Down-regulation of serum miR-151a-3p is associated with renal tissue activity in class IV lupus nephritis

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Abstract

Objective

The aim of this study was to explore the value of serum miRNA for evaluating renal tissue activity in patients with class IV lupus nephritis (LN).

Methods

First, we used a microRNA array to identify miRNAs differentially expressed between class IV LN patients and healthy volunteers (n=4/group). Then, we analysed the association between these identified miRNAs and renal tissue activity in class IV LN patients. Finally, to validate the results, 20 class IV LN patients (confirmed by renal biopsy) and 20 healthy control volunteers were further studied.

Results

We found 23 miRNAs to be significantly differentially expressed between the 2 groups. We selected 5 of these miRNAs (miR-3165, miR-4762-5p, miR-146a-5p, miR-151a-3p, and miR-21-5p) for further experiments. In validation experiments, expression of miRNA-151a-3p was significantly down-regulated in the class IV LN group compared to that in the control group ($p<0.01$) and was negatively correlated with the activity index (AI) in the class IV LN group($r=-0.526$, $p=0.017$); the internal correlation was described with a linear fitting equation ($p<0.01$).

Conclusion

Serum miR-151a-3p expression was decreased in class IV LN patients compared with healthy control volunteers and was negatively correlated with renal tissue activity. Thus, miR-151a-3p may play a employed for diagnosing class IV LN and evaluating renal tissue activity.

Key words

microRNA, biomarker, lupus nephritis, systemic lupus erythematosus

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Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease involving multiple organs that causes serious injury to various organs and systems (1). Lupus nephritis (LN) is a serious complication of SLE that is usually accompanied by a poor long-term prognosis. It affects up to 70% of SLE patients and approximately 20% of these patients eventually develop end-stage renal failure. LN can be divided into 6 classes according to pathology: class I, II, III, IV, V and VI. Among these, class IV LN is usually severe and has a high incidence (2, 3). Appropriate treatment for class IV LN is greatly important for prognosis. Renal tissue activity is a significant reference index for treatment, and when the disease activity is high, usually the treatment measure is aggressive (4).

Renal biopsy is the gold standard for attaining information on the histological LN class and relative degree of activity and chronicity in the renal tissue. As an invasive procedure, renal biopsy carries a risk for bleeding complications, such as haematuria, local haematoma, and internal bleeding, which are potentially life-threatening. Due to this bleeding risk, some patients are unwilling to undergo renal biopsy and refuse this examination (5, 6).

MicroRNAs (miRNAs) are endogenous single-stranded, relatively small noncoding RNAs (21–23 bp) (7). Circulating miRNAs have been acknowledged as biomarkers for early disease diagnosis (8), and miRNAs have been proven to play an important role in the pathogenesis of autoimmune diseases (9, 10). Patient serum samples can be used for miRNA detection and are easy to obtain. Thus, circulating miRNAs can be used as an effective tool for disease diagnosis (11, 12).

This study aimed to explore whether serum miRNAs can assess renal tissue activity in patients with class IV LN and discusses the value of miRNAs in class IV LN patients.

Materials and methods

Participants

All patients were diagnosed with SLE based on the diagnostic criteria for SLE by the American College of Rheumatology (ACR) in 1997 (13); and according to the classification standard of the International Society of Nephrology-Renal Pathology Society (ISN/RPS) in 2003 (14), each LN patient presented with class IV LN, as confirmed by renal biopsy. The following exclusion criteria were used for participants: 1) chronic diseases, such as hepatitis B virus, cancer or diabetes; 2) autoimmune diseases, pregnancy or lactating women; 3) active bacterial or viral infection; 4) immunosuppressive treatment (including cyclosporine A, cyclophosphamide, azathioprine, chloroquine, and mycophenolate mofetil) or high-dose glucocorticoid pulse therapy within the previous 3 months. The estimated glomerular filtration rate (eGFR) was calculated by the Modification of Diet in Renal Disease (MDRD) Study formula. All SLE patient serum samples were collected from the nephrology department of Peking University Shenzhen Hospital. Blood samples were collected early in the morning on the day kidney biopsy was performed. The healthy control serum samples were obtained from the medical examination centre, and the 2 groups were gender and age matched. Blood samples were collected with a heparin-free vacuum tube and were centrifuged at 2500r/min for 15 min to separate the serum; and the samples were stored at -80°C.The study was approved by the ethics committee of the hospital, and all the participants signed an informed consent.

