Regulation of CD4+CD25+FOXP3+ Treg Cells in Systemic Lupus Erythematosus (SLE): Association With miRNAs Expression

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Abstract

Various genetic factors are controlling regulatory cells T (Treg) cell function, such as miRNAs. Interfering in the miRNA synthesis pathway in Treg cells could result in loss of Tregs' regulatory function, leading to the promotion of inflammatory settings and autoimmunity. This study was designed to investigate the role of miRNA in regulating Treg cells in SLE patients. Treg's frequency was determined using flow cytometry in 100 SLE patients' and100 healthy controls. Expression of miR-21, miR-24, miR125, miR-146a, miR-148a, and miR-155 was estimated in peripheral blood mononuclear cells (PBMCs) using quantitative real-time polymerase chain reaction (qRT-PCR). The ROC curve evaluated the diagnostic role of miRNAs in SLE. A significant elevation ($p<0.001$) in Treg cells in SLE patients than controls was observed, with a maximum increase inactive SLE cases. SLE patients exhibit a significant increase in miR-21 ($p<0.01$), miR-148a ($p<0.001$), miR-146a ($p<0.05$) and miR-155 ($p<0.001$) and significant reduction in miR-24 ($p<0.001$). An insignificant decrease in miR-125 was observed in SLE patients. The best sensitivity and specificity were detected in miR-148a (88%, 70%) at a cutoff value of 1. 065. Tregs were positively correlated with miR-21($r=0.333$, $p<0.05$), miR-146a ($r=0.589$, $p<0.01$) and miR-148a ($r=0.309$, $p<0.05$). In conclusion, this research provides a piece of novel information regarding Treg cells' in SLE patients. Our results pointed to the substantial role of miRNAs in controlling Treg cells in lupus. To validate our interesting results, more researches are needed.

1. Introduction

Systemic lupus erythematosus (SLE) is a serious autoimmune multisystem disorder characterized by a lack of self-antigen immune tolerance, resulting in the continuous development of pathogenic autoantibodies, lymphocyte activation, and release of inflammatory mediators[1–3].The subsequent production of autoantibodies by autoreactive B cells is one of the main pathological factors in SLE, contributing to the production and deposition of immune-complexes leading to tissue damage [4–6].Both pathogenesis and autoantibody formation are dependent upon $CD4^+$T cells [7].Based on cytokine patterns, naive CD4 + T cells can be divided into multiple subsets, including Th1, Th2, Th17, and Regulatory T cells (Treg) [7, 8].

Tregs ($CD4^+CD25^+FOXP3^+$) cells are a specialized type of T cells that suppress the immune response, thereby maintaining homeostasis and self-tolerance [9]. It plays a vital role in the tolerance induction and sanctuary against autoimmunity [11, 12].Treg cells regulate the inflammatory activity [12, 13]by suppressing the effector T cells and inducing the release of anti-inflammatory as well as tissue repair cytokines [e.g., transforming growth factor-β (TGF-β),interleukin-10 (IL-10) and IL-35]. TGF-β, working together with IL-10 to induce Treg differentiation from naïve T cells [14]. The equilibrium between the effector and regulatory T cells determines whether an autoimmune response can be triggered and propagated by autoreactive cells or not [12]. While a great deal of effort has been made to shed some light on the Treg imbalance in SLE, contradictory results have been shown [15–24].

Treg cells' epigenetic regulation's molecular mechanism is crucial for understanding SLE pathogenesis. MicroRNAs (miRNAs) are small (only 21–25 nucleotide long) regulatory non-coding RNA molecules which are function as an epigenetic regulator of gene expression and play important roles in various physiologic and pathologic processes [25]. Compared to conventional T cells, Tregs display a distinguished miRNA profile [26]. Via several mechanisms, single miRNA or miRNA clusters could participate in Treg biology [27]. Differentiation, suppressive function, and persistence of thymically derived (tTreg) and periphery-induced (pTreg) or in vitro (iTreg) regulatory T cells might be driven by miRNAs [28–30]. Even the expression of the Treg transcription factor, Foxp3, is based on a particular miRNA profile. Besides, it is now commonly accepted that Foxp3 expression does not grant a terminal differentiation state, and Tregs are malleable, and miRNAs are needed to incorporate the external signals that drive these phenomena [31, 32].

Knowing that abnormalities either in the number or function of Treg cells are associated with the pathogenesis of autoimmune diseases and grasping further pieces of information about changes in certain factors; immunological and

epigenetic, that coordinate the possible divergence of immune cells in lupus, our work performed a flow cytometric analysis of CD4 + CD25 + FOXP3 + Treg cells in SLE patients at various stages of disease activity. The potential impact of multiple expression profiles of microRNAs (miR-21, -24, -125, -146a, -148a, and − 155) on Treg cells was also examined.

2. Patients And Methods

2.1. SLE patients and healthy controls

Our study included 100consecutive patients who met the American College of Rheumatology (ACR) criteria for diagnosing SLE [33, 34]. They were outpatients at the Rheumatology Department at El-Eini Hospitals, Cairo University, Egypt. The mean duration of SLE was 6.97 ± 5.73 years. Disease activity was assessed for all the lupus patients on the day of blood sampling by the SLE disease activity index (SLEDAI), and they were divided into active (SLEDAI score ≥ 6) and inactive (a SLEDAI score of < 6) [35]. The exclusion was made for patients with concomitant malignant diseases, infections, diabetes, abnormal lipid profile, and pregnant women.

One hundred participants matched by age and sex were enrolled as a normal control group with no history of autoimmune disorders or immunosuppressive drug treatment. Through a standardized interview and physical examination, demographic and clinical characteristics were collected. The local Ethics Committee of Cairo University confirmed the study plan. Both patients and healthy controls agreed to participate in this research, and all had received informed consent.

Based on their clinical status, SLE patients were divided into groups with active or inactive organ involvement. Patients were divided based on clinical manifestation into SLE patients with: skin involvement (lupus rash), photosensitivity (discoid lupus); active joint involvement (arthritis with synovial swelling), active hematologic involvement (thrombocytopenia, lymphocytopenia, or leukocytopenia or hemolytic anemia); active renal involvement (nephritis with proteinuria > 0.5 g protein/24 h and/or active nephritic sediment).

2.1. Flow cytometric detection of T-lymphocytes

In sterile ethylenediaminetetraacetic acid (EDTA) tubes, 5 ml of venous blood were withdrawn. Ficoll-Hypaque separating media (Biowest SAS, Nuaillé, France) was used to isolate human peripheral blood mononuclear cells (PBMCs) from the blood, and Treg cells were identified using multiple staining's with three monoclonal antibodies against CD4, CD25, and FOXP3 as previously described [36, 37].

