Peripheral blood circular RNA hsa_circ_0082688-hsa_circ_0008675 can be used as a candidate biomarker of systemic lupus erythematosus with renal involvement

Q. Luo[1], L. Zhang[1], L. Xiong[2], B. Fu[3], Y. Guo[1], Z. Huang[1], J. Li[1]

[1]Department of Clinical Laboratory, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China; [2]Department of Clinical Laboratory, Jiangxi Province Blood Center, Nanchang, Jiangxi, China; [3]Department of Rheumatology, the First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China.

Abstract

Objective

This research aimed to investigate the level of peripheral blood circular RNAs (circRNAs) from systemic lupus erythematosus (SLE) patients with renal involvement (SLE+RI) to identify novel biomarkers for SLE+RI screening.

Methods

circRNAs expression in peripheral blood from 3 SLE+RI patients, 3 SLE patients without renal involvement (SLE-RI) and 3 healthy controls (HC) were performed by microarray. All upregulated expressed circRNAs coming from "circBase" between the three groups were determined by real time-quantitative polymerase chain reaction (qRT-PCR) in SLE+RI, SLE-RI, HC, neprhritis without SLE (NWS) and rheumatoid arthritis (RA) patients. The diagnostic value of these circRNAs for SLE+RI was evaluated by receiver operating characteristic (ROC) curve. A 15-day follow-up was evaluated in 7 newly diagnosed SLE+RI patients to investigate the level change of these circRNAs after treatment.

Results

We confirmed that the level of hsa_circ_0082688, hsa_circ_0082689 and hsa_circ_0008675 were significantly elevated in SLE+RI patients with respect to the SLE-RI, RA, NWS patients and the HC. The level of hsa_circ_0082688, hsa_circ_0082689 and hsa_circ_0008675 were associated with C4, anti-dsDNA, anti-nucleosome. The level of hsa_circ_0008675 was associated with C3, and the level of hsa_circ_0082688 and hsa_circ_0008675 were associated with treatment. ROC curve analysis suggested that hsa_circ_0082688-hsa_circ_0008675 had significant value in the diagnosis of new-onset SLE+RI patients than the controls (new-onset SLE-RI patients, RA patients, NWS patients and HC) with an area under the curve of 0.925, sensitivity of 79.17% and specificity of 96.64%.

Conclusion

This study suggests that peripheral blood hsa_circ_0082688-hsa_circ_0008675 level in SLE+RI patients is upregulated and may also serve as a potential biomarker for SLE+RI patient diagnosis and treatment.

Key words

systemic lupus erythematosus, renal involvement, circular RNAs, microarray assay, peripheral whole blood

*Qing Luo, MM**
*Lu Zhang, MM**
Lihong Xiong, MM
Biqi Fu, MM
Yang Guo, MM
Zikun Huang, MM
Junming Li, PhD

**These authors contributed equally to this study.*

Pleae address correspondence to:
Junming Li,
Department of Clinical Laboratory, First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, China.
E-mail: lisir361@163.com

Zikun Huang,
(address as above)
E-mail: 491353062@qq.com

Received on June 10, 2019; accepted in revised form on September 2, 2019.

Funding: this work was supported by the Key Research and Development Plan Project of Jiangxi Province (20181BBG70013), the Science and Technology Plan Project of the Education Department of Jiangxi Province (170008), the National Natural Science Foundation of China (81360459, 81660277), Jiangxi Provincial Natural Science Foundation of China (20151BAB215031, 20171BAB205113), the Science and Technology Project of Health and Family Planning Commission of Jiangxi Province of China (20165094) and the Foundation for Distinguished Young Scientists of Jiangxi Province of china (20171BCB23087).

Competing interests: none declared.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by the production of autoantibodies, binding with self-antigens to form immune complexes which deposit in various organs and potentially causing various organs or systems damage (1). Renal involvement is a serious complication of SLE leading to significant morbidity and mortality among patients, affecting up to 70% SLE, and about 10–30% of SLE patients with renal involvement will evolve to renal failure (2-3). Despite overall improvement in the care of SLE patients and an increase in 5-and 10-year renal survival rates of SLE patients with renal involvement, its prognosis remains unsatisfactory (4). Hence, early and accurate diagnosis and proper treatment are urgently requisite, which could dramatically change the unpredictable course of renal disease and improve the long-term survival.

Circular RNAs (CircRNAs) are a type of closed circular non-coding RNAs, formed by an exon, an intron, or the reverse splicing of the two, are generally existed in mammalian cells and play an key role in regulation of gene expression through miRNA sponges and interacting with RNA-binding proteins (5-6). Because circRNAs do not have 5′ or 3′ ends, they can resist RNase digestion and more stable than most linear RNAs (7). Moreover, CircRNAs often show tissue/developmental stage-specific expression (8) and circRNAs are widely distributed in the cytoplasm, the nucleus and a variety of body fluids, including saliva and blood (9-10). These make them more appropriate as biomarkers than linear RNAs. Increasing evidence reveals that circRNAs are not only involved in the progress of many diseases, such as atherosclerotic vascular disease risk, neurological disorders, prion diseases, cancer and autoimmune disease (11-14), but can also use as molecular biomarkers in the diagnosis and prognosis of many diseases, such as type 2 diabetes mellitus (15), coronary artery disease (16), and SLE (17). Our previous studies also showed that hsa_circ_0044235 in peripheral blood may serve as potential biomarkers for rheumatoid arthritis (RA) patients diagnosis (18). Recently, only one paper have revealed that plasma circRNAs may use as a novel biomarker in the diagnosis of lupus nephritis (3). Yet, little is known about peripheral blood circRNAs in human SLE with renal involvement.

In this study, the differentially expressed circRNAs in the peripheral blood of SLE patients without renal involvement (SLE-RI) or SLE patients with renal involvement (SLE+RI) and matched control subjects were screened by microarray analysis and then the findings was confirmed in a new cohort.

Patients and controls

Patient variables

Eighty-three patients (40 SLE+RI and 43 SLE-RI) fulfilled the revised American College of Rheumatology criteria for SLE (19) were recruited from the First Affiliated Hospital of Nanchang University from 2016.7 to 2019.3. Renal involvement of SLE was characterised by proteinuria (>0.5g /24h and/or > 3+), haematuria (>10 leukocytes/high power field excluding infection), or pyuria (>10 leukocytes/high power field excluding infection) (20-21). Among them, 54 patients were new-onset SLE that first time diagnosis of SLE and no history of immunosuppressive drugs or corticosteroids use before recruitment (22). Other patients were re-visiting SLE receiving treatment with 0.5~1mg/kg/d corticosteroids or 0.5~1.0mg/kg/d corticosteroids and immunosuppressive drugs including 10~30mg/kg/d mycophenolate mofetil, 0.75~1.0g/m^2/3~4week cyclophosphamide, 3~5mg/kg/d cyclosporine, 0.5~1mg/d tacrolimus, 10~15mg/week methotrexate. Disease activity was assessed by the SLE disease activity index (SLEDAI) (23). The renal SLEDAI (r-SLEDAI) that including the four kidney related parameters of the SLEDAI (haematuria, pyuria, proteinuria and urinary casts) was used to assess the degree of renal involvement. Each parameter in the r-SLEDAI is assigned four points. Yet, the rSLEDAI scores can range from 0 to 16 (3). In addition, 36 healthy controls (HC) without clinical diagnosis of any inflammatory or autoimmune diseases and without relation to patients of autoimmune disease were enrolled from the First Affiliated Hospital

of Nanchang University. Among, 3 new-onset SLE-RI, 3 new-onset SLE+RI and 3 age-matched and sex-matched HC were enrolled for microarray measurement. Other SLE patients and HC specimen were used to validate the results of microarray by real time-quantitative polymerase chain reaction (qRT-PCR) assay. As an autoimmune disease control, 38 RA patients who fulfilled the revised American College of Rheumatology criteria for RA (24) were also enrolled from the First Affiliated Hospital of Nanchang University from 2016.7 to 2019.3. In addition, 24 neprhritis without SLE (NWS) patients were enrolled from the First Affiliated Hospital of Nanchang University from 2016.7 to 2019.8. The demographic characteristics of the study population were shown in Table I. The study had approval from the Ethics Committee of the First Affiliated Hospital of Nanchang University (052) and complied with the Helsinki Declaration. All participants provided signed informed consent before they entered the study.

