IJKD KIDNEY DISEASES

Plasma levels of miR-21, miR-150, miR-423 in patients with lupus nephritis

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Original Paper

Introduction. MicroRNAs (miRNA) are involved in the pathogenesis of systemic lupus erythematosus (SLE), an autoimmune disease, and can be considered as diagnostic and prognostic biomarkers. Lupus nephritis (LN) remains a major challenge of SLE since it damages the kidneys in the course of the disease.
Methods. The aim of this study was to investigate the diagnostic values of circulating miR-21, miR-148a, miR-150, and miR-423 involved in autoimmunity and kidney fibrosis in plasma samples of LN cases (N = 26) and healthy controls (N = 26) using quantitative-PCR (qPCR). The possible associations between the microRNAs and clinical parameters and their diagnostic values were also calculated.
Results. The levels of circulating miR-21 ($P < .001$) and miR-423 ($P < .05$) significantly increased, while miR-150 decreased in LN ($P > .05$) patients as compared with healthy controls. Receiver operating characteristic (ROC) analysis indicated that miR-21 was superior in discriminating LN patients from controls with an Area Under Curve (AUC) of 0.912 [95% CI = 0.83 to 0.99, $P < .001$], whereas the multivariate ROC curve analysis revealed the high accuracy [AUC = 0.93, $P < .001$, 79% sensitivity and 83% specificity] of the miR-21, -150, and -423 to differentiate LN from controls.
Conclusion. The involvement of the studied miRNAs in renal fibrosis and the obtained results make it rational to speculate that they may be used as potential biomarkers in LN.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a prolonged autoimmune disease that is characterized by autoantibody production against a wide range of self-antigens (e.g. DNA, RNA, histones, and other nuclear components). Lupus nephritis (LN) is a serious complication of SLE and continues to be a major challenge for SLE[1] since most of the patients exhibit significant nephritis in the course of their illness.[2] These patients at the active stages of the disease often develop renal damage and kidney dysfunction, both of which are the leading causes of SLE-related mortality and morbidity.[3-5]

The pathogenesis of LN is a complex procedure and consists of cell proliferation and deposition of autoantibodies in the glomerulus.[6,7] Under pathological circumstances, renal glomerular mesangial cells get more activated to produce inflammatory factors, and trigger infiltration of numerous inflammatory cells; finally, lead to further amplified renal inflammatory responses, renal damage, and eventually renal fibrosis.[8] Consequently, the knowledge of LN activeness and the disease condition presents significant role in the treatment and prognosis of patients. So far, there is active ongoing research to identify

improved measures of LN activity that lead to the discovery of several biomarkers.

A growing body of evidence indicates that epigenetic factors like DNA methylation, histone modification, and microRNAs participate in the LN development and kidney fibrosis. Epigenetic alterations can influence gene expression and modify cellular function without altering the genomic sequence.[9,10] As fine-tuning regulators, microRNAs, the endogenous and small non-coding RNAs, can regulate gene expression and DNA methylation by targeting the RNA/proteins and DNA methylation machinery, respectively. Moreover, microRNAs play significant roles in modulation of immune cell function and inflammatory mediator; therefore, they participate in autoimmune diseases.[7] Moreover, their dysregulation can cause variation in a broad range of target genes involved in the development of different diseases. Generally, cell-free miRNAs are considered as informative biomarkers that offer some potential advantages over protein-based biomarkers. While low relative abundance of some proteins of interest in blood can hamper their detection, circulating miRNAs can be detected by polymerase chain reaction (PCR), an acid nucleic-based amplification method.[11] Most importantly, the diversity of post-translational modifications and complexity of protein-based markers can affect the accuracy of measurement; however, miRNA species are homogenous and have conserved sequences. Since miRNAs are available with high stability in body fluids and can be sampled non-invasively, they are reported to be attractive diagnostic and prognostic biomarkers for a wide range of human diseases.[12] Furthermore, the expression of some miRNAs is specific to tissues, biological stages, and the pathology/disease of interest that can be detected before appearance of clinical symptoms.[11] Recently, the role of miRNAs as transcriptional and translational regulators in the pathogenesis of SLE has been revealed and their modulatory effects on immune responses, DNA methylation, and nephrogenesis pathways are becoming clear.[7,13-16]

The involvement of miRNAs in LN molecular pathways like immune responses (miR-21, -148a, and -150), epithelial-mesenchymal transition (miR-148a and -423-3p), and extracellular matrix accumulation (miR-21 and -150) were the basis for studying them. The aim of the present study was to evaluate the expression patterns and diagnostic values of these microRNAs in plasma samples of LN patients.

