

# Expression and clinical significance of miR-181a and miR-203 in systemic lupus erythematosus patients

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**Abstract. – OBJECTIVE:** MiR-181a plays a critical role in modulating T cell and B cell differentiation, as well as immune response. Its abnormal expression probably participates in the pathogenesis of systemic lupus erythematosus (SLE). MiR-203 is involved in regulating Toll-like receptor and inducing immune tolerance. Abnormal expression or function of miR-203 is related to multiple auto-immune diseases but its role in SLE remains unclear. This study, thus, investigated the serum level of miR-181a and miR-203, to analyze their roles in diagnosing and evaluating SLE.

**PATIENTS AND METHODS:** SLE patients were recruited from our hospital, and divided into non-active and active SLE based on disease activity index, along with healthy individuals. qRT-PCR was used to quantify the serum miR-181a and miR-203 expression, and their correlation with clinical features. ROC was used to evaluate the diagnostic value on SLE, while survival curves were compared to show progression-free survival (PFS) between populations with high and low expression.

**RESULTS:** SLE patients had significantly higher serum levels of miR-181a and lower miR-203, both of which were correlated with SLE activity. Expression levels of miR-181a and miR-203 were correlated with erythrocyte sedimentation rate, C reactive protein, anti-dsDNA antibody, complements, and SLEDAI score. Their expression levels had certain values in the differential diagnosis for active SLE (AUC=0.885 and 0.843). PFS in miR-181a high-expression individuals was lower than that in the low-miR-181 group ( $\chi^2=7.474$ ,  $p=0.029$ ). Whilst, miR-203 high-expression SLE patients had higher PFS than low-expression group ( $\chi^2=4.367$ ,  $p=0.037$ ).

**CONCLUSIONS:** SLE patients had higher miR-181a and lower miR-203 expression, which thus may have critical implications in disease diagnosis and evaluation.

Key Words:

Systemic lupus erythematosus, MiR-181a, MiR-203, Disease diagnosis, Progression-free survival.

## Introduction

Systemic lupus erythematosus (SLE) is a chronic auto-immune disease affecting multiple organs or systems, with refractory and recurrent disease course. Major features of SLE include the abundant production of auto-antibody and immune complexes, which are precipitated in tissues to induce an immune inflammatory response, leading to damage of cell and tissue structures<sup>1</sup>. Currently, neither pathogenic mechanism nor the cause of SLE has been fully illustrated, probably containing multiple factors such as genetic background, individual factors, environment, social, endocrine and immunity<sup>2</sup>.

MicroRNA is a highly conserved endogenous non-coding small RNA molecules in eukaryotes and can regulate target gene expression via complete or incomplete binding to 3'-untranslational region (3'-UTR), thus playing an important role in regulating embryonic development, immune cell proliferation and activation, as well as immune response<sup>3</sup>. MicroRNA can also participate in the pathogenesis of autoimmune disease via affecting inflammatory factor release and regulating innate immunity response<sup>4</sup>. With progressions on the study of microRNA in SLE pathogenesis, increasing evidence has implicated the correlation between microRNA and SLE pathogenesis<sup>5-7</sup>. MiR-181a is a member of the human miR-181 family (miR-181a, miR-181b, miR-181c, and miR-

181d), and located on chromosome 1 and chromosome 98. MiR-181a is expressed in human B cells and T cells, and plays important roles in regulating T cell and B cell differentiation and immune response<sup>9</sup>. A previous work<sup>10</sup> showed that about half of SLE sensitive genes were co-regulated by miR-181a, miR-186, and miR-590. The abnormal expression of miR-181a may play a role in the SLE occurrence<sup>11,12</sup>. miR-203 is an important member of microRNA and located in human chromosome 14q32.33 region<sup>13</sup>. miR-203 participates in regulating Toll-like receptor (TLR) expression<sup>14</sup>, release of inflammatory factors<sup>14</sup>, activation of dendritic cells and immune tolerance<sup>15</sup>. The abnormal function or expression of miR-203 is correlated with the occurrence of various autoimmune diseases such as oral lichen planus (OLP)<sup>16</sup>, rheumatoid arthritis (RA)<sup>17</sup> and psoriasis<sup>18</sup>. Whether it plays a role in SLE occurrence, however, is still unclear. This investigation measured the serum expression of miR-181a and miR-203 in SLE patients, to discuss whether miR-181a and miR-203 play a role in predicting the organ damage of SLE patients as well as their prognostic value.