2.2. RNA extraction and quantification of miRNA expression levels

RNA was extracted from PBMCs of all participants using a TRIzol-based miRNA isolation kit (Life Technologies Ltd. UK)as described in the manufacturer's instructions. The purity and concentration of RNA were spectrophotometrically assessed by NanoDrop™ 2000/2000c (Thermo Fisher Scientific, Waltham, MA, USA). The RNA integrity was checked by 1%agarose gel electrophoresis.

miRNAs expression was analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). miScriptII RT Transcription Kit (QIAGEN Valencia, CA, United States) is used to transcribe 100 ng RNA from each sample to cDNA. The reaction was performed at 37ºC for 60 min, followed by incubation at 95ºC for 5 min to inactivate the enzyme. Expression of Hs_miR-21, Hs_miR-24, Hs_miR-125a, Hs_miR-146a, Hs_miR-148a, and Hs_miR-155 was measured using miScript SYBR Green PCR Kit (Qiagen) and miScript Primer Assays (Qiagen) according to the supplemented protocol. The PCR cycling conditions were performed in AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) as follow: 95ºC for 15 min for initial denaturation, followed by 40 cycles at 95°C for 15 s for denaturation, 55°C for 30 s for annealing and 72°C for 30 s for an extension, and a final stage were 95ºC for 15 s, 60ºC for 1 min and 95ºC for 15s.As endogenous housekeeping control, SNORD68 and U6B small nuclear RNA (RNU6B) expression were used for data normalization. All calculations of miRNA expression levels were done as previously reported by El-Maadawy *et al.* [37].

2.3. Statistical analysis

SPSS 21.0 was used for all statistical analysis. Where applicable, data were statistically defined in terms of mean, standard error (SE), or frequencies. The Student's T-test for statistical analysis of parametric data and the Mann–Whitney U test for non-parametric data were used. Comparisons between groups were made using a one-way analysis of variance (ANOVA).By a receiver operating characteristic (ROC) curve, sensitivity versus the false positive frequency (one-specificity) for miRNAs was analyzed. Person's or Spearman's correlation test was used to assessing the correlation between variables. All two-sided values with a P-value of less than 0.05 were considered. significant.

3. Results

3.1. Demographic, biochemical, and clinical characteristic of SLE patients

The present study was conducted on 100 SLE patients; 87 women and 13 men. Their mean age was 32.8 ± 1.0 years. One hundred healthy individuals were run in parallel (85 females and 15 males, with mean age 27.22 ± 7.90). All patients and controls were examined for the biochemical parameters associated with the disease. Table (1) summarized demographic and biochemical data of both patients and controls. The detailed clinical characteristics were summarized in Table (2). The mean disease activity for all patients was 9.37 ± 9.01. Based on the SLEDAI score, SLE patients were divided into 41 active patients (41 %) and 59 patients in an inactive state (59 %).

3.2. Detection of Treg cells by flow cytometry

Figure (1) showed the dot-plots representative of flow cytometric analysis of Treg cells. A significant elevation in the percentage of Treg cells ($p < 0.001$) in SLE patients when compared with healthy controls was observed (Fig. 2). Our results showed a significant increase in active and inactive patients ($p < 0.001$ and $p < 0.05$, respectively) compared to healthy controls with a maximum elevation in inactive ones. Although active SLE patients have more Treg than inactive patients, this increase is statistically insignificant.

3.3. miRNAs expression levels in SLE

As an overview of the whole lupus patients, our data showed a significant diminution in the expression ofmiR-24 in SLE patients compared to healthy controls ($p < 0.001$). miR-125 was also decreased in SLE patients but insignificantly. On the other side, miR-21, miR-146a, miR-148a and miR-155 were significantly elevated ($p < 0.01$, $p < 0.05$, $p < 0.001$and$p < 0.001$; respectively) in lupus patients in relation to normal controls. Concerning the activity of the disease, our data showed a reduction in miR-24 and miR-125 and elevation in miR-21, miR-146a, miR-148a, and miR-155 in both groups (Fig. 3) as compared with normal controls. Regarding active and inactive SLE patients, no significant changes were found in the expression of miRNAs (miR-24 and miR-125 and elevation in miR-21, miR-146a, miR-148a, and miR-155) between both groups.

As shown in Figure (4); positive correlations were found in lupus patients between miR-21 and 3 miRNAs [miR146a ($r = 0.438$; $p < 0.0015$), miR-148a ($r = 0.320$; $p < 0.001$) and miR-155 ($r = 0.255$; $p < 0.01$)]. Moreover, direct correlation between miR-24 and miR-125 ($r = 0.306$; $p < 0.001$) and between miR-148a and miR-155 ($r = 0.0351$; $p < 0.001$) were demonstrated in SLE patients. On the other hand, there was a negative correlation between miR-24 and miR-148a ($r= -0.178$; $p < 0.05$).

To distinguish between SLE patients and healthy control groups, we used ROC curve analysis to estimate the cutoff value for all calculated miRNAs (Fig. 5).According to the ROC curve results, the highest value of the area under the curve (AUC) was found in miR-148a(0.806), followed by miR-155 (0.764), and finally miR-21 (0.625).The highest levels of sensitivity and specificity were discovered (Table 3). The best sensitivity and specificity were detected in miR-148a (88% and 70%; respectively) at a cutoff value of 1.065 (Table 3).

3.4. Correlation between miRNAs and T-lymphocytes

Tregs were directly correlated with miR-21($r = 0.333$, $p < 0.05$), miR-146a ($r = 0.589$, $p < 0.01$) and miR-148a($r = 0.309$, $p < 0.05$).

3.5. Association between SLE clinical manifestations and miRNAs and Tregs.

The association between SLE clinical disease manifestations and miRNAs secretion levels was demonstrated (Table 4). Patients with renal manifestations or neutropenia had significantly higher levels of miR-21 ($p < 0.01$and $p < 0.05$, respectively), while SLE patients with Raynauds phenomena had a significantly lower expression of miR-21 ($p < 0.05$) compared with those without these phenomena. SLE patients with serositis or renal disorder had significantly higher levels of miR-24 ($p < 0.001$and $p < 0.05$, respectively) compared to patients without these manifestations. Vasculitis manifestation was accompanied by a significant elevation ($p < 0.01$) of miR-125. Significantly elevated levels of miR-146a were reported in SLE patients with photosensitivity or lymphopenia ($p < 0.05$). Lupus patients with neutropenia had significantly higher levels of miR-155 than SLE patients without this manifestation ($p < 0.05$). We found a remarkable observation in miR-148a, which is significantly elevated ($p < 0.01$) in all hematological abnormalities (leucopenia, neutropenia, or lymphopenia) in addition to vasculitis ($p < 0.01$), serositis ($p < 0.01$), or renal manifestations ($p < 0.01$).