Assessment of renal tissue and SLE activity

The Austin system was used to evaluate renal tissue activity (15) which is a semiquantitative scoring system that can reflect renal tissue activity via 2 indices: the activity index (AI) and the chronicity index (CI).The AI reflects glomerular proliferation, leucocyte exudation, karyorrhexis/fibrinoid necrosis, cellular crescents, hyaline deposits, and interstitial inflammation, which represent active LN. The chronicity index consists of glomerularsclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis, which represent chronic LN. The following scoring cri-

teria were used: a score of 0, 1, 2, or 3 represented absent (0% lesion), mild (less than 25% lesion), moderate (or 25% to 50% lesion) or severe (or more than 50% lesion) lesions, respectively. In addition, karyorrhexis/fibrinoid necrosis and cellular crescents were double scored. The maximum score for the AI was 24 points and was 12 points for the chronicity index. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used to evaluate SLE activity, which was scored according to clinical manifestation and laboratory examination.

miRNA array screening

To identify the serum miRNAs related to class IV LN, we used a miRNA array to test and screen the serum miRNAs that were differentially expressed between 4 class IV LN patients (confirmed by renal biopsy) and 4 healthy volunteers. Each serum sample was tested by one miRNA array. The RNA samples, which were labelled with the miRCURY™ Array Power Labeling kit, were hybridised with a 7th generation Exiqon miRCURYLNA™ array (v. 18.0) and then washed. The chip contained a total of 3100 capture probes for all miRNAs, according to the miRNA database, in 3 species (human, mouse and rat) and in all the viruses associated with those species. Microarray hybridization and the subsequent analyses were completed at Shanghai KangChen Bio-tech Company. Based on these results, we identified miRNAs appropriate for further verification. After the miRNA array screening, we first identified miRNAs that were differentially expressed between the 2 groups ($p<0.01$). From these, we selected miRNAs that had statistically significant ($p<0.05$) changes in expression that were greater than 3-fold (or below 1/3) between the groups. Then, we selected the miRNAs that had a large fold change and a high level of expression.

Next, we preliminarily analysed the correlation between the expression of the selected miRNAs and renal tissue activity and consulted the literature to select the more common miRNAs. As our target miRNAs, we ultimately chose 5 miRNAs with expression potentially related to renal activity.

MiRNA real-time PCR validation

To validate the screening results, we used real-timePCR to assess miRNA expression in 40 serum samples (20 patients with class IV LN and 20 healthy controls). For total RNA extraction, a miR-Neasy Serum/Plasma kit (Qiagen) was used to extract total RNA from each 200μL serum sample. QIAzol Lysis Reagent (1000μL) was added, and the reaction was mixed well and incubated for 7 min at room temperature. Then, 3.5μL of miR-Neasy Serum/Plasma Spike-In Control (cel-miR-39) was added, which was used as the internal reference standard. Finally, we obtained 12μL of total RNA. The RNA concentration of each sample was tested by a UV spectrophotometer. For real-timePCR, tailing and reverse transcription were performed according to the instructions of the miScript II RT kit. The following reaction conditions were used: 37°C for 60 min and 95°C for 5 min. Each real-time quantitative PCR mix was composed of the following: 10 μL of 2×SYBR Green PCR Master Mix, 2μL of cDNA, 2μL of general primers and upstream primers. All reactions were brought to a 20μL final volume with water. Optimal PCR amplification was achieved with predenaturation at 95°C for 15 min and 45 amplification cycles at 94°C for 15s, 55°C for 30s and 70°C for 30s. MiRNA sequences were searched at the following URL: http://www.mirbase.org.

Real-time PCR was performed on a Light Cycler 480 II (Roche) real-time PCR system. Finally, we verified the assumed target miRNAs. For verification experiments, the relative miRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method, using the miR-39 expression level as the internal reference (16).