## Patients and Methods

### Major Reagent and Materials

Serum RNA extraction kit miRNeasy serum/plasma kit was purchased from QIAGEN (Germantown, MD, USA). Reverse transcription kit ReverTra Ace qPCR RT Kit was purchased from Toyobo (Kita-ku, Osaka, Japan). SYBR Green Real-Time PCR Master Mixes was purchased from Life Technologies (Carlsbad, CA, USA).

### Patients

A total of 100 SLE patients who received surgery in the Second Hospital of Jiaying from February 2013 to July 2015 were recruited. All cases fitted SLE differential diagnostic criteria updated by American College of Rheumatology (ACR) in 1997<sup>19</sup>. There were 22 males and 78 females, with a median age of  $31.5 \pm 14.8$  years. All included patients had not received any immune suppressant drugs, immune modulator or hormonal therapy. Those patients having malignant tumors, acute/chronic infection or other auto-immune disease were excluded. Another 40 healthy individuals were recruited as the control group at the same time.

This research has been pre-approved by the Ethical Committee of the Second Hospital of Jiaying.

All subjects have signed the consent forms before recruitment in this study.

### Plasma Separation and microRNA Assay

EDTA-K2 anti-coagulant tube was used to collect 5 mL fasted blood samples from all research objects. Blood samples were centrifuged to separate plasma under 4°C with 4 000 rpm for 5 min. Plasma RNA was extracted by miRNeasy serum/plasma kit following the manual instructions. RNA was used as the template for generating cDNA by reverse transcription. Using cDNA as the template, PCR was performed under the direction of Taq DNA polymerase under the following conditions: 95°C pre-denature for 5 min, followed by 95°C 15s, 60°C 1 min in 40 cycles. Using U6 as the internal reference gene, data were standardized. Quantitative analysis was performed for miR-181a and miR-203 using comparative Ct method ( $2^{-Ct}$  method), as  $\Delta Ct = Ct^{\text{microRNA}} - Ct^{\text{U6}}$ . Primer sequences were designed as follows: miR-181a<sub>F</sub>: 5'-ATCGT ACGTG GGAAC ATTCA ACGCTGTCG; miR-181a<sub>R</sub>: 5'-GCAGG GTCCG AGGTA TTC-3'; miR-203<sub>F</sub>: 5'-GTCGT TACCA GTGCA GGGTC CGAGG TATTC GCACT GGATA CGACC TAGT-3'; miR-203<sub>R</sub>: 5'-GCCCCG TGAAA TGTTT AGGAC CAC-3'; U6<sub>F</sub>: 5'-CGCTT CACGA ATTTG CGTGT CAT-3'; U6<sub>R</sub>: 5'-GCTTC GGCAG CACAT ATACT AAAAT-3'.

### Clinical Information

Systemic lupus erythematosus disease activity index (SLEDAI) was quantified based on the clinical manifestation and laboratory analysis. Inactive phase was regarded as lower or equal than 4 points (N = 36), whilst activity phase was identified with higher or equal than 5 points (N = 64). Erythrocyte sedimentation rate (ESR) was tested, whilst ELISA was used to test the content of dsDNA antibody. IMMAGE 800 fully automatic protein analyzer (Beckman Coulter, Fullerton, CA, USA) was employed to test the serum levels of C-reactive protein (CRP), and complement C3 and C4.

### Statistical Analysis

SPSS18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean  $\pm$  standard deviation (SD). Comparison of plasma microRNA was performed by Mann-Whitney U rank-sum test. Spearman rank correlation was used to analyze the correlation among measurement data.

**Table I.** Correlation between plasma miR-181a/miR-203 and clinical feature of SLE patients.

|                    | miR-181a |       | miR-203 |       |
|--------------------|----------|-------|---------|-------|
|                    | r        | p     | r       | p     |
| ESR (mm/h)         | 0.766    | 0.029 | -0.792  | 0.017 |
| CRP (mg/L)         | 0.831    | 0.014 | -0.643  | 0.039 |
| anti-dsDNA (IU/mL) | 0.787    | 0.023 | -0.625  | 0.044 |
| C3 (g/L)           | 0.329    | 0.067 | -0.773  | 0.026 |
| C4 (g/L)           | 0.759    | 0.030 | -0.435  | 0.058 |
| SLEDAI score       | 0.714    | 0.031 | -0.692  | 0.035 |

Kaplan-Meier approach plotted patient survival curve. Comparison of survival rate was done by Log-rank test. Receiver operating characteristic (ROC) was used to evaluate the diagnostic value of miR-18a and miR-203 on SLE. A statistical significance was defined when  $p < 0.05$ .