Table I. Demographic characteristics of the study population.

Study set	Categories	SLE+RI	SLE-RI	HC	RA	NWS
Discovery	n	3	3	3		
set	New-onset, n (%)	3 (100.0)	3 (100.0)			
	Females, n (%)	3 (100.0)	3 (100.0)	3 (100.0)		
	Age, mean (SD), years	28.7±16.0	38.7±23.1	34.0±7.2		
	SLEDAI, mean (SD)	20.7±1.5	11.0±4.5			
Validation	n	37	40	33	38	24
set	New-onset, n (%)	24 (64.9)	19 (47.5)			
	Females, n (%)	34 (91.9)	38 (95.0)	29 (87.9)	32 (84.2)	19 (79.2)
	Age, mean (SD), years	36.9±13.3	43.5±16.2	42.5±13.3	48.6±12.7	37.0±13.6
	SLEDAI, mean (SD)	19.8±7.6	8.9±6.0			
	Lupus encephalopathy (n)	7	5			
	Vision disorder (n)	2	1			
	Vasculitis (n)	0	3			
	Arthritis (n)	14	16			
	Myositis (n)	2	1			
	Fever (n)	15	11			
	Skin rash (n)	10	13			
	Alopecia (n)	8	7			
	Ulcer (n)	3	1			
	Pleurisy (n)	18	6			
	Pericarditis (n)	14	7			

HC: healthy controls, RA: rheumatoid arthritis, S.D.: standard deviation, SLE: systemic lupus erythematosus, NWS: neprhritis without SLE, SLEDAI: SLE disease activity index, SLE-RI: SLE patients without renal involvement, SLE+RI: SLE with renal involvement.

Table II. The PCR primer sequence.

CircRNAs	Sequence (5′-3′)
hsa_circ_0046995	F: TCAGTTCCATCCGGGTCATC R: AGCTTGGAAATGATTCTTCTGCT
hsa_circ_0082626	F: TCACCAAGCCAGCCAATTCT R: TCAGTCCAGAGAGTTCGTGA
hsa_circ_0082689	F: GTCCCCAAACACTCTTAGCCA R: CACACTCAGGTTGTGTTCGG
hsa_circ_0082688	F: TGCCGTATCGATGGCAATTC R: ATAGCTCAGGTGGTCAACGC
hsa_circ_0001093	F: CTGACACCCAAGCAGTCAAT R: TCTCCAGGGAAGAGTCCCAAA
hsa_circ_0002715	F: GCAAACCTCCTCTCCATGCT R: GTGAAAAGGCTGTGCCTGTG
hsa_circ_0004156	F: TGCAGCATCCCAGTTTTTGG R: GCCTGCTTCATCTTTATGCACT
hsa_circ_0031482	F: ACACTTTAACCACCAGCCTCA R: TCCAAAAGCAGAGGCCCAGT
hsa_circ_0022383	F: CCACAAGGATCCCGATGTGAA R: TTCACCAATCAGCAGGGGTT
hsa_circ_0008675	F: GGAAGCCTTGCAGTTTGCTC R: AGCATTGGCTGGTGGGTTAT
hsa_circ_0027070	F: GCACAAGAGCCTTGATTGAAGA R: TGGCCAGGGTAGGCTGATA
β-actin	F: TACTGCCCTGGCTCCTAGCA R: TGGACAGTGAGGCCAGGATAG

CircRNAs: circular RNAs, PCR: polymerase chain reaction.

Blood samples collection and total RNA isolation

The blood samples collection and total RNA isolation are consistent with our previous research (18).

Microarray analysis

Sample labelling and array hybridisation were executed by KANGCHEN (Shanghai, China) according to the manufacturer's protocol (Arraystar Inc., Rockville, MD, USA). The specific steps are consistent with the report of Ouyang Q (3)).

qRT-PCR analysis

Complementary DNA(cDNA) was acquired by reverse transcription using a PrimeScript™ RT reagent kit (Takara Bio Inc, Japan). The relative expression of circRNA was then performed on an ABI 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR® Premix Ex Taq™ II (Takara Bio Inc, Japan), with the following PCR assay: an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, then melt curve were detected. β-action was used as an internal control. The PCR primer sequences were presented in Table II. The relative expression of circRNAs were derived by $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Kolmogorov-Smirnov method was used to assess the normality of data. Student's *t*-test or Mann-Whitney U-test were used to compare the data ac-

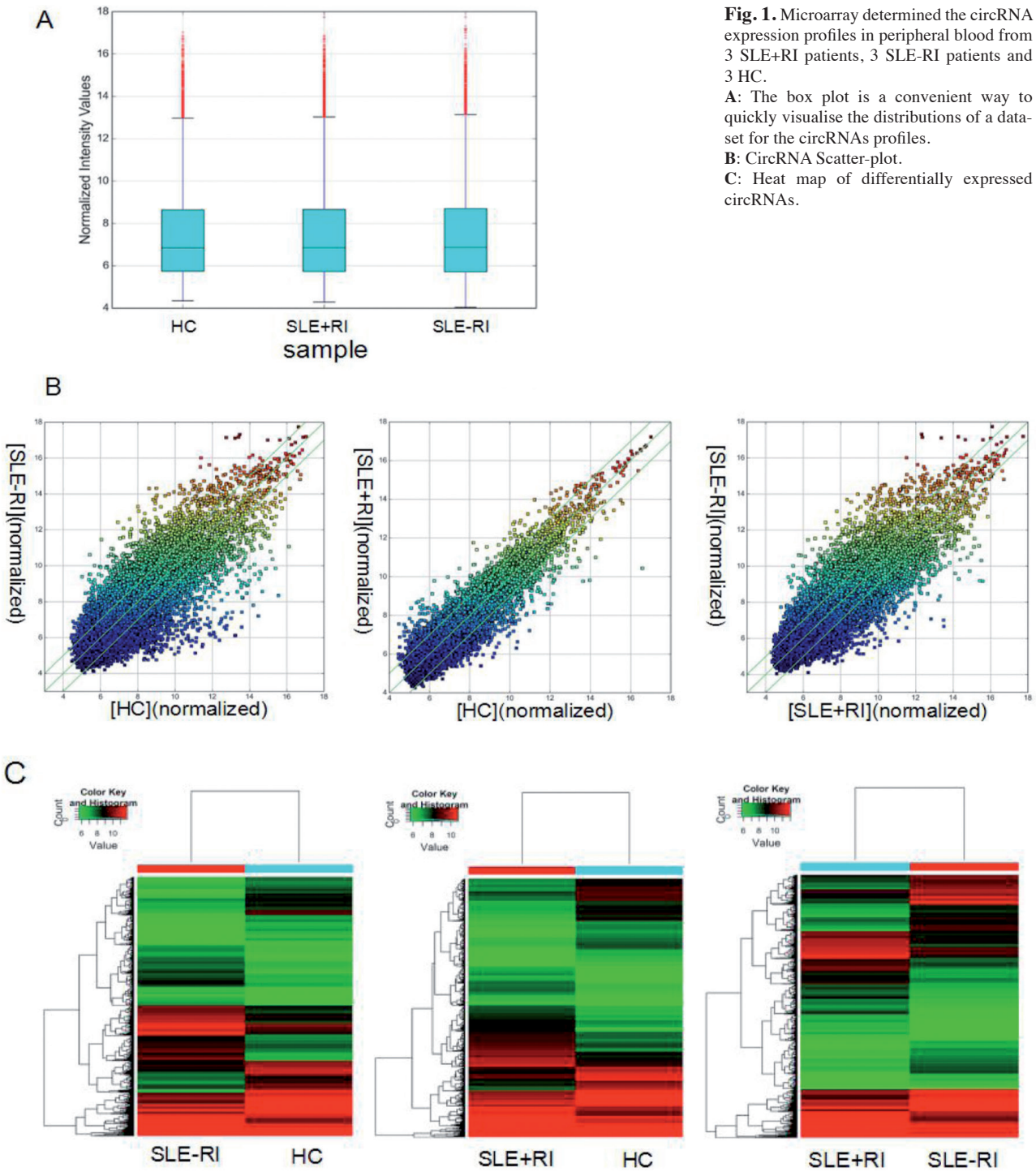

Fig. 1. Microarray determined the circRNA expression profiles in peripheral blood from 3 SLE+RI patients, 3 SLE-RI patients and 3 HC.
A: The box plot is a convenient way to quickly visualise the distributions of a dataset for the circRNAs profiles.
B: CircRNA Scatter-plot.
C: Heat map of differentially expressed circRNAs.

cording to the normality. The paired t test was employed to evaluate the changes with treatment. The nonparametric Spearman method was used for correlation analysis. A receiver operating characteristic (ROC) curve was built to evaluate the diagnostic value of circRNAs that were dysregulated in the peripheral blood of SLE+RI patients compared to SLE-RI patients and HC. All statistical tests were carried out with GraphPad Prism v. 5.0 (GraphPad Software, San Diego, CA) and SPSS v. 17.0 (SPSS Inc., Chicago, IL). A *p*-value of <0.05 was considered as significant difference.