According to the type of treatment (Table 5), SLE patients treated with Endoxan, Imuran, and biologic had significantly higher levels ($p < 0.01$) of miR-21 compared to SLE patients without these treatments. A reduction in miR-125 ($p < 0.001$), miR-146a ($p < 0.01$), and miR-155 ($p < 0.05$) was observed in response to the treatment with Endoxan, Imuran, and Biologic.

A significant increase in Treg cells was observed in SLE patients with photosensitivity ($p < 0.01$) and with Raynauds ($p < 0.001$) as compared with patients without these manifestations (Table 6). Alternatively, Treg cells didn't show any statistical significance with the other clinical manifestations of the disease or any treatment regimens.

4. Discussion

Tregs suppress self-reactive T cells and inhibit their number and functions, helping to maintain peripheral tolerance and prevent the onset of autoimmune diseases [38, 39]. Tregs suppress the functions of a number of cell types, including CD4 + TH cells, B cells, CD8 + cytotoxic T lymphocytes (CTLs), and antigen-presenting cells(APC), to effectively block immune responses, inflammation, and tissue destruction [40–42].In the present study, alteration in Treg cells' frequency in SLE has been analyzed in the light of miRNAs expression, which possibly influences Tregs.

Our study observed a substantial increase in Treg cells in both active and inactive SLE patients compared to controls, with the most significant increase in active ones. This result agreed with Singla *et al.'s*(2017) [43] results, who reported a significant increase in Tregs in childhood SLE and mentioned that active lupus patients had a higher percentage than inactive lupus patients do. A previous study by Suarez*et al.* (2006)[44] also observed a significant elevation in both active and inactive SLE patients with a maximum increase in inactive ones. In contrast, Kailashiya *et al.* (2019)[45]found an insignificant difference in Treg cells' percentage in SLE patients. Knowing that corticosteroids (glucocorticoids and cyclophosphamide) were used as a regular treatment of lupus due to their suppressing effect on the immune response, specifically the development of pro-inflammatory cytokines, the observed rise in Tregs in our SLE patient might be returned to the impact of immunosuppressive therapies. These therapeutics have been proven to augment Tregs frequency in several conditions, including lupus [46–49].

Several miRNAs have been discovered to be essential in immune homeostasis. The role of microRNAs in immune cell lineage differentiation and their physiological functions in maintaining normal innate and adaptive responses is well known [50, 51]. Aberrations in the miRNA-mediated immune-cell development and function regulation have been related to

autoimmune diseases [52–54]. Intuitively, miRNA dysregulation is one of the main contributors to the collapse of self-tolerance, leading to autoimmunity [55].

In the current study, there was a lowering in miR-125a expression level in both active and inactive phases of the disease compared to the healthy group. These findings agree with Zhao*et al.* (2010) [56] and Wang *et al.* (2012) [57], who reported a reduction in miR-125 level in SLE patients. In T cells isolated from lupus patients, diminished levels of miR-125a had been reported [58]. miR-125a promotes the up-regulation of the inflammatory chemokine RANTES, which is needed for the adverse effects of inflammatory processes. Its deficiency impairs Treg maintenance and immunoregulatory capacity, while over expression of miR-125a stabilizes Treg-mediated self-tolerance [59].

A significant diminution in miR-24 expression levels was observed in SLE patients, either active or inactive, compared to normal controls. No previous studies have been performed on the change in miR-24 level in lupus patients to the best of our knowledge. Murata *et al.* (2013)[58] reported an increase in the expression level of miR-24 in Rheumatoid Arthritis (RA) patients. Both miR-24 and miR-125a can play a role in the inflammation's enhancement. Via direct targeting of Furin, miR-24 might control the processing of latent transforming growth factor (TGF-1) [60], and miR-125 targets the tumor necrosis factor-alpha-induced protein 3 (TNFAIP3) [61]. TGF-β1 plays a suppressive role in immune system regulation[62]. Moreover, furin expression in T-cells is also essential for maintaining peripheral immune tolerance [63, 58].

Our results recorded a significant elevation in miR-146a in active and inactive SLE patients compared to normal controls. Our data agree with Chen *et al.* (2017) [64] and Zheng *et al.* (2017) [65], who observed over expression of miR-146a in SLE patients. On the other hand, Luo *et al.* (2011) [66] reported a reduction in miR-146a expression in lupus patients. The TLR4/NFB signaling pathway is negatively regulated by miR146a, and its down regulation causes inflammatory responses to be activated. Over expression of miR-146a reduced TRAF6 and consequently inhibited the activity of NF-κB, resulting in simultaneous inhibition of TNF-α, IL-1β, and IL-8 synthesis [67].

Similar to previously published studies of Chen *et al.* (2017) [64] and Shumnalieva *et al.* (2018) [68] who found over expression of miR-155 in SLE patients; our data showed a significant increase miR-155 in the peripheral blood of SLE patients with a maximum elevation in inactive patients. This data disagrees with Wang *et al.* (2012) [57], who reported an unexpected reduction in the expression level of miR-155 in SLE patients. Over expression of miR-155 contributes to the development of antibodies, irregular T cell differentiation, kidney failure, and lupus-like symptoms [69, 70]. Some miRNAs, such as miR-155, commonly associated with a compromised immune response and increases disease activity, were differentially expressed in multiple autoimmune diseases [71].

We demonstrated a significant increase in miR-21 was observed in both active and inactive status of the disease compared to healthy controls. In accordance with this data, the study of Wang*et al.* (2012)[57] on SLE patients pointed to the up-regulation of miR-21 expression in SLE patients. The same observation was previously mentioned by Pan *et al.* (2010) [72], who observed a significant increase in miR-21.In accordance, patients with active disease have substantially higher levels of miR-21 in their PBMC than normal subjects and patients with inactive disease [73]. Elevated miR-21 levels promoted CD4 + T cell activation, B cell hyper-responsiveness, and over expression of autoimmune-associated methylation-sensitive genes through repression of DNMT1, PDCD4, or PTEN expression [72, 74, 75]. Besides, the inhibition of miR-21 in CD4 + T cells from SLE patients might reverse T cells' activation [74, 76].

A significant elevation in miR-148a expression levels in SLE patients was observed in the present study, with the maximum increase in the active group. This finding was in agreement with Wang *et al.* (2012) [57] and Chen *et al.* (2017)[64], who observed an increase in miR-148alevel in SLE patients. Moreover, our finding was consistent with Pan *et al.* (2010)[72], who observed that miR-148a was up-regulated in SLE patients. miR-148a expression was up-regulated in $CD4^+$ T cells from patients with SLE patient. miR-21, miR-126, and miR-148a over-expression resulted in DNA hypomethylation in CD4 + T cells by direct inhibition of DNMT1 protein expression, thus inducing CD4 + T cell activation and secretion of autoimmune-related proteins, such as CD70, CD11a, and LFA-1[72, 77, 76].In females, DNA methylation serves as a housekeeping mechanism

for physiological X-chromosome inactivation [78–81].It might be estimated that increased circulating miR-21 and miR-148a, in turn, might also accelerate disease progression through the cell-cell communication processes between these apoptotic bodies, exosomes, and target cells, such as quiescent lymphocytes[76]. Zhang *et al.* (2020)[71] pointed to the elevation of miR-148a, which is generally associated with the immune response and increases the disease's activity.