MATERIALS AND METHODS

Sample Collection

Twenty-six lupus patients with biopsy-proven nephritis were included in the present cross-sectional study. The patients were recruited from the Kidney Ward of Imam Reza Hospital, Tabriz, Iran within 1 year (2015-2016). We excluded patients with any infection, inflammatory diseases, diabetes, malignancy, and other glomerulopathy and autoimmune diseases. Based on the clinical parameters, the LN activity and chronicity were determined as our previous work.[11] Moreover, twenty-six sex- and age-matched healthy individuals recruited as control group. These healthy controls did not have any evidence of kidney diseases, cardiovascular diseases, infection or cancer.

A written informed consent was obtained from all the participants. The consent forms and study approved by the ethics committee of the Tabriz university of medical sciences, Tabriz, Iran (Ethical code: *TBZMED.REC.1395.494*). Samples were collected close to the time of the biopsy and at this time, our patients did not receive any LN-related therapy. Venous blood samples (4 mL) were collected from the subjects in vacutainers with anticoagulant Ethylenediaminetetraacetic acid. Levels of serum chemistry, serum creatinine, complement (C3 and C4), antinuclear antibodies (ANA), anti-dsDNA antibody (anti-dsDNA), and the amount of proteinuria in 24-hour urine samples were measured.

Evaluation of microRNAs in Plasma

RNA extraction and quantification were done by Exiqon Kits (Vedbaek, Denmark) according to the manufacturer's instruction. Circulating RNAs were isolated from plasma specimens using biofluid microRNA isolation kit (Cat. Number: 300112) and were subjected to reverse-transcription using the Universal RT cDNA Synthesis Kit (Cat. Number: 203301). Quantitative PCR (qPCR) was also performed using SYBR® Green master mix kit (Cat. Number: 203403) and specific LNA (locked nucleic acids) primers; miR-21-5p (assay ID: 204230), miR-148a-3p (assay ID: 205867), miR-150-5p (assay ID: 204660), miR-423-3p (assay ID: 204488). Moreover, miR-191-5p (assay ID: 204306)

was used as normalization control. The expression of the studied microRNAs calculated in the relative expression using $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The IBM SPSS software version 17.0 used for statistical analyses. Normally and non-normally distributed data were shown as Mean (SD) and Median (Interquartile Range), respectively. To compare statistical significances between different groups the independent t-test and Mann–Whitney U test and Kruskal-Wallis tests were used for normally distributed and nonparametric values, respectively. Spearman's rank correlation used to analyze the correlation between clinical parameters and plasma levels of miRNAs. Receiver operating characteristic (ROC) analysis was applied to test the diagnostic performance of miRNAs. The area under the curve (AUC) with 95% confidence intervals (CI) was employed to evaluate the diagnostic performance of the selected miRNAs.[11,17-19] A P value < .05 was considered statistically significant.

RESULTS

Demographic characteristics, disease activity, and laboratory data of the studied subjects are shown in Table 1.

Differential microRNA Expression in Plasma Samples of Patients with LN

The levels of circulating microRNAs including miR-21, miR-148a, miR-150, and miR-423 were evaluated in plasma samples of LN and healthy groups. Compared to controls, circulating miR-21 (P < .001) and miR-423 (P < .05) were elevated significantly while miR-150 diminished in plasma samples of LN patients, it was not statistically significant (P > .05), Figure 1. miR-148a was not detected in plasma samples of LN patients. Moreover, there were significant differences in miR-21 (P < .001) and miR-423 (P < .05) but not miR-150 (P > .05) expression levels between LN stages (Figure 2A, B, and C). miR-21 expression was significantly higher in patients with stage 5 than stages 3 and 4.