Results

Circular RNA expression profiling in peripheral blood from patients with SLE+RI or SLE-RI

To identify circRNAs that were differentially expressed in patients with SLE-RI and SLE+RI, we performed microarray analysis with circRNAs in the peripheral blood from SLE-RI patients, SLE+RI patients and healthy controls (HC) using an Arraystar Human circRNA Microarray version 2.0. Based on the criteria of a fold change >2.0 and a *p*-value <0.05 (Fig. 1A and 1B), Compared with HC group, 4235 circRNAs (2197 of them upregulated) and 1566 circRNAs (753

Table III. Differentially expressed circRNAs between three groups.

circRNAS	source	Alias	SLE-RI *vs*. HC	SLE+RI *vs*. HC	SLE+RI *vs*. SLE-RI
hsa_circRNA_406581	25070500		4.153144	5.855354	2.184304
hsa_circRNA_102306	circBase	hsa_circ_0046995	3.739935	5.575620	2.411116
hsa_circRNA_405138	25070500		3.525192	11.90579	6.084413
hsa_circRNA_406168	25070500		3.387955	11.56061	6.652412
hsa_circRNA_082626	circBase	hsa_circ_0082626	3.071274	4.848695	2.329550
hsa_circRNA_401055	25242744		3.024274	5.647978	2.903562
hsa_circRNA_082689	circBase	hsa_circ_0082689	2.818399	4.870480	2.700472
hsa_circRNA_082688	circBase	hsa_circ_0082688	2.814211	4.619195	2.637428
hsa_circRNA_406408	25070500		2.603037	3.780046	2.164676
hsa_circRNA_001093	circBase	hsa_circ_0001093	2.563266	3.964081	2.122166
hsa_circRNA_103150	circBase	hsa_circ_0002715	2.499029	5.398658	3.229399
hsa_circRNA_103583	circBase	hsa_circ_0004156	2.331397	7.852232	6.071249
hsa_circRNA_101335	circBase	hsa_circ_0031482	2.254306	5.104165	3.716669
hsa_circRNA_100833	circBase	hsa_circ_0022383	2.188690	3.454638	2.256307
hsa_circRNA_008675	circBase	hsa_circ_0008675	2.162192	5.138098	3.645753
hsa_circRNA_027070	circBase	hsa_circ_0027070	2.123394	7.228973	5.538308
hsa_circRNA_400718	25242744		2.035996	3.337168	2.238255
hsa_circRNA_403068	25242744		-12.909637	-16.164994	-2.649568
hsa_circRNA_101286	circBase	hsa_circ_0002473	-9.067349	-13.35173	-2.974871
hsa_circRNA_010932	circBase	hsa_circ_0010932	-8.042356	-13.52340	-3.470771
hsa_circRNA_038651	circBase	hsa_circ_0038651	-6.365426	-11.00553	-2.911778
hsa_circRNA_000367	circBase	hsa_circ_0000367	-4.534204	-5.873599	-2.092669
hsa_circRNA_105034	circBase	hsa_circ_0001947	-3.307064	-6.520617	-3.242595
hsa_circRNA_001226	circBase	hsa_circ_0001226	-3.181497	-70.819917	-11.656439
hsa_circRNA_001729	circBase	hsa_circ_0000691	-3.115667	-9.1103534	-5.432180
hsa_circRNA_068784	circBase	hsa_circ_0068784	-2.678247	-4.6201621	-2.637489
hsa_circRNA_101515	circBase	hsa_circ_0035197	-2.251106	-3.6859097	-2.402207
hsa_circRNA_079787	circBase	hsa_circ_0079787	-2.088063	-6.2241987	-4.217075
hsa_circRNA_401418	25242744		-2.067624	-3.380782	-2.273286
hsa_circRNA_103845	circBase	hsa_circ_0072568	-2.003793	-3.308580	-2.293905

CircRNAs: circular RNAs, HC: healthy controls, SLE: systemic lupus erythematosus, SLE-RI: SLE patients without renal involvement, SLE+RI: SLE with renal involvement.

of them upregulated) were dysregulated in SLE-RI group and SLE+RI group, respectively, and 828 circRNAs (234 of them upregulated) were differentially expressed in both SLE-RI group and SLE+RI group. Moreover, compared with SLE-RI group, 3957 circRNAs (1982 of them up-regulated) were differentially expressed in SLE+RI group. It was worth noting that, 17 circRNA were upregulated between three groups, 13 circRNA were downregulated between three groups (SLE-RI group compared with HC group, SLE+RI group compared with HC group, SLE+RI group compared with SLE-RI group), and these differently expressed circRNAs were listed in Table III. A heat map was created to group the circRNAs based on their expression levels among the samples (Fig. 1C).

Validation of circRNA expression

Because the main objective of this study was to identify diagnostic markers of SLE+RI in peripheral blood, we focused our attention on the up-regulated circRNAs in SLE+RI patients. To verify the microarray data, we selected all upregulated expressed circRNAs between three groups and come from "circBase", including hsa_circ_0046995, hsa_circ_0082626, hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0001093, hsa_circ_0002715, hsa_circ_0004156, hsa_circ_0031482, hsa_circ_0022383, hsa_circ_0008675, hsa_circ_0027070. We validated their expression levels via qRT-PCR in an independent set of samples from 55 SLE patients (24 SLE+RI patients and 31 SLE-RI patients) and 33 HC. Consistent with the microarray data, the average expression levels of hsa_circ_0082626, hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0002715, hsa_circ_0004156, hsa_circ_0008675 in the peripheral blood of patients with SLE were significantly increaesd than those of the HC (all $p>0.05$) (Fig. 2A-2F), while the expression of hsa_circ_0001093 and hsa_circ_0031482 did not show any remarkable differences between patients with SLE and the HC (all $p>0.05$) (Fig. 2G-2H). The expression levels of hsa_circ_0046995, hsa_circ_0027070 and hsa_circ_0022383 were not detectable in peripheral blood from SLE patients and HC. Moreover, we analysed the expression differences of these dysregulated circRNAs between SLE+RI patients, SLE-RI patients and HC. The data suggested that the expression level of hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 were significantly higher in SLE+RI patients than in SLE-RI and HC (all $p>0.05$) (Fig. 2J, 2K, 2N), while the expression level of hsa_circ_0004156, hsa_circ_0002715 and hsa_circ_0082626 did not show any remarkable differences between SLE+RI patients and SLE-RI patients (all $p>0.05$) (Fig. 2I, 2L, 2M).