In conclusion, we approved the numeric rising in Treg cells' frequency in SLE patients, especially those in an active state. Although, we stressed the idea that these elevated cells might be malfunctioning. Studying the expression of some miRNAs associated with Treg cells pointed to the increase in miR146a, miR155 miR148a, and miR-21, coinciding with the reduction of miR-24.We hypothesized that the increase in miR-21, miR-148a, and miR-155 (Treg positive regulators) accompanied by a decrease of miR-24 (Treg negative regulators) favors the elevation of Treg cells, leading to this observed increase of Treg cell frequency. There is a lack of consensus in the research on the relationship between Treg and rheumatic diseases. The majority of evidence proposes Treg cells' impairment, quantitatively and/or qualitatively. Herein, our results provide a novel insight into Treg-miRNA's role in lupus patients' regulation network.

However, our study has some potential limitations, such as the lack of previous studies on some miRNAs in SLE patients (such as miR-24) and using a sorter to test the function of detected Treg cells. Thus, further studies are needed to confirm our findings. We performed the same research on another important autoimmune disease (RA) to examine our assumption's strength in view of this hope.

Declarations

Conflict of interest: The authors declare that they have no conflict of interest.

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Availability of data and material: Data available by corresponding author on request.

Informed consent: All patients and healthy controls agreed to be enrolled in this study, and informed consent was obtained from all participants.

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Tables

Table (1). Demographic and biochemical characteristics of controls and SLE patients.

Parameters	Control (N=100)	SLE (N=100)	P value
Age	27.22± 7.90	32.8± 1.0	NS
Sex (female/male)	85/15	87/13	NS
ESR	6.2 ± 0.2	56.4 ± 3.4	P<0.01
WBC (X1000/µl)	8.0 ± 0.14	8.1 ± 0.4	NS
HGB (g/dl)	13.2 ± 0.14	11.0 ± 0.2	P<0.01
Platelets (X1000/µl)	285.5 ± 6.4	257.1 ± 12.3	P<0.01
Creatinine (mg/dl)	0.9 ± 0.23	0.9 ± 0.7	NS
ALT (IU/L)	21.09 ± 0.6	21.2 ± 1.6	NS
AST (IU/L)	23.8 ± 0.6	23.5 ± 1.5	NS

All data are presented as mean ± standard Error (mean ± SE). NS = not significant.

White Blood Cells (WBCs); Alanine aminotransferase (ALT); Aspartate aminotransferase (AST); ESR (erythrocyte sedimentation rate); HGB (Hemoglobin).

Table (2): Clinical and laboratory characteristics of SLE patients

Demographic data	Mean ± SD	Laboratory Data	Mean ± SD
Age (years)	32.75±10.31	Serum albumin	3.19 ± 0.7
Disease duration (years)	6.97 ±5.73	C3titre	77.4 ± 44.6
Female/Male	87/13	C4 titre	20.6 ± 24.3
SLEDAI	9.37±9.01	Cholestrol	187.5 ± 72.1
ACR criteria of SLE	No (%)	Treiglyceride	162.97 ± 92.9
Malar rash	73 (72.3)	HDL	47.2 ± 12.9
Photosensitivity	53 (52.5)	LDL	118.3 ± 47.3
Oral Ulcers	63 (62.4)	Consumed C3	34 (33.7)
Arthritis	50 (49.5)	Consumed C4	20 (19.8)
Serositis	39 (38.6)	Treatment	N (%)
Renal disorders	62 (61.4)	HCQ	89 (94.6)
Neuropsychiatric disorders	13 (14.9)	Endoxan	62 (65.9)
PanCytopenia	19 (18.8)	Imuran	67 (71.2)
Anti-nuclear Ab	71 (70.3)	Biological	6 (6.3)
Anti-dsDNA Ab	71 (70.3)		
Other clinical manifestations	No (%)		
Constitutional symptoms	81 (80.2)		
Mucocutaneous manifestation	84 (83.2)		
Vasculities	28 (28.7)		
Raynauds phenomena	19 (18.8)		
Alopecia	30 (29.7)		
Hypertension	29 (28.7)		
Thrombocytopenia	29 (28.7)		
Haemolyticanaemia	16 (15.8)		
Leucopenia	34 (33.7)		
Neutropenia	13 (12.9)		
Lymphopenia	31 (30.7)		

Table (3). ROC curve results of selected microRNAs in SLE

Parameters	Cut-off	AUC	Sensitivity (%)	Specificity (%)	95% CI	P-value
miR_21	1.345	0.628	78	48	0.724-0.532	P<0.01
miR_24	0.115	0.374	70	42	0.473-0.274	P<0.001
miR_125	5.065	0.502	80	86	0.604-0.399	P<0.001
miR_146	1.185	0.610	65	60	0.712-0.509	P<0.001
miR_148	1.065	0.806	88	70	0.884-0.728	P<0.001
miR_155	0.815	0.764	81	64	0.848-0.581	P<0.001

(AUC): Area under the curve, (CI): Confidence interval

Table (4). miRNA expression in SLE patients with different clinical manifestations