## Results

### Expression Characteristic of Plasma miR-181a and miR-203 in SLE Patients

The qRT-PCR test showed significantly higher plasma miR-181a expression level in SLE patients compared with healthy population, with much higher miR-181a level in active SLE patients (Figure 1A). The MiR-203 expression level in SLE patient's plasma was remarkably lower than healthy control, with much lower level in active SLE patients, compared with inactive SLE patients (Figure 1B).

### Correlation Between miR-181a and miR-203 Levels in SLE Patient's Plasma and Clinical Features

Spearman rank correlation analysis showed si-

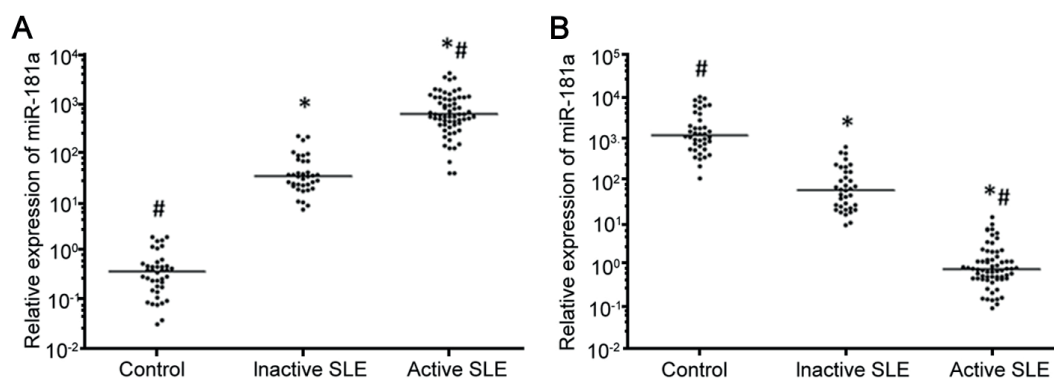
gnificantly positive correlation between miR-181a expression and ESR, CRP, anti-dsDNA, complement C4 or SLEDAI ( $p < 0.05$ ) but not with complement C3 ( $p > 0.05$ ). Plasma miR-203 level was negatively correlated with ESR, CRP, anti-dsDNA, complement C3 or SLEDAI ( $p < 0.05$ ) but not with complement C4 ( $p > 0.05$ , Table I).