Double validation peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 expression in SLE+RI patients

Based on the above analysis, the expression level of hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675

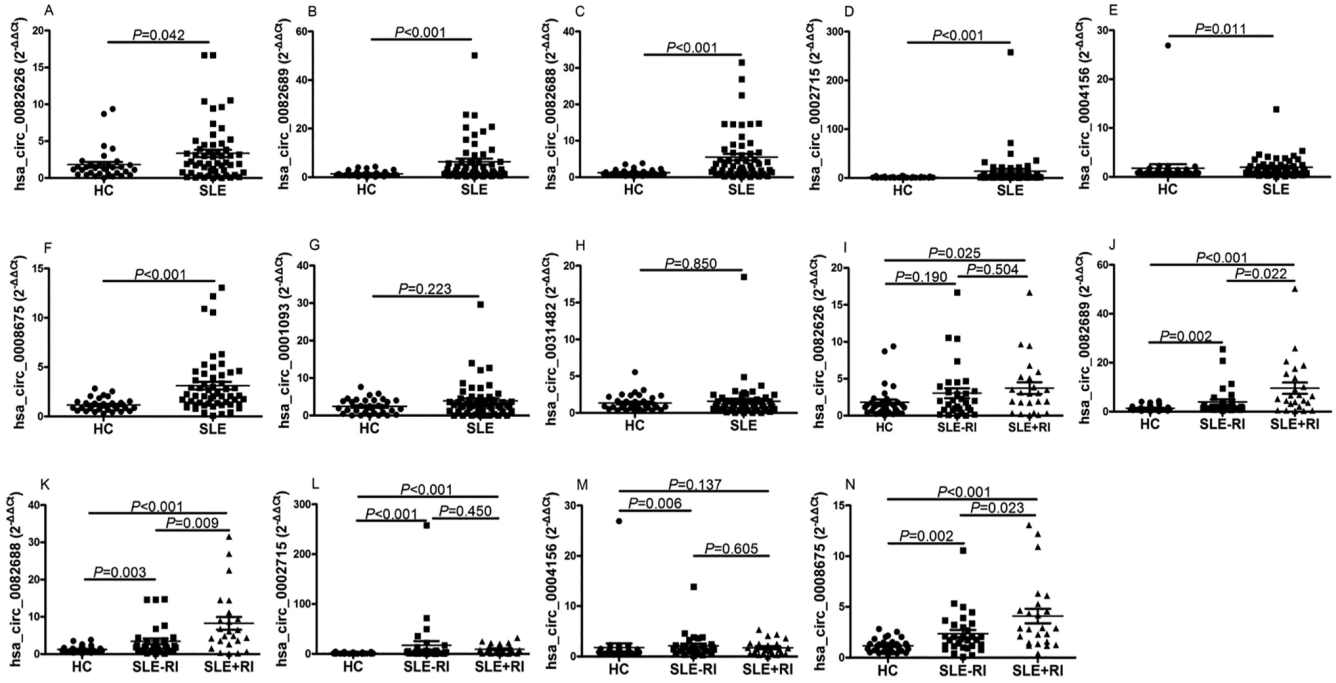

Fig. 2. qRT-PCR determined the relative levels of circRNAs in peripheral blood from SLE patients and comparison group.
Hsa_circ_0082626 (**A**), hsa_circ_0082689 (**B**), hsa_circ_0082688 (**C**), hsa_circ_0002715 (D), hsa_circ_0004156 (**E**) and hsa_circ_0008675 (**F**) in SLE patients were significantly increased than HC. Hsa_circ_0001093 (**G**) and hsa_circ_0031482 (**H**) did not show any remarkable differences between SLE patients and HC. Hsa_circ_0082626 (**I**), hsa_circ_0002715 (**L**) and hsa_circ_0004156 (**M**) did not show any remarkable differences between SLE+RI and SLE-RI. Hsa_circ_0082689 (**J**), hsa_circ_0082688 (**K**) and hsa_circ_0008675 (**N**) in SLE+RI were significantly increased than SLE-RI, HC.

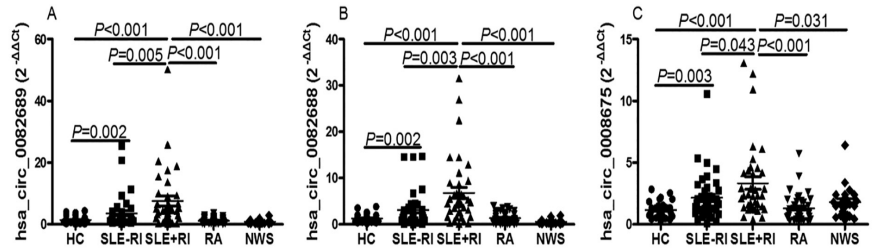

Fig. 3. Double validation peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 expression in SLE+RI patients.
Hsa_circ_0082689 (**A**), hsa_circ_0082688 (**B**) and hsa_circ_0008675 (**C**) in SLE+RI was significantly increased than SLE-RI, HC, RA, NWS.

were significantly higher in SLE+RI patients than in SLE-RI patients and HC. Thus, to validate the results, we collected 22 SLE patients (13 SLE+RI and 9 SLE-RI). From the data of all the SLE patients and HC, the expression level of hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 in peripheral blood from 37 SLE+RI patients were significantly higher compared to 40 SLE-RI patients and 33 HC (all $p>0.05$) (Fig. III). Moreover, we found that the expression level of hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 in peripheral blood from SLE+RI patients were significantly higher compared to RA patients and NWS (all $P < 0.05$) (Fig. III).

Correlation between peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 and clinical variables in SLE+RI patients

To determine whether the aforementioned differentially expressed peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 were relevant biomarkers for the disease activity or severity of SLE in SLE+RI patients, the clinical indicators related to inflammation and renal damage (ESR, CRP, IgG, C3, C4 and 24-hour proteinuria, proteinuria, hematuria, pyuria, blood urea nitrogen, blood creatinine) were collected and the SLEDAI, rSLEDAI of all SLE+RI patients were calculated, and the correlations between this dataset and the levels of the verified differentially expressed circRNAs were analysed. As shown in Figure 4, the level of peripheral blood hsa_circ_0082688 in SLE+RI patients correlated with IgG (r_s =0.400, p=0.032), the level of peripheral blood hsa_circ_0008675 in SLE+RI patients correlated with C3 (r_s = 0.344, p=0.040), and the level of peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 in SLE+RI patients correlated with C4 respectively (r_s =0.335, p=0.049; r_s =0.340, p=0.047; r_s = 0.442, p=0.012), while theirs level did not correlate with blood urea nitrogen, blood creatinine, 24-hour proteinuria, proteinuria, haematuria, pyuria, rSLEDAI and SLEDAI (data no shown).

Production of multiple autoantibodies such as anti-dsDNA and anti-ENAs is one of important characteristics of SLE. And, these autoantibodies are markers for detecting ongoing disease activity in lupus kidneys and early relapse of nephritis. In 37 SLE+RI patients, 35 SLE+RI patients were tested for anti-dsDNA and 28 SLE+RI patients were