Statistical analysis

All statistics were analysed by SPSS (v. 22.0) software, and diagrams were made by Origin (v. 2017) software. For the miRNA array screening, we used Student's *t*-test to compare the differences between 2 groups and the Spearman correlation coefficient to assess the correlation between miRNA expression and renal tissue activity (AI and CI). For correction for multiple testing, the False Discovery Rate (FDR) was calculated according to the Benjamini-Hochberg (BH) procedure. For real-time PCR validation, we used scatter diagrams to describe the expression level of miRNA and ROC (receiver operating characteristic) curves for evaluating the diagnostic value of candidate miRNAs by AUCs (areas under thecurve). For normally distributed data, differences between 2 groups were compared by Student's *t*-test, and for non-normally distributed data, the Mann-Whitney U-test was used. The correlation between miRNA expression and clinical parameters was evaluated with the Pearson and Spearman correlation coefficients, and the latter was used as a non-parametric test for non-normally distributed data. To describe the intrinsic correlation between miRNA expression and clinical parameters, the least squares method (LSE) was used for regression analysis and linear fitting of the processed data. A good fit was indicated by an adjusted R-squared value above 0.8.

Results

miRNAs related to renal tissue activity, as determined by miRNA array

According to the miRNA array experiment, we found that 23 miRNAs were significantly differentially expressed between the 2 groups; The FDR was below 0.01.Among these 23 miRNAs, we selected 5 serum miRNAs that were potentially related to renal tissue activity, including miR-3165, miR-4762-5p, miR-146a-5p, miR-151a-3p, and miR-21-5p (Fig. 1).

Different serum miR-151a-3p expression levels between the class IV LN and control group

For the validation experiment, from January 2010 to August 2015, a total of 40 serum samples were collected from 20 LN patients and 20 healthy volunteers. According to the general clinical patient data (Table I), there were no differences in age or gender between the 2 groups.

The expression of 1 miRNA (miR-151a-3p) was significantly different between the class IV LN group and the control group. The expression of miR-151a-3p

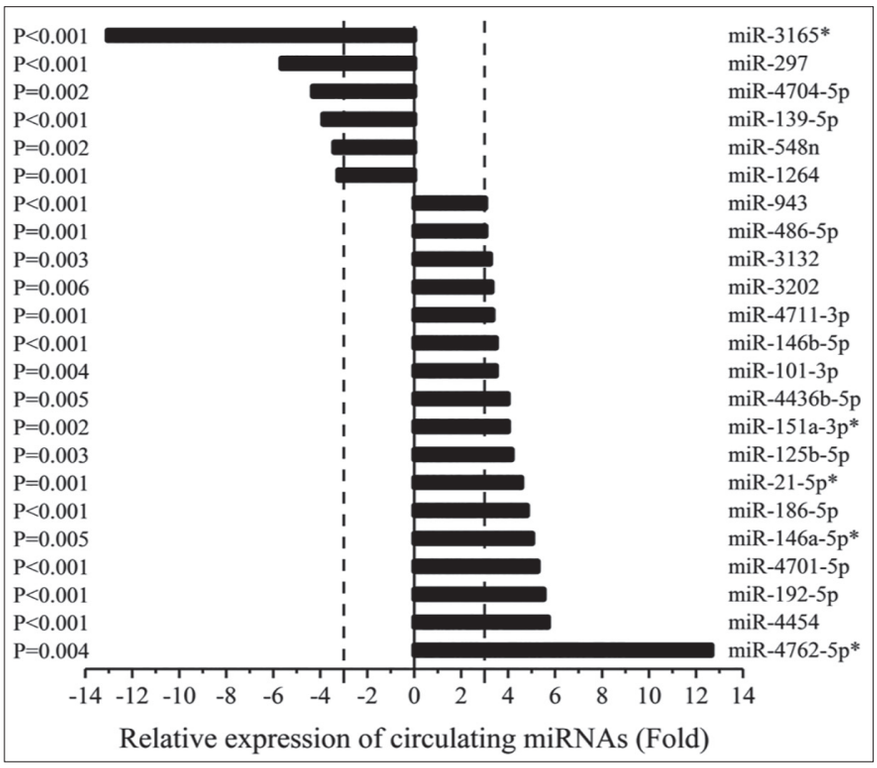

Fig. 1. The fold changes in serum miRNA expression of class IV LN patients *vs*. healthy controls, as determined by miRNA arrays. When compared between the groups, the fold change in the expression of the listed miRNAs was above 3 or below 1/3.

Table I. General clinical data of subjects ($\bar{x}\pm s$).