### Diagnostic Value of miR-181a and miR-203 Expressions on SLE

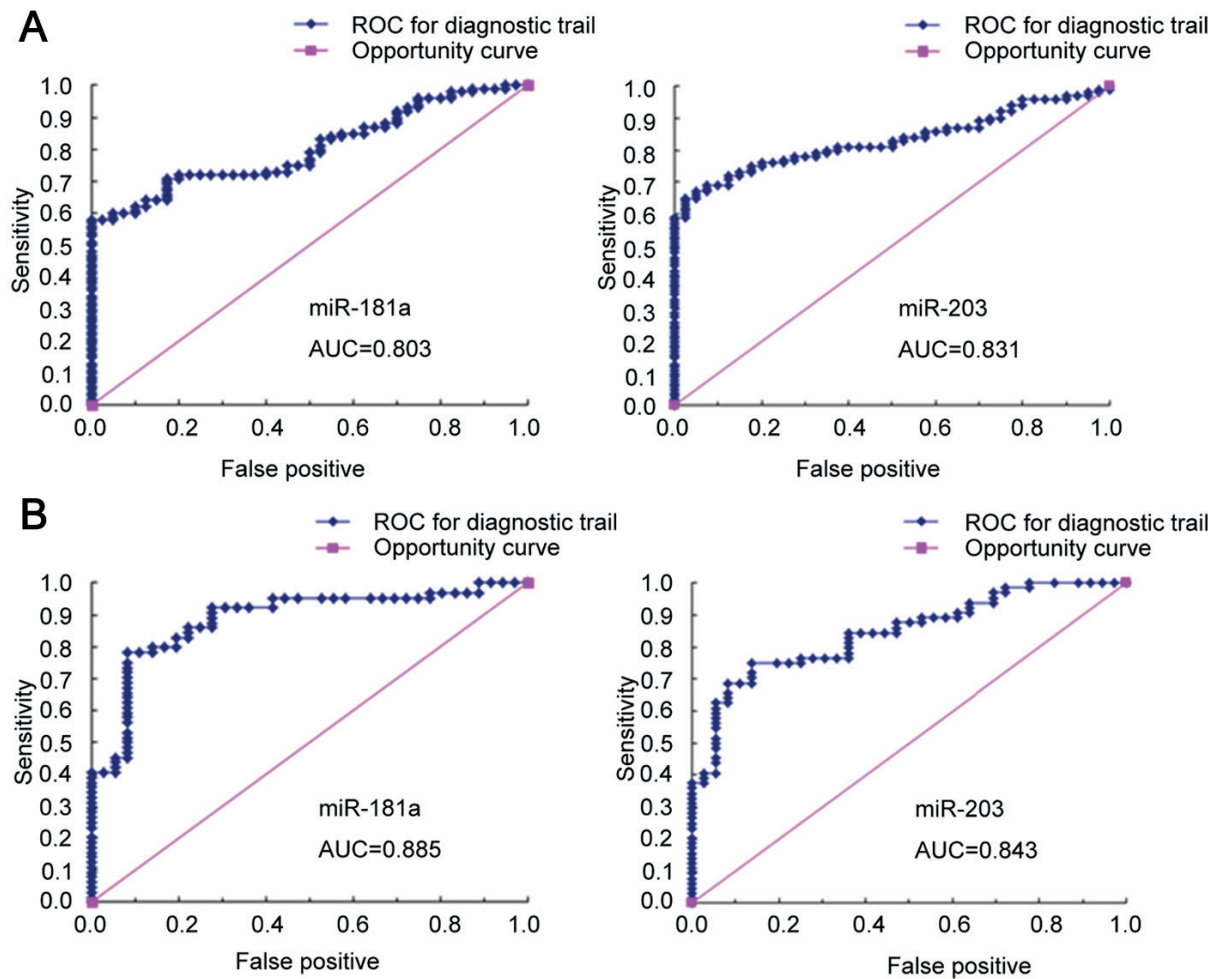
By constructing ROC, we obtained diagnostic value of miR-181a and miR-203 on SLE. Results showed that plasma miR-181a and miR-203 levels all had certain diagnostic values on SLE (AUC = 0.803 and 0.831, Figure 2A). We further divided SLE patients into inactive and active disease groups, in which plasma levels of miR-181a and miR-203 were evaluated for their diagnostic values between active and inactive SLE (AUC = 0.885 and 0.843, Figure 2B).

### Expression of miR-181a and miR-203 Affects Patient's Survival

Using inactive SLE patients as the research subjects, we divided them into high-expression and low-expression groups using the median le-



**Figure 1.** Expressional profiles of miR-181a and miR-203 in plasma of SLE patients. **A**, qRT-PCR for miR-181a expression; **(B)** qRT-PCR for miR-203 expression. \*,  $p < 0.05$  compared with control group; #,  $p < 0.05$  compared with inactive SLE patients.



**Figure 2.** Diagnostic value of miR-181a and miR-203 on SLE. (A) Diagnostic value of miR-181 and miR-203 on SLE by ROC; (B) Diagnostic value of miR-181a and miR-203 on active SLE by ROC.

vel of miR-181a and miR-203 expression as the threshold. Progression-free survival (PFS) was compared between two groups. Log-rank test showed significantly shorter PFS in miR-181a high-expression groups compared with that in low-expression individuals ( $\chi^2=4.747$ ,  $p = 0.029$ , Figure 3A). Patients with high-expression of miR-203, on the other hand, had significantly longer PFS compared with those with low-expression ( $\chi^2=4.367$ ,  $p = 0.037$ , Figure 3B).