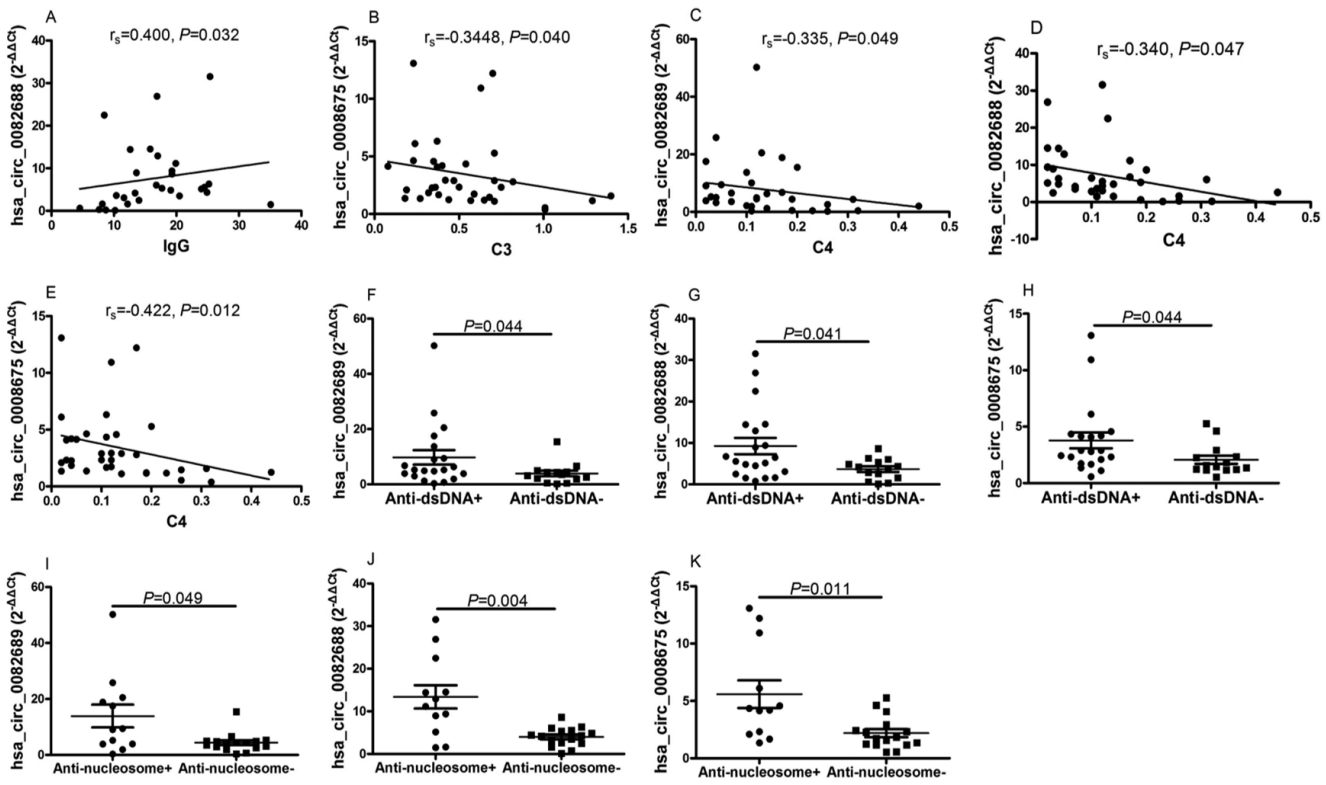

Fig. 4. Correlation of the levels of peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 in SLE+RI patients with SLE clinical characteristics.

IgG correlated with hsa_circ_0082688 (**A**) in SLE+RI patients. C3 correlated with hsa_circ_0008675 (**B**) in SLE+RI patients. C4 correlated with hsa_circ_0082689 (**C**), hsa_circ_0082688 (**D**), hsa_circ_0008675 (**E**) in SLE+RI patients. Anti-dsDNA correlated with hsa_circ_0082689 (**F**), hsa_circ_0082688 (**G**), hsa_circ_0008675 (H) in SLE+RI patients. Anti-nucleosome correlated with hsa_circ_0082689 (I), hsa_circ_0082688 (**J**), hsa_circ_0008675 (**K**) in SLE+RI patients.

tested for anti-ENAs. Then, the correlation between the level of these verified differentially expressed circRNAs and autoantibodies were investigated in SLE+RI patients. As shown in Figure 4, the level of of peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 in SLE+RI patients correlated with anti-dsDNA (p=0.044; p=0.041; p=0.044) and anti-nucleosome (p=0.049; p=0.004; p=0.011) respectively, while theirs level did not correlate with other anti-ENAs (data no shown).

In addition, we found the expression level of of peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 in new-onset SLE+RI patients were significantly higher than new-onset SLE-RI patients (p=0.001; p<0.001; p=0.016) (Fig. 5A-5C) , while the expression level of peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 did not show any remarkable differences between re-visiting SLE+RI patients and re-visiting SLE-RI patients (all p>0.05) (Fig. 5 D-F). These results indicated that the expression level of peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 are associated with drug therapy. Therefore, we investigated the relationship between the expression levelof these circRNAs in SLE+RI patients and drug therapy. As shown in Fig. 5H, the expression level of peripheral blood hsa_circ_0082688 in new-onset SLE+RI patients was significantly higher than re-visiting SLE+RI patients (p=0.003). There was a trend towards elevated peripheral blood hsa_circ_0082689 and hsa_circ_0008675 in new-onset SLE+RI patients, but the difference was not significant (all p>0.05) (Fig. 5G, 5I). Subsequently, we performed a 15-day follow-up evaluation in 7 new-onset SLE+RI patients who received regular treatment with 0.5~1.0mg/kg/d corticosteroids or 0.5~1.0mg/kg/d corticosteroids and immunosuppressive drugs (10~30mg/kg/d mycophenolate mofetil or 3~5mg/kg/d cyclosporine). After treatment, the proteinuria in 5 new-onset SLE+RI patients were decreased, while 2 new-onset SLE+RI patients were no changed. Notably, the expression level of peripheral blood hsa_circ_0008675 decreased following treatment when compared with those prior to treatment (p=0.028) (Fig. 5L), but there was no difference in peripheral blood hsa_circ_0082689 and hsa_circ_0082688 (Fig. 5J, 5K). These results show that the expression level of peripheral blood hsa_circ_0082688 and hsa_circ_0008675 correlates with disease severity.

ROC curve analysis of peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 as SLE+RI diagnosis

To investigate the potential value of peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 for SLE+RI diagnosis, we performed ROC curve analysis. The area under