Correlations Between Clinical Parameters and microRNAs in LN Patients

The relationship between level of the studied microRNAs and clinical parameters were assessed in the cases. A significant correlation was detected between miR-150 and activity index (r = -0.419, P < .05) and miR-21 and anti-dsDNA (r = 0.460, P < 0.05). Additionally, there were associations between the studied miRNAs and clinical parameters like proteinuria, anti-dsDNA, activity index, and ESR; however, they were not significant (Table 2).

Correlations Between the Studied microRNAs in LN Patients

Associations between levels of the studied

Table 1. Demographic and Baseline Clinical Data

Characteristics	Control Group (n=26)	LN Group (n=26)	*P**
Age, y	30.00 ± 7.22	32.61 ± 8.79	> .05
Number of Cases			
Male	9	6	> .05
Female	17	20	
C3 (mg/dL)	89.94 ± 17.34	27.00 ± 9.25	< .001
C4 (mg/dL)	45.83 ± 16.18	13.27 ± 5.74	< .001
ANA	0.60 ± 0.20	7.37 ± 3.41	< 0.001
Anti-dsDNA	14.11 ± 4.24	61.00 ± 25.34	< 0.001
Creatinine (mg/dL)	0.91 ± 0.10	1.46 ± 0.32	< 0.001
Proteinuria (mg/24h)	94.66 ± 15.55	2107 ± 1094	< 0.001
ESR (mm/h)	12.66 ± 3.67	33.61 ± 12.15	< 0.001
Chronicity Index	-	6.46 ± 3.1	-
Activity Index	-	10.0 ± 3.4	-
Stages (3, 4, 5)	-	9, 11, 6	-

ANA: anti-nucleic acid, anti-dsDNA: anti-double strand DNA, ESR: erythrocyte sedimentation rate. The quantity data are expressed as mean ± SD.
P < .05 was considered significant.
*P values indicate comparison between groups (independent sample t test).

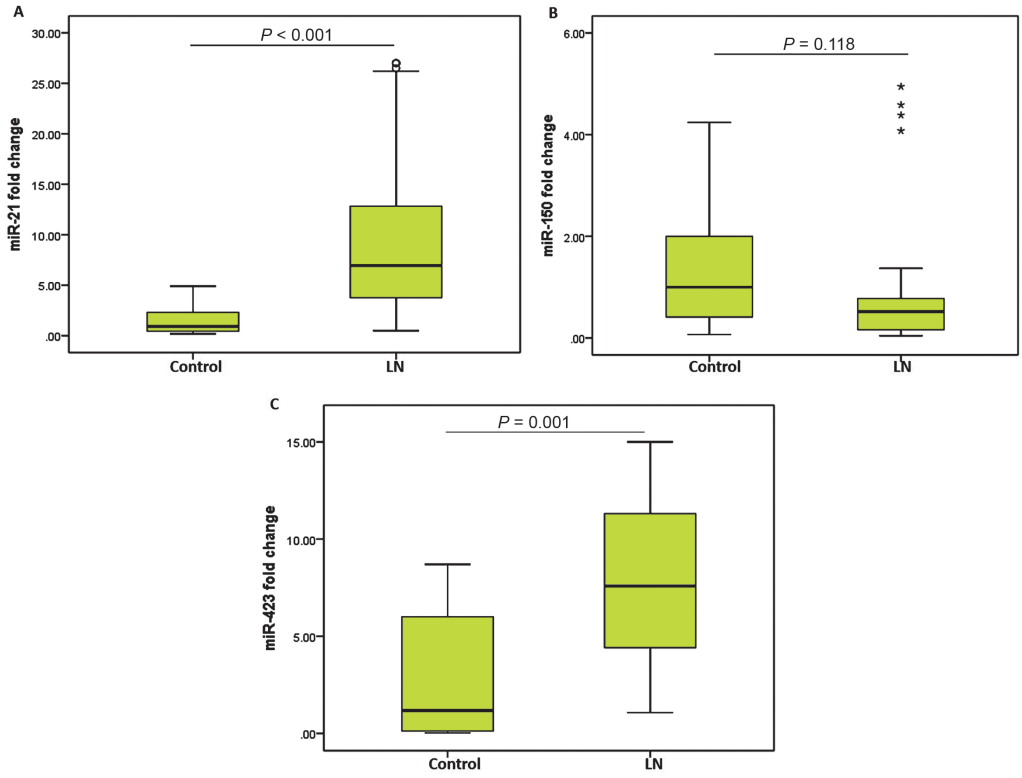

Figure 1. The RT-qPCR Analysis. Differentially Expressed (A) miR-21, (B) miR-150, and (C) miR-423 in LN Patients in Comparison with the Control Group. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression (fold change) between sample groups.

