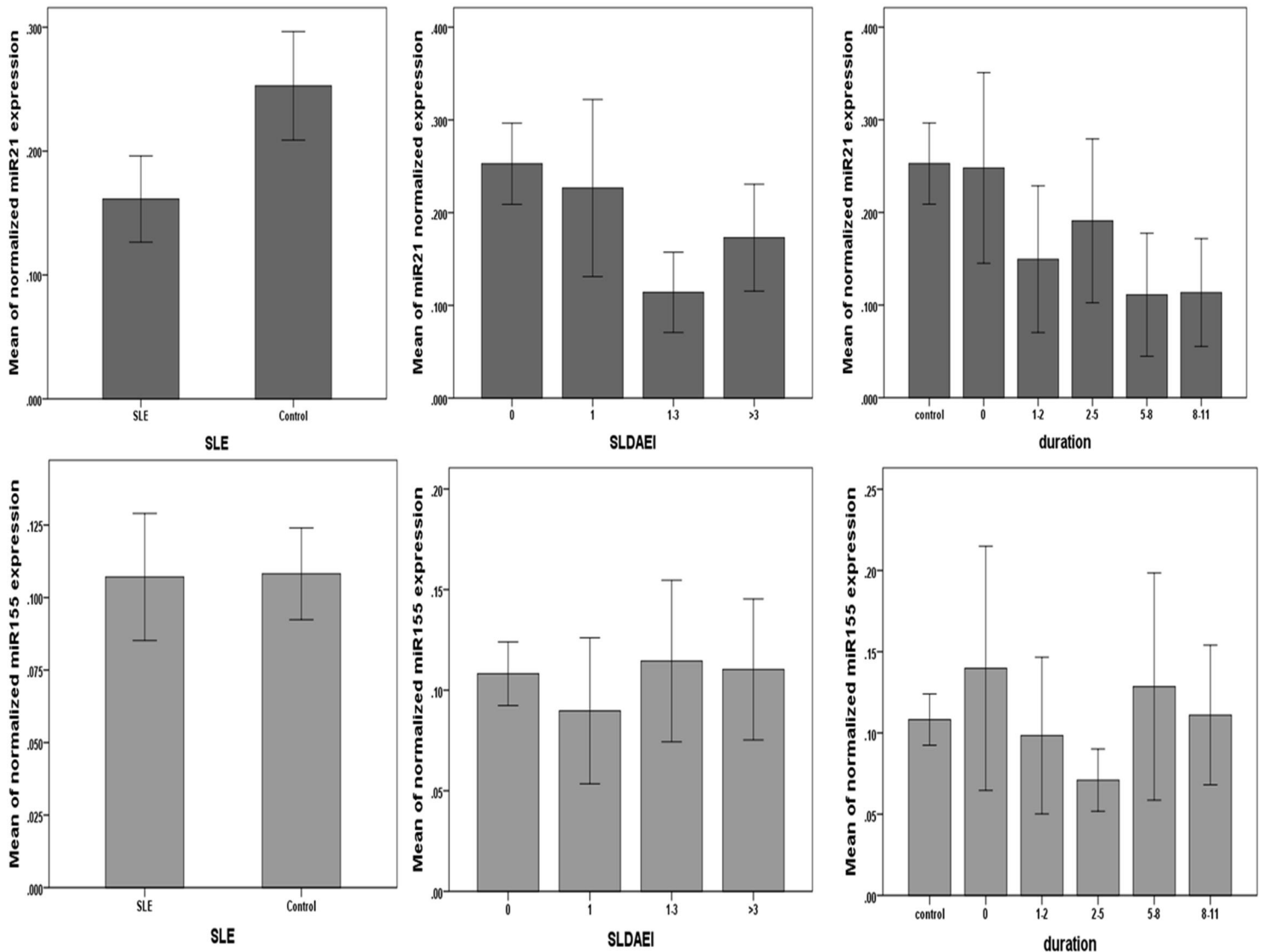
Another target micro-RNA was miR-155; its deficiency was reported as protective against SLE development in the lupus mouse model [26]. MiR-155 is reported to be deregulated in serum or urine samples of SLE patients [27,28]. Dysregulation of miR-155 is reported to be associated with the inflammation by targeting the sphingosine-1-phosphate receptor 1 (S1PR1) or

suppressor of cytokine signaling 1 (SOCS1) [29,30]. The results indicated that the expression of miR-155 is deregulated in patients suffering from SLE for a period of 1-5 years, but the overall mean miR-155 expression changes in all patients were not significant.