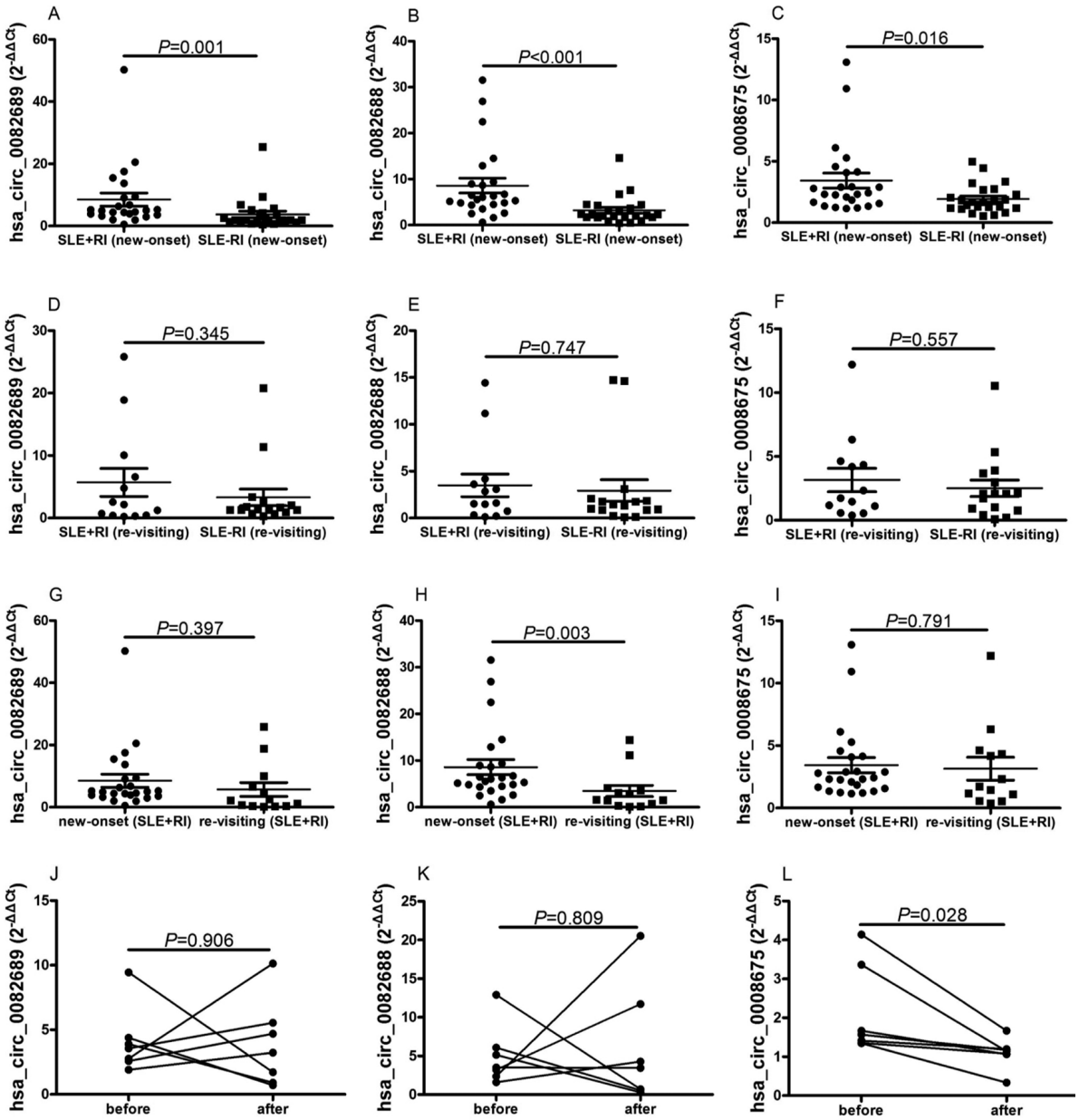

Fig. 5. Correlation of the levels of peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 in SLE+RI patients with treatment. Hsa_circ_0082689 (**A**), hsa_circ_0082688 (**B**) and hsa_circ_0008675 (**C**) in new-onset SLE+RI patients was significantly increased than new-onset SLE-RI patients. Hsa_circ_0082689 (**D**), hsa_circ_0082688 (**E**) and hsa_circ_0008675 (**F**) did not show any remarkable differences between re-visiting SLE+RI patients and re-visiting SLE-RI patients. Hsa_circ_0082689 (**G**) and hsa_circ_0008675 (**I**) did not show any remarkable differences between new-onset SLE+RI patients and re-visiting SLE+RI patients. Hsa_circ_0082689 in new-onset SLE+RI patients was significantly increased than re-visiting SLE+RI patients. There was no difference in hsa_circ_0082689 (**I**) and hsa_circ_0082688 (**K**) following treatment. hsa_circ_0008675 (**L**) decreased following treatment.

curve (AUC) values showed that peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 could separate the patients with SLE+RI from the controls (SLE-RI, RA, NWS, HC). The highest AUC was found for hsa_circ_0082688 (AUC: 0.812, 95% CI: 0.721–0.904, $p<0.0001$) with sensitivity of 75.68%, specificity of 83.70% and cut-off of 2.471, followed by hsa_circ_0082689 (AUC: 0.796, 95% CI: 0.695–0.897, $p<0.0001$) with sensitivity of 70.27%, specificity of 88.15% and cut-off of 2.969, hsa_circ_0008675 (AUC: 0.731, 95% CI: 0.637–0.824, $p<0.0001$) with sensitivity of 59.46%, specificity of 79.26% and cut-off of 2.085 (Table IV and Fig. 6A). To evaluate the cumulative performances of the three circRNAs in discriminating SLE+RI from the controls (SLE-RI, RA, NWS, HC), a binary logistic regression was performed. The logistic regression model showed that combination of hsa_circ_0082688 and hsa_circ_0008675 could provide the better diagnostic accuracy with the AUC of 0.814 (95% CI: 0.722–0.905, $p<0.0001$, sensitivity: 72.97%, specificity: 85.19%), which equal to the AUC (AUC: 0.814, 95% CI: 0.722–0.905, $p<0.0001$, sensitivity: 72.97%, specificity: 85.19%) of combination of hsa_circ_0082688, hsa_circ_0082688 and hsa_circ_0008675 (Table IV and Fig. 6B).

Table IV. ROC curve analysis of peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 as SLE+RI diagnosis.

Variables	AUC	*p*-value	95% C.I.	Sensitivity	Specificity	Cut-off
SLE+RI *vs*. (SLE-RI, RA, HC)						
hsa_circ_0082689	0.796	<0.0001	0.695-0.897	70.27	88.15	2.969
hsa_circ_0082688	0.812	<0.0001	0.721-0.904	75.68	83.70	2.471
hsa_circ_0008675	0.731	<0.0001	0.637-0.824	59.46	79.26	2.085
hsa_circ_0082689+hsa_circ_0082688	0.813	<0.0001	0.721-0.905	75.68	84.44	-
hsa_circ_0082689+hsa_circ_0008675	0.802	<0.0001	0.711-0.893	75.68	80.74	-
hsa_circ_0082688+hsa_circ_0008675	0.814	<0.0001	0.722-0.905	72.97	85.19	-
hsa_circ_0082689+hsa_circ_0082688 +hsa_circ_0008675	0.814	<0.0001	0.722-0.905	72.97	85.19	-
new-onset SLE+RI *vs*. (new-onset SLE-RI, RA, HC)						
hsa_circ_0082689	0.913	<0.0001	0.842-0.984	87.50	89.08	2.969
hsa_circ_0082688	0.924	<0.0001	0.859-0.989	91.67	84.03	2.471
hsa_circ_0008675	0.806	<0.0001	0.720-0.892	66.67	81.51	2.085
hsa_circ_0082689+hsa_circ_0082688	0.923	<0.0001	0.860-0.987	79.17	95.80	-
hsa_circ_0082689+hsa_circ_0008675	0.902	<0.0001	0.834-0.970	87.50	85.71	-
hsa_circ_0082688+hsa_circ_0008675	0.925	<0.0001	0.861-0.988	79.17	96.64	-
hsa_circ_0082689+hsa_circ_0082688 +hsa_circ_0008675	0.922	<0.0001	0.859-0.985	79.17	96.64	-

AUC: area under curve, HC: healthy controls, RA: rheumatoid arthritis, ROC: receiver operating characteristic, SD: standard deviation, SLE: systemic lupus erythematosus, NWS: neprhritis without SLE, SLEDAI: SLE disease activity index, SLE-RI: SLE patients without renal involvement, SLE+RI: SLE with renal involvement.

The aforementioned results have indicated that drug therapy could influence the expression levels of peripheral blood circRNAs. Therefore, we assessed the potential value of peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 for new-onset SLE+RI diagnosis. ROC curves of peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 showed that they had an AUC of 0.913 (95% CI: 0.842-0.984, $p<0.0001$, sensitivity: 87.50%, specificity: 89.08%, cut-off: 2.969), 0.924 (95% CI: 0.859-0.989, $p<0.0001$, sensitivity: 91.64%, specificity: 84.03%, cut-off: 2.471), 0.806 (95% CI: 0.720-0.892, $p<0.0001$, sensitivity: 66.67%, specificity: 81.51%, cut-off: 2.085) to discriminate individuals with new-onset SLE+RI patients from controls (SLE-RI, RA, NWS, HC), respectively (Table IV and Fig. 6C). The logistic regression model showed that combination of hsa_circ_0082688 and hsa_circ_0008675 could provide the better diagnostic accuracy, with the AUC of 0.925 (95% CI: 0.861–0.988, $p<0.0001$), sensitivity of 79.17% and specificity of 96.64% (Table IV and Fig. 6D). The combination of all these three circRNAs (Table IV and Fig. 6D) and any two circRNAs had no improvement in new-onset SLE+RI diagnosis when compared with the aforementioned combination of 2 circRNAs. These results indicated that hsa_circ_0082688-hsa_circ_0008675 may be used as a novel biomarker in the diagnosis of SLE with renal involvement.

hsa_circ_0082689/miRNA, hsa_circ_ 0082688/miRNA, hsa_circ_0008675/miRNA interaction analysis

To confirm the function of hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675, potential miRNA targets of the circRNAs were predicted by aligning with the miRNA response elements (MREs) using miRanda software. The details of the relationships between hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 and above target miRNAs are presented in Figure 7.

Discussion

At present, there was only one paper investigating the circRNAs level in serum from SLE+RI patients (3), no study investigating the circRNAs level in peripheral blood from SLE+RI patients. For the first time, we performed a microarray analysis of dysregulated circRNAs by comparing the transcriptome profiles of peripheral blood from SLE+RI patients, SLE-RI patients and those from HC. Microarray data showed 17 circRNA were upregulated and 13 circRNA were downregulated between three groups. A subsequent validation research showed that hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 in the peripheral blood of SLE+RI patients were significantly higher in SLE+RI patients than in SLE-RI and HC. These information indicated that peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 may participate in the renal involvement of SLE. We also found that peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 were significantly higher in SLE patients than HC, which indicating that peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 may be related to the pathophysiology of SLE. Moreover, our results demostrated that peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 were significantly higher in SLE+RI patients than RA patients and NWS patients. But, the expression of these three circRNAs did not show any remarkable differences between patients with RA and HC, which was consistent with the results in our previous research (18). These results indicated that peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 may be the relatively specific biomarker for SLE+RI. The following ROC curves analysis showed that these validated peripheral blood circRNAs have better diagnostic performance for SLE+RI patients.