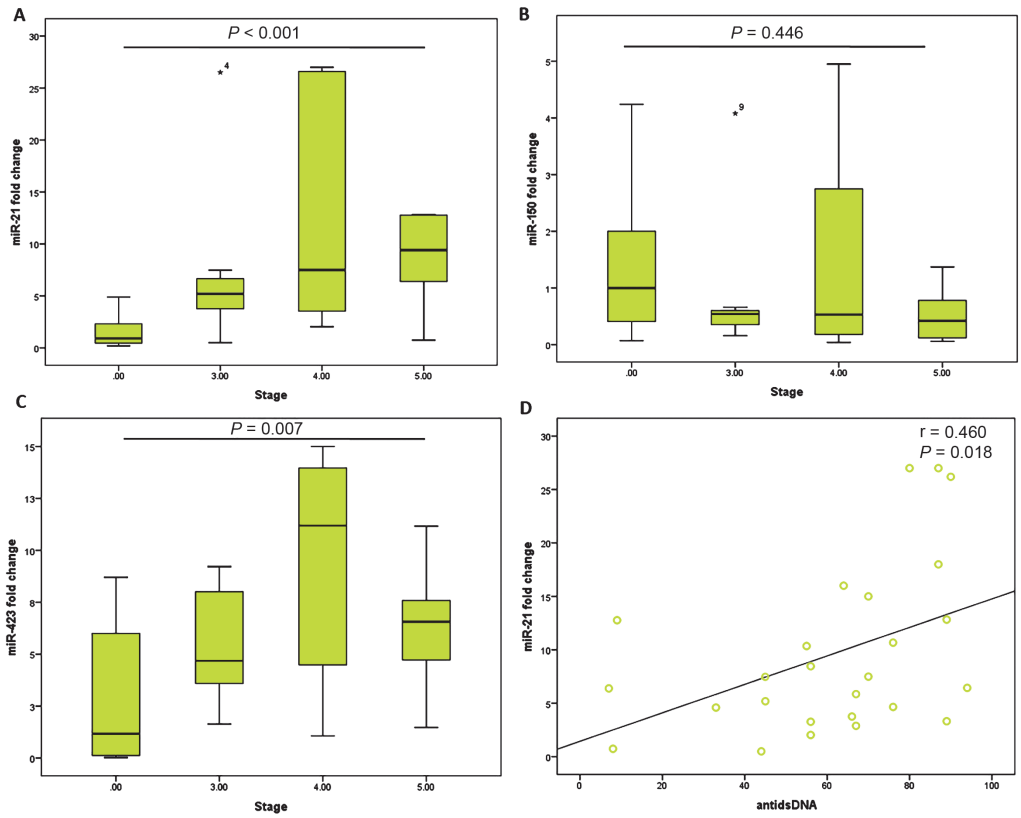

Figure 2. Differentially Expressed microRNAs. Expression levels of (A) miR-21, (B) miR-150, and (C) miR-423 in patients with Different LN Stages (D) A Correlation Between miR-21 and Anti-double Strand DNA

Table 2. Correlations Between Studied miRNAs and Clinical Parameters in LN Patients