Clinical parameter		miR_21 (Mean ± SE)	miR_24 (Mean ± SE)	miR_125 (Mean ± SE)	miR_146 (Mean ± SE)	miR_148 (Mean ± SE)	miR_155 (Mean ± SE)
ACR criteria of SLE							
Malar rash	No	0.30±0.05	2.17±0.43	1.74±0.21	3.05±0.63	3.10±0.81	3.82±0.57
	Yes	0.32±0.04	2.15±0.23	1.99±0.23	3.15±0.34	3.11±0.51	4.37±0.65
Photosensitivity	No	0.28±0.047	2.02±0.32	1.82±0.25	2.69±0.43	3.63±0.74	3.55±0.64
	Yes	0.34±0.05	2.27±0.27	2.01±0.25	3.49±0.42*	2.67±0.48	4.75±0.69
Oral Ulcers	No	0.34±0.06	2.03±0.28	1.99±0.25	2.81±0.48	2.79±0.61	3.79±0.58
	Yes	0.29±0.05	2.27±0.30	1.91±0.25	3.39±0.40	3.36±0.60	4.54±0.71
Arthritis	No	0.29±0.44	2.29±0.34	1.89±0.24	2.62±0.31	3.22±0.61	3.93±0.67
	Yes	0.31±0.06	2.02±0.24	1.95±0.27	3.70±0.51	3.09±0.64	4.48±0.72
Serositis	No	0.34±0.05	1.92±0.16	1.91±0.23	2.91±0.37	2.42±0.43	4.11±0.52
	Yes	0.27±0.05	2.53±0.47***	1.96±0.29	3.47±0.53	4.26±0.85***	4.37±0.95
Renal disorders	No	0.21±0.03	1.70±0.21	1.80±0.29	3.25±0.56	2.37±0.37	4.04±0.78
	Yes	0.36±0.05**	2.41±0.31*	1.98±0.23	3.09±0.36	3.63±0.67**	4.28±0.63
Neuropsychiatric disorders	No	0.31±0.04	2.22±0.23	1.81±0.19	3.07±0.30	3.12±0.49	3.94±0.49
	Yes	0.32±0.06	1.82±0.43	2.46±0.49	3.35±0.99	3.04±0.70	5.50±1.47
PanCytopenia	No	0.31±0.04	2.27±0.26	1.83±0.19	3.29±0.39	3.11±0.48	3.95±0.51
	Yes	0.33±0.09	1.78±0.25	2.21±0.42	2.63±0.38	3.09±0.96	5.01±1.19
Other clinical Manifestations							
Constitutional symptoms	No	0.37±0.06	1.88±0.35	1.91±0.34	2.43±0.32	3.13±1.07	3.73±0.69
	Yes	0.29±0.04	2.24±0.24	1.93±0.21	3.34±0.38	3.10±0.46	4.35±0.59
Mucocutaneous manifestation	No	0.34±0.08	2.44±0.73	1.91±0.37	3.16±1.10	3.93±1.34	3.20±0.75
	Yes	0.31±0.04	2.09±0.19	1.93±0.20	3.11±0.29	2.94±0.44	4.42±0.55
Vasculities	No	0.32±0.05	2.07±0.24	1.71±0.18	3.26±0.34	2.50±0.31	3.80±0.51
	Yes	0.31±0.06	2.37±0.41	2.44±0.42**	2.81±0.63	4.56±1.21***	5.18±1.07
Raynauds phenomena	No	0.34±0.04	2.19±0.24	1.90±0.21	3.27±0.37	3.01±0.45	4.07±0.48
	Yes	0.18±0.03*	2.03±0.37	2.02±0.33	2.50±0.26	3.55±1.21	4.80±1.53
Alopecia	No	0.32±0.05	2.24±0.27	2.06±0.25	3.18±0.42	3.57±0.61	4.31±0.68
	Yes	0.31±0.06	2.05±0.31	1.73±0.22	3.09±0.39	2.27±0.43	4.09±0.55
Hypertension	No	0.28±0.04	2.16±0.23	1.96±0.22	2.95±0.34	3.06±0.52	3.94±0.48
	Yes	0.39±0.09	2.15±0.44	1.84±0.31	3.57±0.67	3.22±0.78	4.91±1.18
Thrombocytopenia	No	0.29±0.04	2.19±0.04	1.81±0.22	3.25±0.41	3.23±0.54	4.37±0.67
	Yes	0.34±0.08	2.07±0.25	2.13±0.30	2.88±0.42	2.88±0.71	3.91±0.57

Haemolyticanaemia	No	0.33±0.04	2.29±0.24	1.87±0.18	3.17±0.38	3.18±0.52	3.94±0.47
	Yes	0.26±0.06	1.61±0.21	2.16±0.52	2.96±0.34	2.82±0.55	5.27±1.49
Leucopenia	No	0.29±0.04	2.20±0.29	1.75±0.20	3.12±0.38	2.53±0.34	3.79±0.60
	Yes	1.12±0.15	2.08±0.29	2.18±0.33	3.28±0.52	4.06±0.94**	4.81±0.83
Neutropenia	No	0.29±0.04	2.10±0.22	1.81±0.21	3.06±0.29	2.56±0.38	3.76±0.46
	Yes	0.37±0.05*	2.28±0.60	2.39±0.31	3.59±1.09*	5.82±1.48**	6.17±1.69*
Lymphopenia	No	0.27±0.04	2.22±0.29	1.75±0.20	2.74±0.23	2.59±0.33	3.73±0.60
	Yes	0.36±0.06	2.05±0.31	2.18±0.35	3.79±0.67*	4.11±0.97**	4.93±0.85

P<0.05 (*), P<0.02 (**); P<0.001 (*)

Table (5). miRNA expression in SLE patients with different treatments

Clinical parameter		**miR_21** (Mean ± SE)	**miR_24** (Mean ± SE)	**miR_125** (Mean ± SE)	**miR_146** (Mean ± SE)	**miR_148** (Mean ± SE)	**miR_155** (Mean ± SE)
Treatment							
HCQ	No	0.23±0.06	1.97±0.56	1.97±0.41	3.08±0.94	3.12±1.02	2.47±0.62
	Yes	0.31±0.04	2.16±0.22	1.89±0.19	3.17±0.33	3.14±0.47	4.42±0.54
Endoxan	No	0.18±0.02	1.99±0.28	2.23±0.44	2.52±0.29	2.97±0.84	4.09±0.67
	Yes	0.37±0.05**	2.21±0.28	1.74±0.15***	3.49±0.43	3.22±0.49	4.23±0.65
Imuran	No	0.18±0.03	2.29±0.53	1.87±0.43	3.49±0.86	3.58±0.98	4.11±1.09
	Yes	0.35±0.05**	2.08±0.21	1.92±0.19	3.05±0.29**	2.97±0.47	4.21±0.53
Corticosteroids	No	0.32± 0.03	1.04± 0.11	1.05±0.15	1.80± 0.21	6.21± 0.22	3.17±0.45
	Yes	0.30±0.04	2.15±0.21	1.91±0.18	3.18±0.31	3.09±0.44	4.19±0.49
Biologic	No	0.27±0.03	2.19±0.23	1.92±0.19	3.06±0.33	3.26±0.49	4.19±0.53
	Yes	0.49±0.24**	0.81±0.23	1.62±0.37	1.80±0.53	2.30±0.39	1.30±0.08*

P<0.05 (*), P<0.02 (**); P<0.001 (*)

Table (6). Percentage of Treg cells in SLE patients with different clinical manifestations and different treatments