Group	Class IV LN Group	Control Group
Number	20	20
Age	29.60 ± 6.27	32.65 ± 6.70
Female/Male	18/2	18/2
Disease duration	23.13 ± 38.86	--
Mean arterial pressure (mmHg)	97.70 ± 16.43	90.62 ± 13.97
Serum creatinine* (μmol/L)	128.14 ± 107.81	57.94 ± 6.05
Ds-DNA	508.69 ± 276.54	--
C3 (g/L)	0.31 ± 0.16	0.40 ± 0.25
C4 (g/L)	0.06 ± 0.06	0.11 ± 0.10
24-hour urinary protein (g/L)	3.14 ± 2.28	--
Activity Index	11.15 ± 3.88	--
Chronicity Index	2.35 ± 2.18	--
SLEDAI	12.15 ± 4.63	--
eGFR*(ml/mi)	81.78 ± 35.42	138.75 ± 15.42

SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; eGFR: estimated glomerular filtration rate; *significantly different between the 2 groups; -- represents a negative status.

was significantly down-regulated in the class IV LN group compared to that in the control group ($p<0.01$) (Fig. 2). The AUC was 0.888 (95%, CI:0.770-1.000, $p<0.01$), which was statistically significant ($p<0.01$). The ROC revealed that the cut-off point was 0.203,and the optimal sensitivity and specificity of the ROC were 80% and 100%, respectively (Fig. 3).

Correlation between serum miR-151a-3p expression and AI in class IV LN patients

In the class IV LN group, the expression of miR-151a-3p was negatively correlated with AI (r=-0.526, $p=0.017$), indicating that low miR-151a-3p expression may indicate severe kidney injury. Since the adjusted R-squared value of the linear fitting was 0.858 (>0.8), the linear fitting results properly represent the correlation between renal AI and serum miR-151a-3p expression. The linear fitting equation was y=10.075x, and the slope was 10.075±0.913 (99%CI: 9.162–10.988) (Fig. 4). Finally, between the 2 groups, no statistically significant correlations were identified for CI, SLEDAI, serum creatinine, eGFR, dsDNA, C3, C4 and other clinical parameters (Table II).

Correlation between other miRNAs and clinical parameters

There was a positive correlation between serum miR-21-5p and SLEDAI (r=0.443, $p=0.050$) in the class IV LN group, but the expression of this miRNA was not different ($p=0.416$) between the patient and control group. Although serum miR-4762-5p was significantly correlated with C3(r=0.559, $p=0.01$), serum creatinine (r=0.880, $p<0.01$) and eGFR (r=-0.565, $p<0.01$), its expression level in the patient group was not significantly different from that in the control group ($p=0.269$) (Fig. 2).

Discussion

This study explored the potential ability of serum miRNA to assess renal tissue activity in class IV LN patients. First, we used a miRNA array to identify several miRNAs that were potentially related to class IV LN patients, and then, we further narrowed down the list of possible class IV LN-related miRNAs. Ultimately, we validated the ability of several miRNAs (mentioned above) to assess renal tissue activity. To our knowledge, we are the first to report miRNA as a biomarker of renal activity in class IV LN patients.

During this study, we explored target miRNAs through a gradual series of experiments. We used a miRNA array as a preliminary test, which roughly estimates the expression of serum miRNAs. To increase the reliability of the experiment, we used real-time PCR to validate the miRNA expression results. Before the validation test, miR-3165, miR-4762-5p, miR-146a-5p, miR-151a-3p, and miR-21-5p were all potential target miRNAs; But after this process, we found that only miR-151a-3p met the specified requirements. And