Parameters	miR-21	miR-150	miR-423
C_3	0.027	-0.159	0.007
P	> .05	> .05	> .05
C_4	0.116	-0.021	-0.105
P	> .05	> .05	> .05
ANA	0.042	-0.047	0.210
P	> .05	> .05	> .05
Anti-dsDNA	0.460	-0.013	0.375
P	< .05	> .05	> .05
Creatinine	-0.167	-0.022	0.031
P	> .05	> .05	> .05
Proteinuria 24h	-0.176	0.218	-0.219
P	> .05	> .05	> .05
ESR	-0.131	-0.214	0.298
P	> .05	> .05	> .05
Activity Index	0.065	-0.419	-0.103
P	> .05	< .05	> .05
Chronicity Index	-0.044	0.050	-0.008
P	> .05	> .05	> .05
Stage	0.220	-0.138	0.121
P	> .05	> .05	> .05

r: correlation coefficient, ANA: anti-nucleic acid, anti-dsDNA: anti-double strand DNA, ESR: erythrocyte sedimentation rate.
P < .05 was considered significant.
P values indicate Spearman's rank correlation.

miRNAs were tested in LN and control groups. No correlations were found between miRNAs expression in the control group, except for miR-21 and miR-423 (r = 0.762, *P* < .001). However, in LN group, significant correlations were found between miR-423 and miR-150 (r = -0.604, *P* < 0.05) as well as miR-21 and miR-423 (r = 0.463, *P* < .05).

ROC Curve Analysis

To examine the relationship between miRNAs and LN and evaluate their ability to discriminate patients from controls, the ROC analysis was performed. miR-21 showed a AUC = 0.912 [95% CI = 0.83 to 0.99], 86% sensitivity and 63% specificity. The AUC value of 0.64 [CI = 0.46 to 0.82] with 69% sensitivity and 72% specificity were produced by miR-150 (Figure 3B). miR-423 yielded a AUC of 0.81 [0.68 to 0.94] with 96% sensitivity and 66% specificity. The studied miRNAs together indicated the high accuracy; 79% sensitivity, 83% specificity, and AUC of 0.93 [CI = 0.56 to 1.00] for separating LN patients from controls (Table 3, Figure 3D).

DISCUSSION

The kidney involvement is the foremost cause of morbidity and mortality in SLE patients and LN remains a potential complication for SLE. Different immunologic (immune cells, cytokines) and epigenetic factors especially microRNAs have been implicated in the pathogenesis of LN.[20] A growing body of data has revealed that the abnormally expressed microRNAs are contributors of LN.[21,22]

miR-21 regulates multiple gene targets including the programmed cell death 4 (PDCD4), a protein translation inhibitor of genes involved in immune responses.[23] Abnormal expression of miR-21 causes inflammation, cellular transformation, tissue fibrosis, and appearance of different kidney diseases.[17,18,24,25] In lupus, miR-21 exerts a pluripotent role and promote B and T cell activation. In peripheral blood mononuclear cells (PBMCs) of patients with SLE, up-regulated miR-21 can disturb PDCD4 expression and regulate aberrant T cell responses in patients.[21] Up-regulation of miR-21 has been reported in different samples of SLE patients compared to healthy controls.[26-28] Consistent with these data, a significant increase

Table 3. ROC curve analysis of miRNAs expression to discriminate LN patients from healthy controls.

microRNAs	miR-21	miR-150	miR-423	The Studied miRs
Sensitivity (95% CI)	0.86 [0.81 to 0.99]	0.69 [0.50 to 0.83]	0.96 [0.82 to 0.99]	0.79 [0.58 to 0.89]
Specificity (95% CI)	0.63 [0.43 to 0.80]	0.72 [0.44 to 0.84]	0.66 [0.43 to 0.84]	0.83 [0.64 to 0.93]
PPV (95% CI)	0.76 [0.59 to 0.87]	0.75 [0.55 to 0.88]	0.81 [0.65 to 0.91]	0.83 [0.641 to 0.93]
NPV (95% CI)	0.93 [0.70 to 0.989]	0.60 [0.38 to 0.78]	0.98 [0.76 to 1.00]	0.77 [0.579 to 0.89]
LR^+ (95% CI)	2.64 [1.51 to 4.62]	2.07 [1.03 to 4.19]	3.00 [1.56 to 5.76]	4.60 [1.84 to 11.57]
LR^- (95% CI)	0.06 [0.01 to 0.42]	0.46 [0.24 to 0.89]	0.05 [0.00 to 0.39]	0.28 [0.13 to 0.57]
Youden Index J	0.7350	0.4145	0.5171	
Associated Criterion	> 3.14	≤ 0.55	> 1.35	

Circulating microRNAs fold change values were used to assess the diagnostic value of the selected miRNAs. ROC; receiver operating characteristic, PPV; positive predictive value, NPV; negative predictive value, LR; likelihood ratios. The studied miRs: combination of miR-21, miR-150 and miR-423.