Demographic data		Mean ± SD			Mean ± SD
ACR criteria of SLE			Treatment		
Malar rash	NO	6.03±0.89	HCQ	NO	7.91±4.28
	Yes	6.99±1.21		Yes	6.85±1.16
Photosensitivity	NO	3.75±0.98	Endoxan	NO	6.16±2.11
	Yes	8.04±1.38**		Yes	7.51±1.32
Oral Ulcers	NO	6.71±2.29	Imuran	NO	8.60±1.34
	Yes	6.98±1.26		Yes	6.49±1.42
Arthritis	NO	7.40±1.52	Corticosteroids	NO	6.55±1.09
	Yes	6.43±1.72		Yes	6.99±1.13
Serositis	NO	6.95±1.28	Biologic	NO	6.74±1.10
	Yes	6.80±2.08		Yes	6.71±1.09
Renal disorders	NO	6.06±1.67			
	Yes	7.42±1.47			
Neuropsychiatric disorders	NO	6.89±1.09			
	Yes	5.08±1.02			
PanCytopenia	NO	6.91±1.33			
	Yes	3.61±1.47			
Other clinical manifestations					
Constitutional symptoms	NO	5.25±0.21			
	Yes	7.08±1.21			
Mucocutaneous manifestation	NO	6.30±1.47			
	Yes	6.94±1.17			
Vasculities	NO	6.49±1.05			
	Yes	10.49±6.09			
Raynauds phenomena	NO	6.35±0.98			
	Yes	22.66±0.92***			
Alopecia	NO	6.77±1.28			
	Yes	7.21±2.19			
Hypertension	NO	6.87±1.14			
	Yes	6.95±2.45			
Thrombocytopenia	NO	7.44±1.73			
	Yes	6.28±1.31			
Haemolyticanaemia	NO	6.87±1.28			
	Yes	7.05±1.67			

Leucopenia	NO	7.16±1.57
	Yes	6.63±1.16
Neutropenia	NO	6.87±1.16
	Yes	10.63±1.12
Lymphopenia	NO	7.16±1.57
	Yes	6.63±1.16

P<0.01 (**), P<0.001 (***)