## Discussion

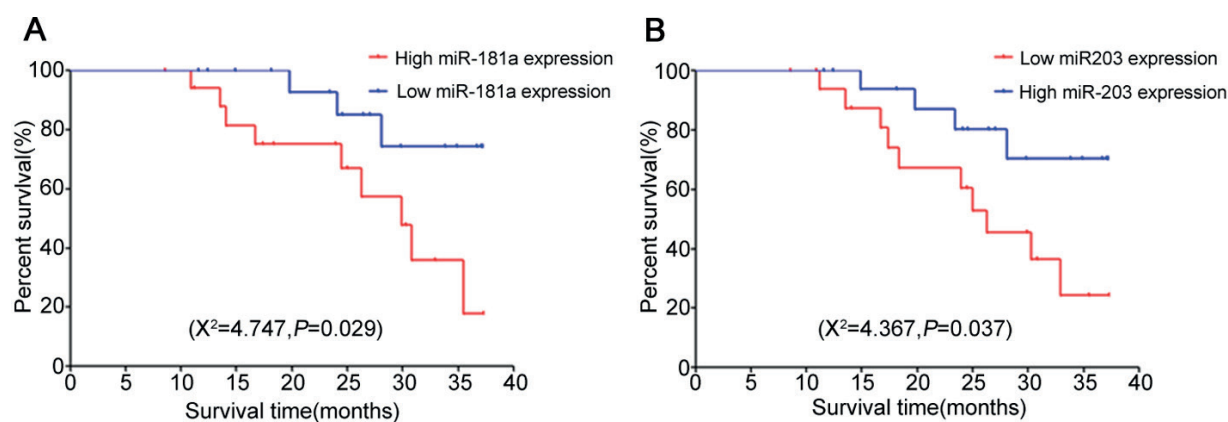
SLE is an auto-immune disease with multiple organs affected. Abnormal proliferation and activation of T and B lymphocytes, abundantly production of auto-antibody, and precipitation of im-

mune complex in organs are major features and pathological processes of SLE<sup>20</sup>. It is commonly believed that SLE pathogenesis is mediated by genetic risk factors, which can induce immune body abnormality under the influence of environment, sex hormone and infection, leading to dysregulation of T cell modulation, imbalance of T cell sub-populations, abnormal proliferation and activation of auto-reactive B lymphocytes, thus producing large amounts of auto-antibodies against body's antigen, and then forming immune complex with those antigens to precipitate in multiple organs and tissues, leading to initiation of immune inflammation, damages of cells and tissues, and eventually chronic auto-immune disease<sup>7</sup>. The tissue injury caused by precipitation of immune complex affects multiple organs including skin, joints, heart, kidney, brain and blood system<sup>21</sup>. The worldwide incidence of

SLE is about 4 to 25 per 100 000. It is estimated that about 520 000 to 910 000 people in China suffer from SLE, which was significantly higher (40 to 70 per 100 000) than the average number<sup>23</sup>. Female has a higher incidence than males (8:1 to 10:1 sex ratio). Fertility women are high-risk populations of SLE<sup>24</sup>. The pathogenic mechanism of SLE is a major challenge in the field of autoimmunity. Currently, no effective treatment has been developed against SLE. Therefore, studies on the pathogenesis and disease course mechanism are of critical importance for the diagnosis, treatment, and prognosis. Although the application of corticosterone and cytotoxic drugs or immune suppressant agents can relieve clinical symptoms to certain extents and improve long-term prognosis, these drugs, however, may cause severe side effects after long-term application. A previous work<sup>25</sup> showed that 5-year, 10-year and 15-year survival rates of SLE are 92%, 83% and 80%, whilst late onset SLE patients had significantly lower survival rates which were 66%, 44% and 44% within 5, 10 and 15 years.

Previous investigations found the presence of microRNA in tissue/cells, and also in extracellular fluids including plasma, serum<sup>26</sup>, urine<sup>27</sup>, saliva<sup>28</sup> and milk<sup>29</sup>. Circulated microRNA is distributed in vesicles with membrane structures, thus protecting them from RNAase degradation<sup>30</sup>. With such stable structure, its stability can be maintained after repeat freeze and thawing<sup>31</sup>. In recent years, circulated microRNA has become a research hotspot as the disease marker<sup>32</sup>. miR-181a plays an important role in regulating T cell and B cell differentiation and innate immunity response<sup>9</sup>. A previous study<sup>33</sup> indicated lower expression level in hematological precursor cells, and higher levels in differentiated