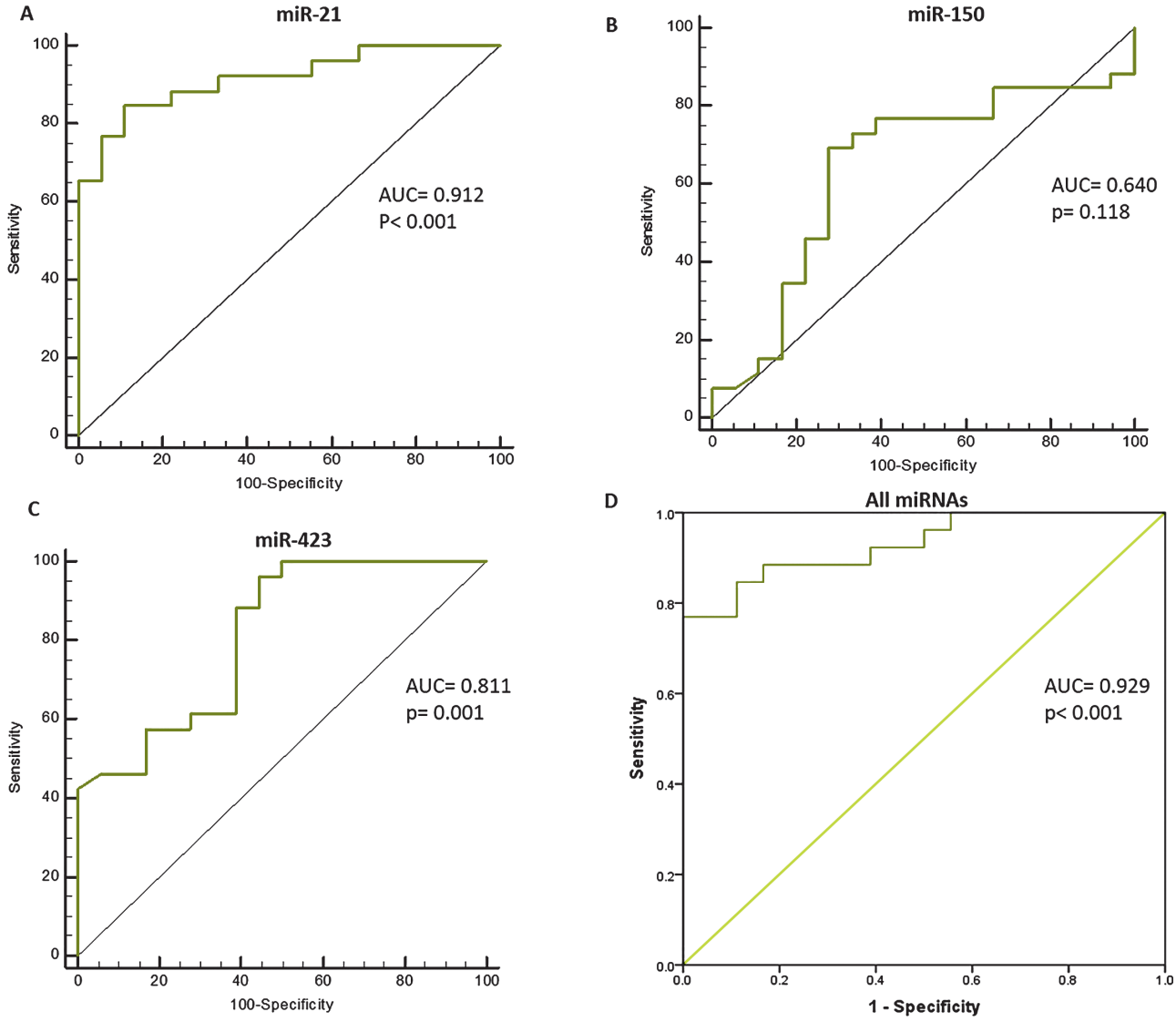

Figure 3. Roc Curve Analysis. ROC Curve Analysis of (A) miR-21, (B) miR-150, and (C) miR-423 (D). The Multivariate ROC Curve with the miRNAs (miR-21, miR-150, and miR-423). ROC, receiver operating characteristic; AUC, the areas under the ROC curve