Figures

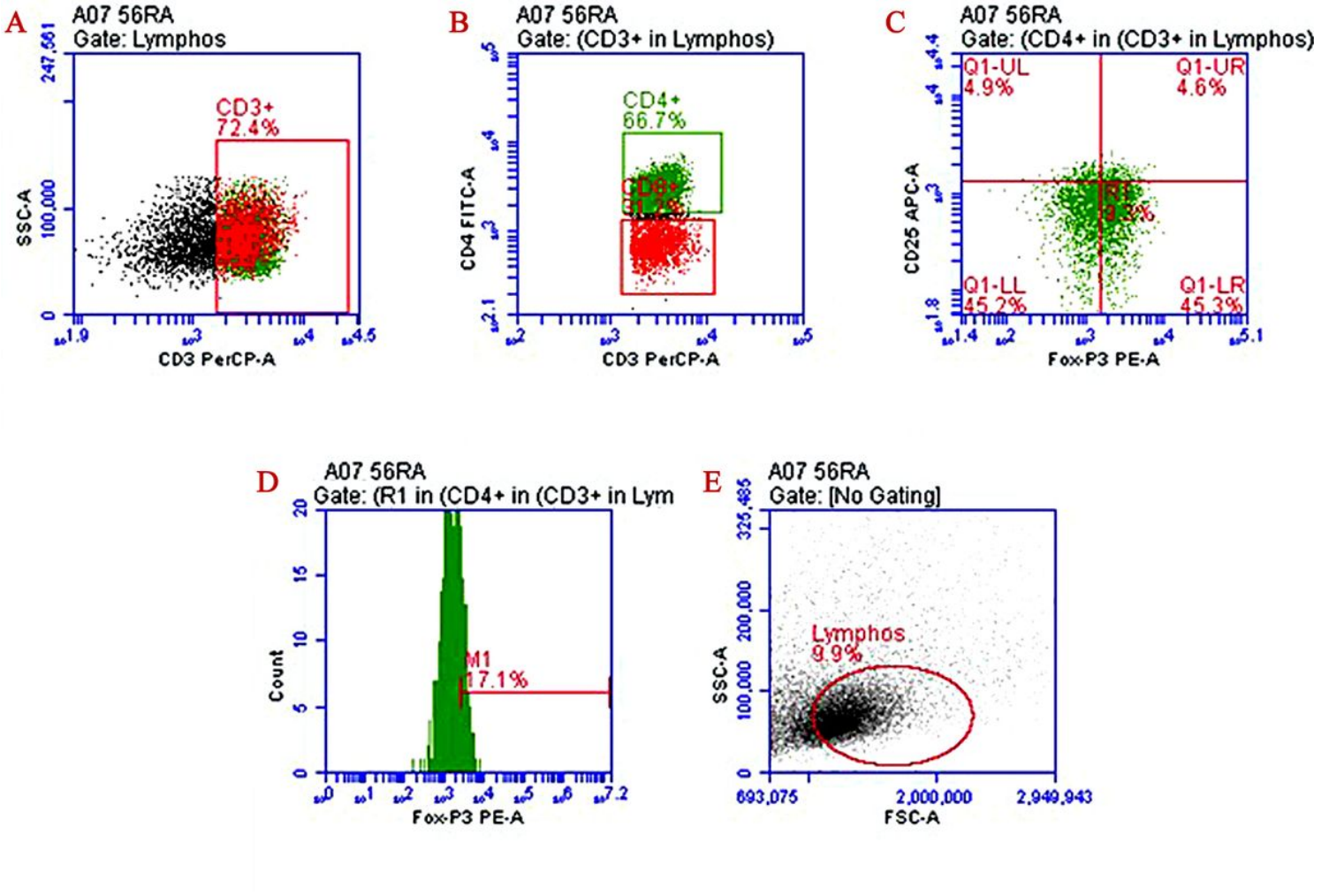

Figure 1

Flow cytometric detection of Treg cells

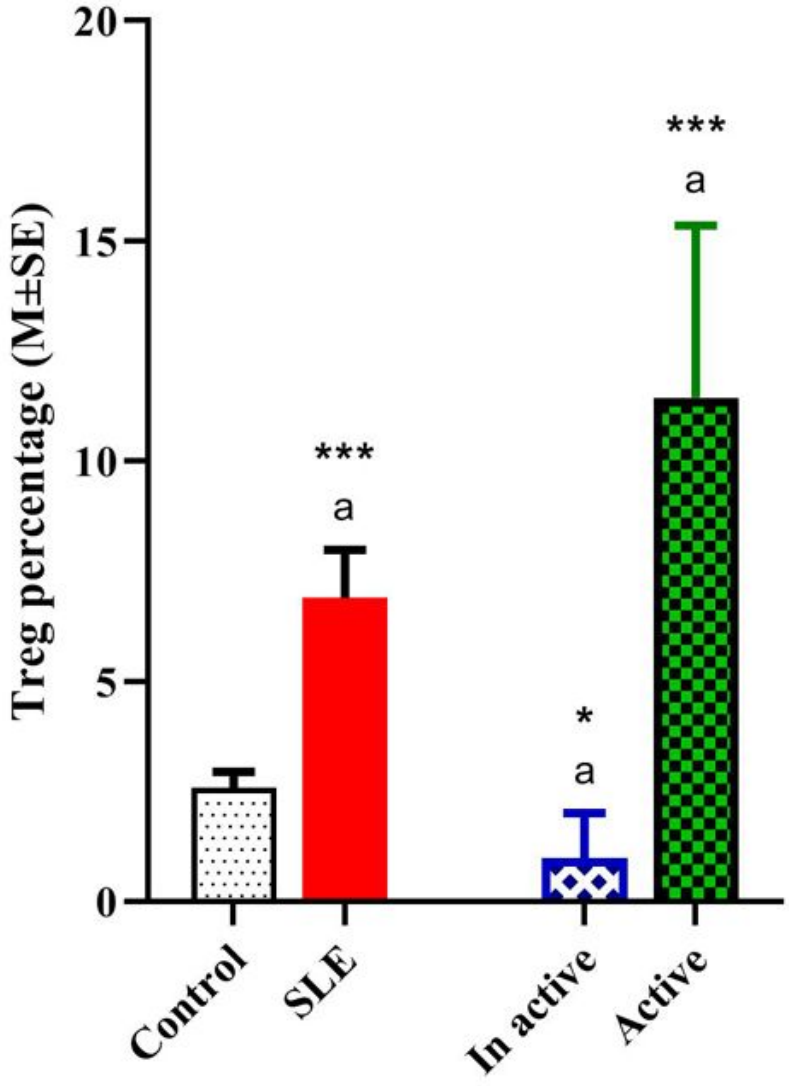

Figure 2

Percentages of Treg in controls and SLE patients (active and inactive patients). p<0.05 (*), p<0.02 (**); p<0.001 (***)

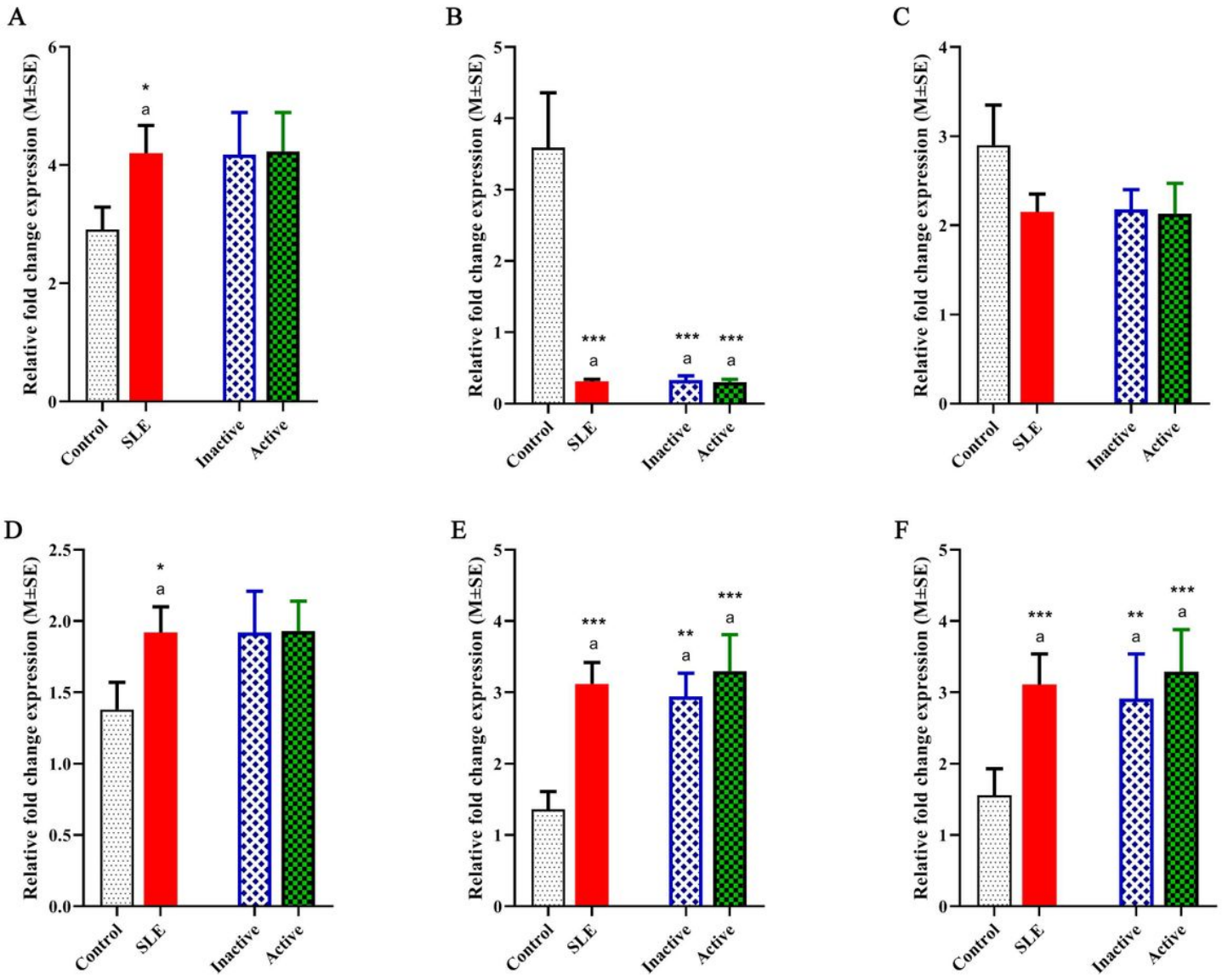

Figure 3

The relative fold change expression level of miRNAs in controls and SLE patients (active and inactive patients). p<0.05 (*), p<0.02 (**); p<0.001 (***)