Albeit renal biopsy is the gold criteria for diagnosing and defining “severity and activity” of SLE+RI, its invasiveness makes it inapplicable for conventional monitoring (2). As we known, C3/C4, anti-dsDNA and anti-nucleosome not only are the traditional biomarkers of SLE, but also are associated with renal disease activity (2, 25-26). And, this study found that the level of peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 in SLE+RI patients correlated with C3/C4,

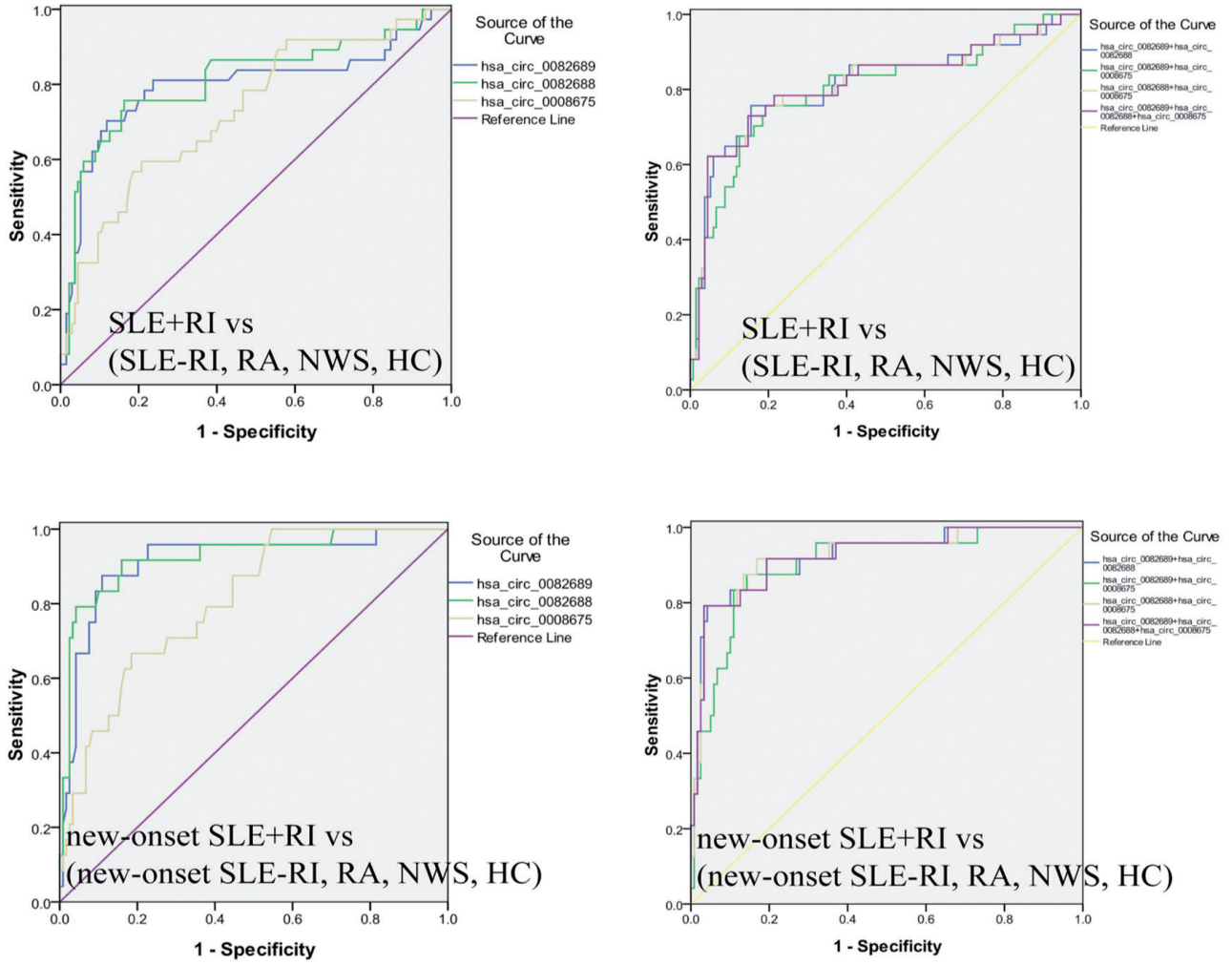

Fig. 6. ROC analysis of peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 from SLE+RI patients.
The AUC separating SLE+RI from the controls (SLE-RI, RA, NWS, HC) was found for hsa_circ_0082688/ hsa_circ_0082689/ hsa_circ_0008675 (**A**) and combination CircRNAs (**B**). The AUC separating new-onset SLE+RI from the controls (new-onset SLE-RI, RA, NWS, HC) was found for hsa_circ_0082688/ hsa_circ_0082689/ hsa_circ_0008675 (**C**) and combination CircRNAs (**D**).

anti-dsDNA, anti-nucleosome, while theirs level did not correlate with 24-hour proteinuria, which is not necessarily related with histological index activity changes in renal involvement of SLE and cannot be considered the most reliable marker of renal involvement of SLE severity or activity, in spite of its correlation with the eventual renal outcome (27). Although theirs level did not correlate with 24-hour proteinuria, proteinuria, haematuria, pyuria, rSLEDAI and SLEDAI. Considering the correlation between the level of peripheral blood hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0008675 and renal disease activity (C3/C4, anti-dsDNA and anti-nucleosome), our results indicate that peripheral blood hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 are potential biomarker of the activity of renal involvement in patients with SLE+RI.

Furthermore, we found that the expression level of peripheral blood hsa_circ_0082688 in new-onset SLE+RI patients was significantly higher than re-visiting SLE+RI patients and the expression level of peripheral blood hsa_circ_0008675 decreased following treatment when compared with those prior to treatment. These results showed that peripheral blood hsa_circ_0082688 and hsa_circ_0008675 are associated with disease severity of SLE+RI, indicating peripheral blood hsa_circ_0082688 and hsa_circ_0008675 are potential biomarker of monitoring treatment in patients with SLE+RI. In addition, in this study, we found that the peripheral blood hsa_circ_0082688-hsa_circ_0008675 combination model had an AUC of 0.814 to distinguish SLE+RI patients from controls (SLE-RI, RA, NWS, HC) with high sensitivity and specificity. Moreover, peripheral blood hsa_circ_0082688-hsa_circ_0008675 combination model was found have the more better potential to discriminate new-onset SLE+RI and controls (SLE-RI, RA, NWS, HC) with AUC=0.925, sensitivity=79.17, specificity=96.64, indicating its high potential as a non-invasive diagnostic biomarker and treatment for SLE+RI.