CONCLUSION

Altogether, our findings illustrated the expression of miR-21 in PBMC of SLE patients is significantly less than healthy individuals, suggesting it as a potential biomarker.

Figure 2: A comparison of miR-21 and miR-155 gene expression changes in SLE patients and normal individuals. The overall expression levels of miR-155 (top) and miR-21 (bottom) in controls and SLE patients are represented in a bar chart (A). The SLE patients are categorized according to disease duration (B) and disease severity score (C) and then the gene expression level of micro-RNAs were compared with the control group. The bar represents mean \pm SE of gene expression level normalized to U6.



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Figure 3: The ROC curve analysis for miR-21 expression level between SLE patients and normal individuals. The area under the curve (AUC=0.67) shows the suitability of miR-21 to correctly classify SLE groups of the normal samples (pvalue = 0.01).

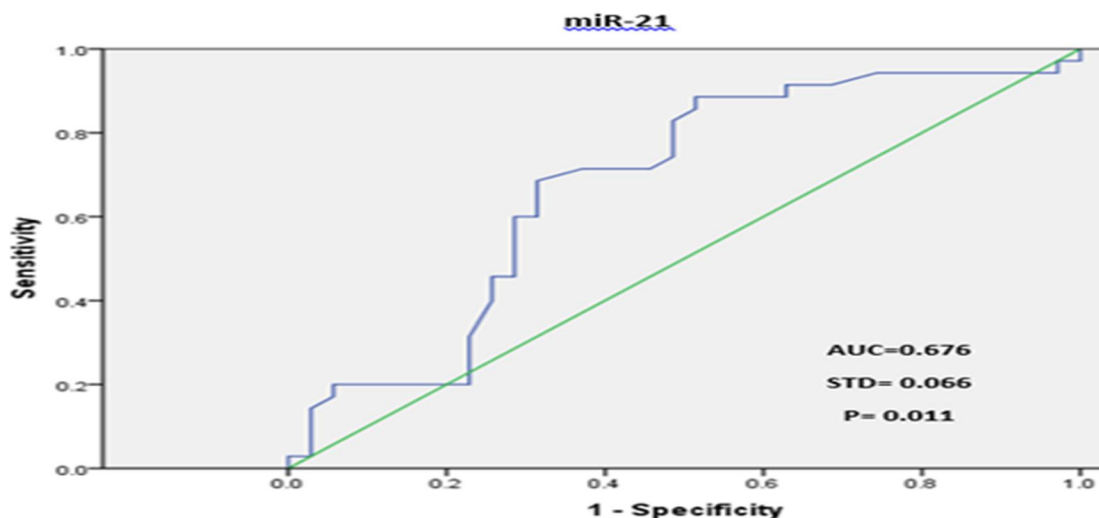


Table 1: Clinical-pathological feature of the SLE patients

Samples characteristics		
Gender	Female Male	n=33 n=2
Age	Mean	31
Time Duration Diagnosis	New Case <1 Years 2-5 Years 6-8 Years 9-11 Years	6 6 8 7 8
SLEDAI	Controlled phase (<1) Weak phase (1-3) Active phase (>3)	9.26% 14.40% 12.34%

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Table 2: The microRNA expression values according the clinical-pathological characteristics

		miR-21 *(mean±SE)	P-value	miR-155* (mean±SE)	P-value
Disease duration	Control	0.22 ± 0.40	0.21	0.10 ± 0.01	0.92
	New case	0.21 ± 0.11		0.13 ± 0.07	
	1-2 years	0.14 ± 0.07		0.09 ± 0.04	
	2-5 years	0.19 ± 0.08		0.06 ± 0.01	
	5-8 years	0.11 ± 0.06		0.12 ± 0.06	
	8-11 years	0.11 ± 0.05		0.11 ± 0.04	
Gender	M	0.04 ± 0.02	0.18	0.15 ± 0.06	0.45
	F	0.20 ± 0.02		0.09 ± 0.01	
SLDAIE	Control	0.22 ± 0.04	0.06	0.10 ± 0.01	0.75
	1	0.22 ± 0.09		0.08 ± 0.03	
	1-3	0.11 ± 0.04		0.11 ± 0.04	
	>3	0.14 ± 0.05		0.11 ± 0.03	

* The normalized gene expression toU6 ($2^{-\Delta\Delta CT}$)

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