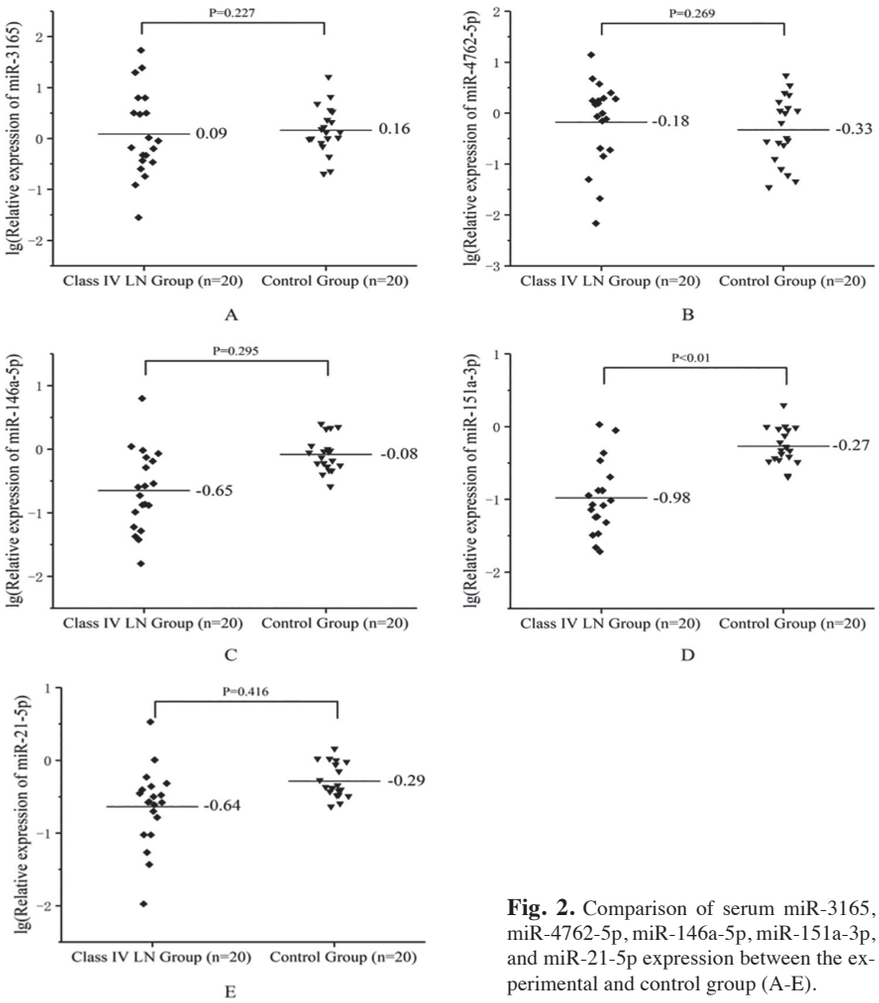

Fig. 2. Comparison of serum miR-3165, miR-4762-5p, miR-146a-5p, miR-151a-3p, and miR-21-5p expression between the experimental and control group (A-E).

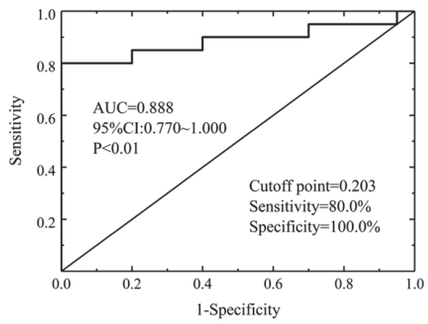

Fig. 3. The AUC for miR-151a-3p's ability to diagnose class IV LN.

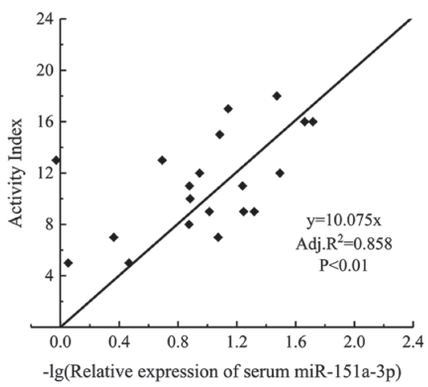

Fig. 4. Correlation between the relative serum miR-151a-3p expression (fold change) and the renal activity index.

the results also show that the expression of the other four miRNAs between the LN group and control group was not different, suggesting that these miRNAs were not able to diagnose class IV lupus nephritis.

We discovered that the expression of serum miR-151a-3p was decreased in the class IV LN group compared with that in the healthy control group. Analysis of the ROC curves showed that the AUC was equal to 0.888 (>0.8, p<0.01). Moreover, when the critical value (cutoff point) was 0.203, the optimum sensitivity and specificity were 80.0% and 100.0%, respectively, indicating that miR-151a-3p effectively differentiated between healthy volunteers and class IV LN patients. We found that miR-151a-3p was negatively correlated with AI (r=-0.526, p=0.017), and regression analysis was used to describe the quantitative relationship between the 2 groups. These results showed that miR-151a-3p expression was able to evaluate renal tissue activity in class IV LN patients but was not correlated with mean arterial pressure, dsDNA, C3, C4, 24-hour urinary protein, serum creatinine, or glomerular filtration rate. Thus, miR-151a-3p may play a role for diagnosing class IV LN and evaluating renal tissue activity.