and matured B lymphocytes. Chen et al<sup>33</sup> reported that over-expression of miR-181a significantly facilitated the differentiation of precursor cells toward B lymphocytes. Li et al<sup>34</sup> showed that miR-181a modulated B cell differentiation via targeted inhibition on Lin<sup>28</sup> expression. miR-181a is one of microRNAs with profound expression in T cells. Li et al<sup>35</sup> showed over-expression of miR-181a could significantly enhance the sensitivity of T cell receptor (TCR), whilst down-regulation of miR-181a significantly reduced the sensitivity of immature T cells on antigen peptide, and disrupting positive and negative selection of T cells. In addition, Liu et al<sup>36</sup> found that miR-181a could enhance TCR signal strength and sensitivity, thus modulating the strength of agonists. They also found that elevation of miR-181a expression could activate T cell response against TCR antagonist, and miR-181a genetic defect mice showed immune deficiency including absence of NKT cells, fewer T cell number, and reduced proliferation potency<sup>37</sup>. Moreover, miR-181a also plays important roles in mediating the release of inflammatory factors including IL-1 $\beta$ , IL-6<sup>38</sup>, IL-8<sup>39</sup>, and TNF- $\alpha$ <sup>40</sup>. Carlsen et al<sup>12</sup> showed higher miR-181a expression level in peripheral blood of SLE patients compared with healthy individuals. Li et al<sup>41</sup> found significantly elevated serum miR-181a levels in lupus nephritis (LN) patients. This study observed higher plasma miR-181a expression level in SLE patients compared with healthy individuals, as correlated with SLE activity, consistent with previous researches conducted by Carlsen et al<sup>12</sup> and Li et al<sup>41</sup>. Zhou et al<sup>13</sup> found that miR-203 effectively inhibited the synthesis and release of inflammatory factors TNF- $\alpha$  and IL-12 via targeting TLR4 expression in inhibit maturation of dendritic



**Figure 3.** PFS of SLE patients under the effect of miR-181a and miR-203 expression. (A) Analysis of PFS in patients with different miR-181a expression levels; (B) Analysis of PFS in patients with different miR-203 expression levels.

cells, thus exerting negative regulation on innate immunity. Primo et al<sup>14</sup> showed that miR-203 directly targeted mRNA expression of inflammatory factors TNF- $\alpha$  and IL-24, exerting negative regulatory effects on immunity. Stumpfova et al<sup>15</sup> found that miR-203 was specifically expressed in tolerogenic dendritic cells (tDCs), and its expression was increased during the process of imDCs differentiation towards tDCs cells under the induction of IL-10 and TGF- $\beta$ . All these studies indicated the role of miR-203 in inducing immune tolerance and inhibiting immune inflammation. Our work showed significantly lowered miR-203 expression in plasma of SLE patients compared with healthy control, plus lower miR-203 levels in active SLE patients than inactive ones, indicating the association of lower miR-203 expression with compromised immune tolerance of SLE patients. CRP, ESR, anti-dsDNA antibody, complement and SLEDAI are important markers for evaluating disease course of SLE and its activity. We showed positive correlation between miR-181a expression in plasma of SLE patients and their ESR, CRP, anti-dsDNA, complement C4 and SLEDAI levels, plus negative correlation between miR-203 levels and ESR, CRP, anti-dsDNA, complement C3 and SLEDAI. Therefore, we speculated that plasma miR-181a and miR-203 were probably potential markers for evaluating disease progressions of SLE. ROC analysis showed certain diagnostic values of miR-181a and miR-203 in differentiating SLE, inactive and active cases. Survival curve analysis showed lower PFS period in patients with high-expression of miR-181 compared to those with low miR-181a expression, plus higher PFS in patients with high-expression of miR-203 than those with low-expression, suggesting that abnormal expression of miR-181a and miR-203 might be markers for evaluating SLE disease progression. Their expression levels may provide evidences for SLE diagnosis, evaluating disease course and predicting disease progression. The mechanism of miR-181a and miR-203 in immune modulation and SLE pathogenesis, however, is still unclear and requires further investigation.

## Conclusions

Plasma level of miR-181a is significantly elevated in SLE patients, with lower miR-203 expressions. The detection of miR-181a and miR-203, thus, has important values for diagnosing SLE, evaluating disease, and predicting progression.

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## Conflict of interest

The authors declare no conflicts of interest.

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