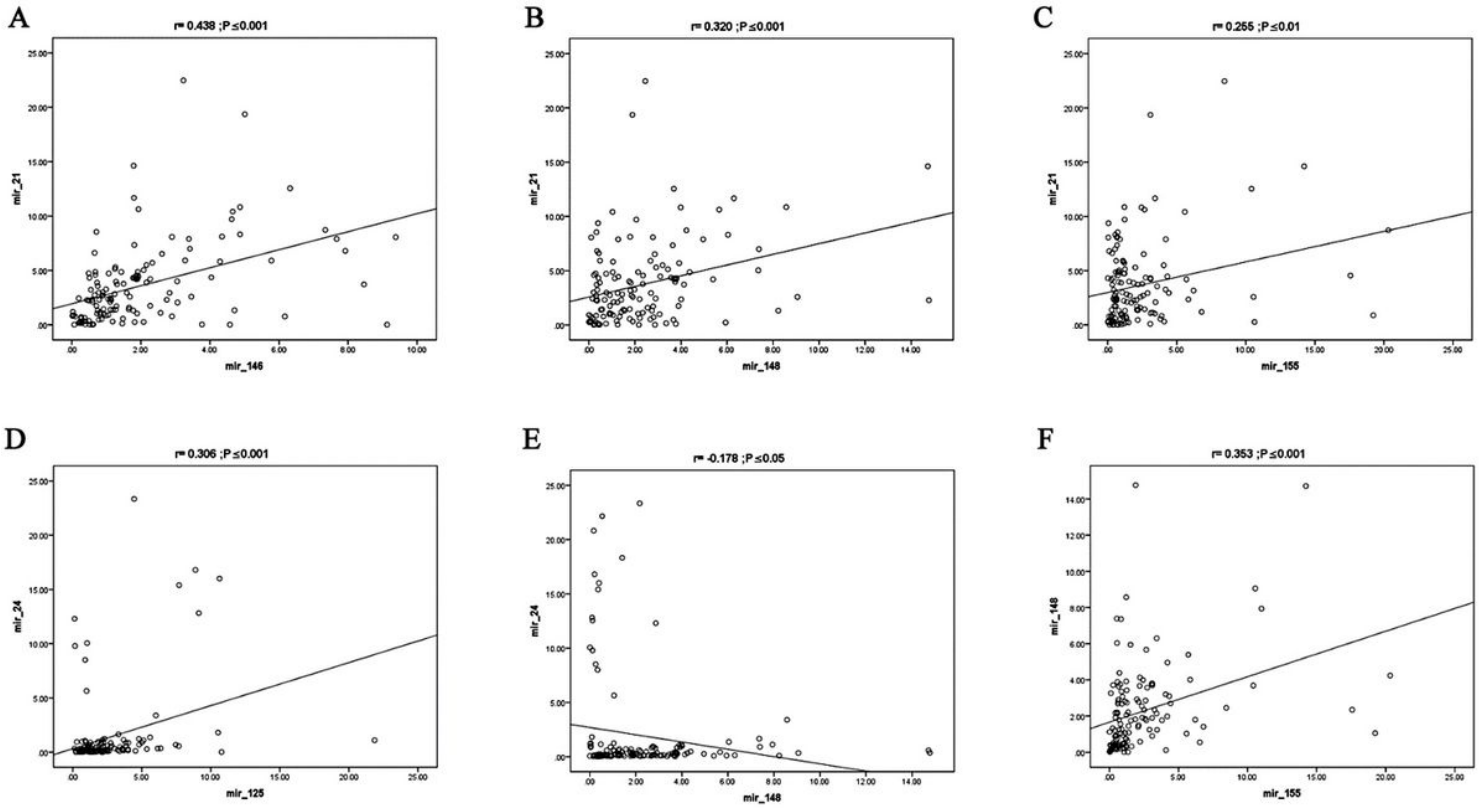

Figure 4

Correlation between miRNAs in SLE patients

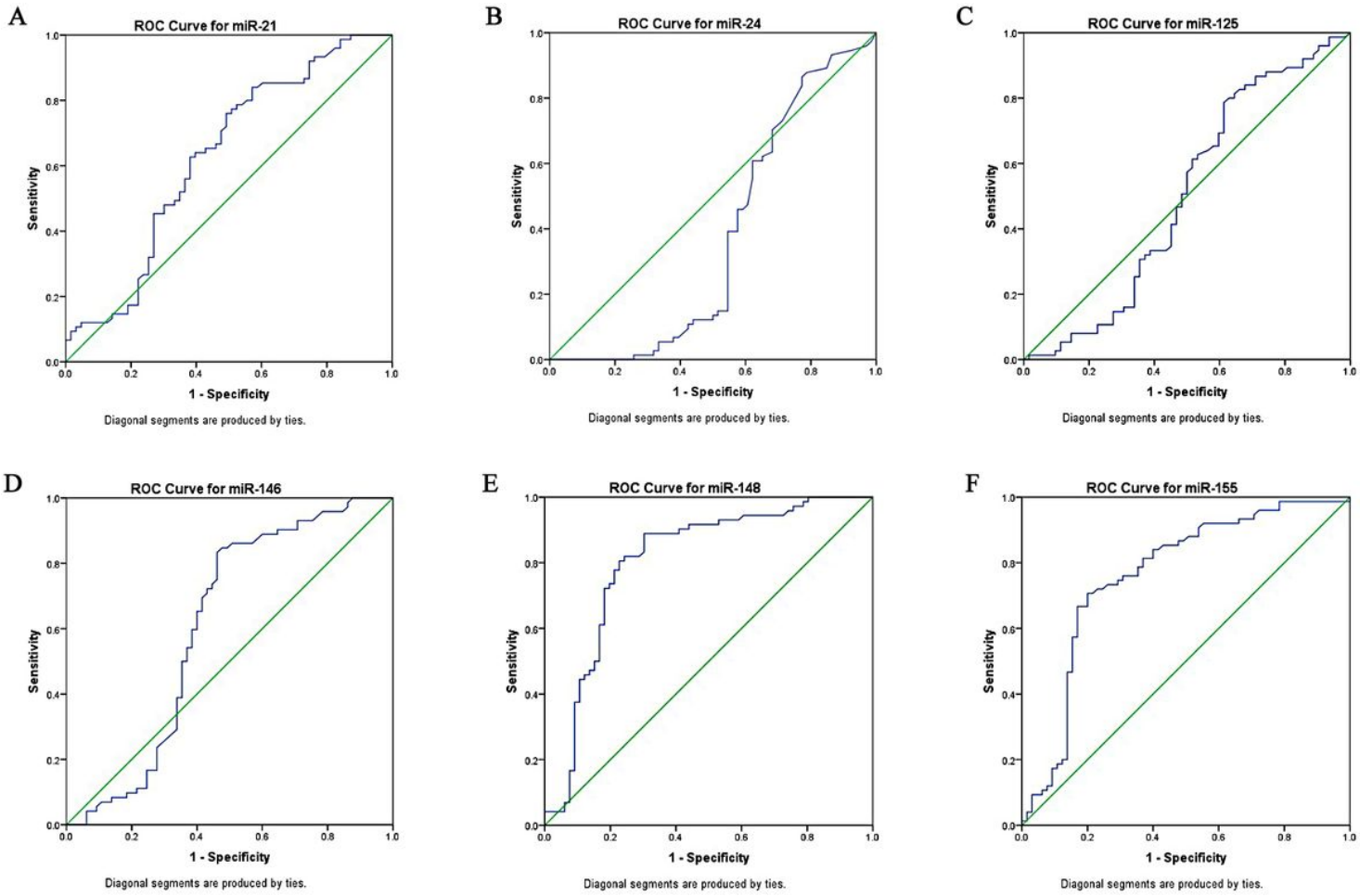

Figure 5

The Roc curve of miRNAs in SLE patients