To gain insight into the function of miR-151a-3p and the other four miRNAs, we mined the miRBase miRNA database (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) and Target scan database 7.1 (http://www.targetscan.org/vert_71/) to identify gene targets of these miRNAs. We found that TWIST1 and IL12Rβ2 are potential gene targets of miR-151a-3p. TWIST1 is a transcription factor, with a role in the process by which Interleukin 17 (IL-17) regulates B cell biology and the pathophysiology of SLE (17). In addition, TWIST1 is involved in the renal epithelial-to-mesenchymal transition (EMT) and plays significant roles in renal fibrosis (18, 19). IL-12Rβ2 is a receptor of Interleukin 12(IL-12), and its polymorphisms are associated with the risk of SLE in the Chinese population (20), Administration of IL-12 to mice can render them vulnerable to induction of experimental SLE (21). Accordingly, miR-151a-3p may be involved in the pathogenesis of SLE and LN, and the mechanism may involve regulation of fibrosis and immune or inflammation, however this speculation needs to be explored. Some literature reports that miR-146a has a function in the pathogenesis of SLE (22, 23), and that its expression is decreased in SLE compared to healthy controls (24). In our study, we found no difference in miR-146a expression in class IV LN compared with controls, and the reason for this difference may be the two different disease populations.

This study does have shortcomings, such as the small sample size, and we did not test the expression of these

Table II. Correlation between serum miRNA expression and clinical parameters in the class IV LN group.

Subjects	miR-151a-3p		miR-21-5p		miR-146a-5p		miR-3165		miR-4762-5p	
	R-value	*p*-value	R-value	*p*-value	R-value	*p*-value	R-value	*p*-value	R-value	*p*-value
Age	-0.375	0.103	-0.417	0.068	-0.488	0.029*	0.076	0.751	0.281	0.230
Mean arterial pressure	-0.345	0.137	-0.199	0.401	-0.264	0.260	-0.459	0.042*	0.012	0.962
C-reactive protein	0.373	0.189	0.204	0.484	0.200	0.494	-0.032	0.914	0.013	0.964
dsDNA	-0.235	0.398	0.043	0.878	-0.040	0.887	-0.137	0.626	-0.102	0.718
C3	-0.269	0.251	-0.249	0.289	-0.270	0.250	0.039	0.871	0.559	0.010*
C4	-0.113	0.667	-0.057	0.828	-0.072	0.784	-0.214	0.409	0.481	0.050
Total protein	0.196	0.450	0.478	0.052	0.485	0.048*	0.542	0.024*	-0.240	0.353
Albumin	-0.067	0.779	0.080	0.739	0.126	0.597	0.526	0.017*	0.061	0.799
Globulin	0.253	0.326	0.465	0.060	0.439	0.078	0.462	0.062	-0.296	0.249
24-hour urinary protein	-0.322	0.166	-0.287	0.220	-0.270	0.249	-0.342	0.140	-0.379	0.099
Serum creatinine	-0.083	0.728	-0.116	0.627	-0.173	0.465	0.067	0.779	0.880	<0.001*
Blood urea nitrogen	-0.016	0.951	-0.139	0.582	-0.192	0.445	0.262	0.294	0.782	<0.001*
Serum uric acid	0.050	0.844	-0.134	0.596	-0.076	0.763	0.264	0.290	0.068	0.788
eGFR	-0.004	0.987	0.178	0.453	0.268	0.253	-0.188	0.428	-0.565	0.009*
AI	-0.526	0.017*	-0.301	0.197	-0.414	0.070	-0.294	0.209	-0.242	0.303
CI	-0.248	0.293	-0.232	0.325	-0.227	0.337	0.208	0.379	0.439	0.053
SLEDAI	0.234	0.321	0.443	0.050*	0.058	0.809	0.235	0.319	0.259	0.270

AI: Activity Index, CI: Chronicity Indices, SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.
*The correlation reached a significant level.

miRNAs in other classes of LN. Therefore, the value of miR-151a-3p in diagnosing class IV LN still requires further research.

Conclusion

This study showed that serum miR-151a-3p expression was decreased in the class IV LN group compared to that in the healthy control group and was negatively correlated with the renal tissue activity of class IV LN patients. Therefore, miR-151a-3p can potentially diagnose class IV LN and evaluate renal tissue activity.

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