in the levels of circulating miR-21 was observed in our study. Moreover, miR-21 expression was even higher in patients with severe LN stages. Tang *et al*. found a significant correlation between miR-21 and the level of plasma complement C_3 and C_4 in SLE patients.[29] However, we could not observe a significant correlation between miR-21 and C_3 nor C_4 in LN cases. The levels of circulating miR-21 in our LN patients could be a part of active renal injury, and fibrosis.

miR-148a serves as a tumor suppressor[30] and acts as a novel EMT and metastasis suppressor in many tumor types.[22,31-33] Qingjuan *et al*. (2016) reported that the overexpression of miR-148a in glomeruli and the blood serum may promote cell proliferation and contribute to LN progression by targeting phosphatase and tensin homology deleted on chromosome ten (PTEN) and the proapoptotic protein Bim.[34] Moreover, in lupus patients and animal models, the elevated levels of miR-148a can ease the development of a lethal autoimmune disease that offers an immune regulatory function for miR-148a; B cell tolerance and autoimmunity.[34] Increased levels of miR-148a-3p have been also observed in the PBMCs of SLE[15,21] and LN[35] patients as well as glomeruli of patients and mice with LN.[16] It is demonstrated that miR-21 and miR-

148a can indirectly or directly modulate DNA methyltransferase 1 (DNMT-1) expression and participate in inhibiting DNA methylation in T cells and the global hypomethylation noted in SLE.[21,22] In contrast to the previous studies, we did not detect the previously reported upregulation of miR-148a in plasma samples of LN patients. The decreased level of miR-148a in the present study was confirmed by a lack of strong amplification signal during Real-time PCR.

miR-150 regulates B cell development via targeting the c-myb, a transcription factor.[36] Controversial data is available on miR-150 expression levels in SLE patients with or without LN. For example, in renal biopsies of LN patients, an elevated level of miR-150 was observed that was correlated with kidney fibrosis, nephritic chronicity score, and an anti-fibrotic protein SOCS1, a suppressor of cytokine signaling.[37] However, Abulaban *et al*. could not find a significant difference in miR-150 levels of children's urine samples (pellet and supernatant) with active LN when compared to those of SLE patients without LN or controls.[38] Conversely, decreased levels of miR-150 have been reported in PBMCs[21] and renal biopsies[39] of LN patients when compared to controls. Likewise, we found diminished levels of circulating miR-150 in plasma samples of LN cases. The inconsistency between the studies may rise from differences in sample size, clinical specimen (cells/biopsy *vs*. body fluids), genetic background, and environmental factors (life style, exposure to infectious).[40,41]

miR-423 is associated with EMT and elevated levels of miR-423-5p and -3p have been reported in urine and plasma samples of kidney recipients with active fibrosis.[19,42] For the first time, Te *et al*. (2010) reported that miR-423-5p was differentially expressed in PBMCs and associated with LN in African/European American racial patients.[35] Likewise, in the present study, an increased level of this microRNA was observed in patients with LN that may present active EMT and renal fibrosis. In the percent study, a negative and positive correlations were observed between miR-423 and respectively miR-150 and miR-21.

Some limitations existed in this study; small sample size mainly due to the incidence of LN. Moreover, we did not include the lupus patients to compare the results between groups.

CONCLUSION

Abnormal levels of circulating miR-21, miR-150, and miR-423-3p in plasma samples of patients with LN were observed that might be associated with renal dysfunction. The multivariate ROC curve analysis with miR-21, miR-150, and miR-423-3p indicated that the studied microRNAs with high levels of accuracy could discriminate LN from controls (AUC = 0.93). The involvement of the studied miRNAs in renal fibrosis and the obtained results make it rational to speculate that these miRNAs may be used as potential biomarkers in LN. Further molecular and clinical follow-up with large sample size studies are needed to confirm these findings.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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