However, several limitations in this study should be acknowledged. First is the relatively small sample size and this study is limited by the patients from only one hospital, which may restrict the generalisability of our results. Second, we did not analyse circRNAs

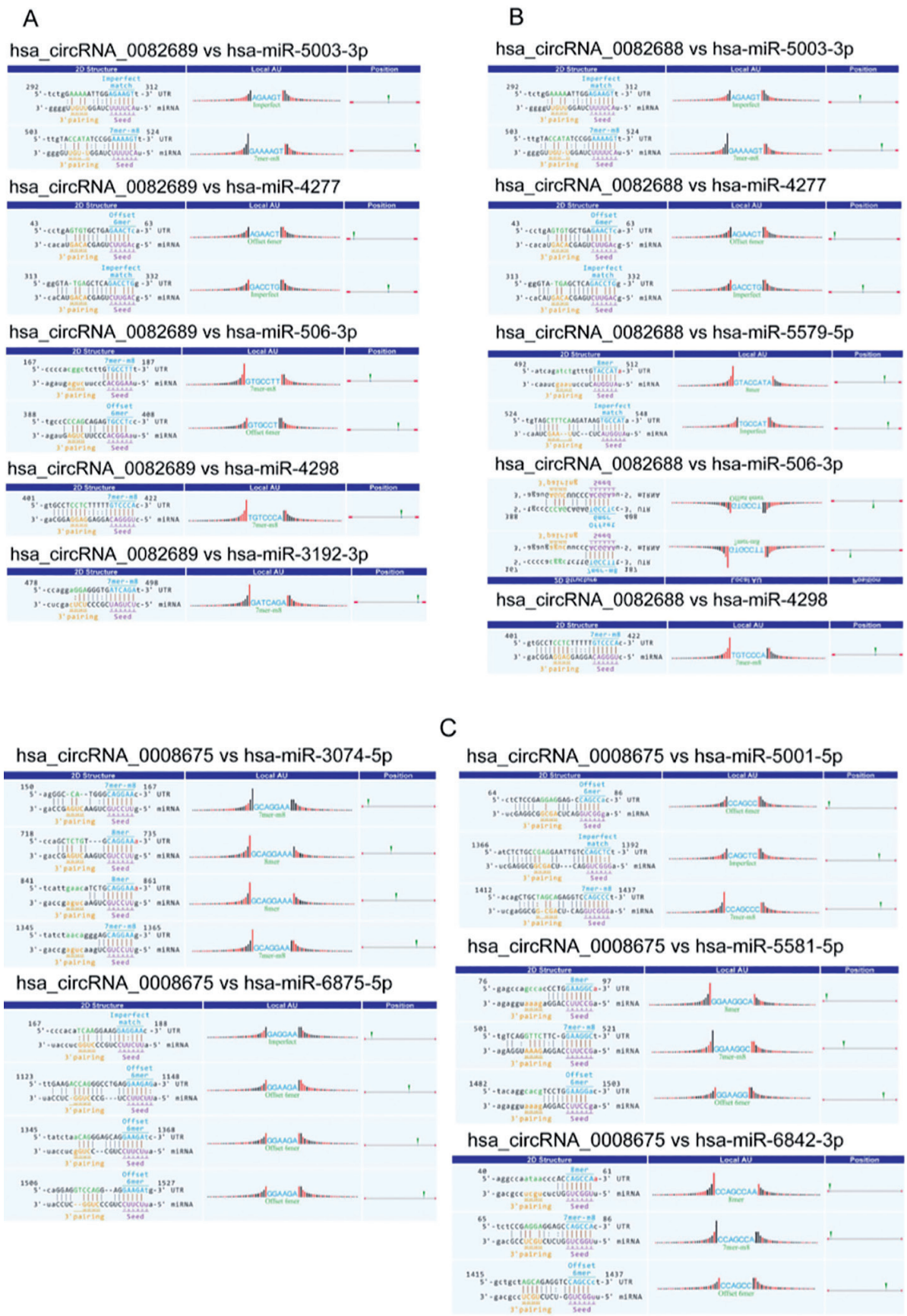

Fig. 7. The detailed notes for hsa_circ_0082689/miRNA, hsa_circ_0082688/miRNA, hsa_circ_0008675/miRNA interaction. Hsa_circ_0082689/miRNA (**A**), hsa_circ_0082688/miRNA (**B**), hsa_circ_0008675/miRNA (**C**) interaction.

in the plasma or serum. Third, due to the lack of data on C3/C4/anti-dsdna/anti-nucleosome in HC, we were unable to evaluate the values of circRNAs plus C3/C4/anti-dsdna/anti-nucleosomein SLE+RI diagnosis. Fourth, we did not analyse the role of hsa_circ_0082689, hsa_circ_0082688 and hsa_circ_0008675 in the pathogenesis of SLE+RI, thus functional researches are required.

It is well known that circRNAs could function as microRNA sponges and regulate target genes to change the SLE development. For instance, Zhang and his colleagues have demonstrated that hsa_circ_0012919 was the competitive endogenous RNA (ceRNA) for miR-125a-3p, which regulated DNA methylation of CD11a and CD70 in $CD4^+$ T cells of SLE (14). Wang's team quantified that CircIBTK inhibits DNA demethylation and activation of AKT signalling pathway via miR-29b in peripheral blood mononuclear cells in SLE (28). And, the research of Li's team indicated that hsa_circ_0045272 acted as a sponge of hsa-miR-6127 in SLE (29). Bioinformatics identified that hsa-miR-5003-3p, hsa-miR-4277, hsa-miR-506-3p and hsa-miR-4298 might be potential common targets of hsa_circRNA_0082689 and hsa_circRNA_0082688. In addition, hsa-miR-3192-3p and hsa-miR-5579-5p may be potential other targets of hsa_circRNA_0082689 and hsa_circRNA_0082688, respectively. hsa_circRNA_0008675 was indicated to potentially bind hsa-miR-3074-5p, hsa-miR-6875-5p, hsa-miR-5001-5p, hsa-miR-5581-5p and hsa-miR-6842-3p. However, the precise functions of the circRNA-miRNA axis in SLE+RI require further investigation.

In conclusion, the current study firstly measured the circRNA expression in peripheral blood from SLE+RI patients, from SLE-RI patients and from HC using circRNAs microarray, and depicted that dysregulated circRNAs associated with SLE+RI. In addition, we found that the combination of peripheral blood hsa_circ_0082688 and hsa_circ_0008675 could be used as novel, non-invasive biomarkers for SLE+RI diagnosis and treatment. The precise molecular mechanisms underlying circRNA functions in SLE+RI still require further investigation.

Acknowledgements

We would like to acknowledge the help given by Dr Rui Wu at the Department of Rheumatology, the First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China.

References

1. DI BATTISTA M, MARCUCCI E, ELEFANTE E *et al*.: One year in review 2018: systemic lupus erythematosus. *Clin Exp Rheumatol* 2018; 36: 763-77.
2. SOLIMAN S, MOHAN C: Lupus nephritis biomarkers. *Clin Immunol* 2017; 185: 10-20.
3. OUYANG Q, HUANG Q, JIANG Z *et al*.: Using plasma circRNA_002453 as a novel biomarker in the diagnosis of lupus nephritis. *Mol Immunol* 2018; 101: 531-8.
4. ABUJAM B, CHEEKATLA S, AGGARWAL A: Urinary CXCL-10/IP-10 and MCP-1 as markers to assess activity of lupus nephritis. *Lupus* 2013; 22: 614-23.
5. BARRETT SP, WANG PL, SALZMAN J: Circular RNA biogenesis can proceed through an exon-containing lariat precursor. *Elife* 2015; 4: e07540.
6. LI Z, HUANG C, BAO C *et al*.: Exon-intron circular RNAs regulate transcription in the nucleus. *Nat Struct Mol Biol* 2015; 22: 256-64.
7. JECK WR, SORRENTINO JA, WANG K *et al*.: Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 2013; 19: 141-57.
8. MEMCZAK S, JENS M, ELEFSINIOTI A *et al*.: Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 2013; 495: 333-8.
9. LIN X, LO HC, WONG DT *et al*.: Noncoding RNAs in human saliva as potential disease biomarkers. *Front Genet* 2015; 6: 175.
10. MEMCZAK S, PAPAVASILEIOU P, PETERS O *et al*.: Identification and characterization of circular RNAs as a new class of putative biomarkers in human blood. *PLoS One* 2015; 10: e0141214.
11. BURD CE, JECK WR, LIU Y *et al*.: Expression of linear and novel circular forms of an INK4/ARF-associated non-coding RNA correlates with atherosclerosis risk. *PLoS Genet* 2010; 6: e1001233.
12. HANSEN TB, KJEMS J, DAMGAARD CK: Circular RNA and miR-7 in cancer. *Cancer Res* 2013; 73: 5609-12.
13. CHEN LY, ZHI Z, WANG L *et al*.: NSD2 circular RNA promotes metastasis of colorectal cancer by targeting miR-199b-5p-mediated DDR1 and JAG1 signalling. *J Pathol* 2019; 248: 103-15.
14. ZHANG C, WANG X, CHEN Y *et al*.: The down-regulation of hsa_circ_0012919, the sponge for miR-125a-3p, contributes to DNA methylation of CD11a and CD70 in $CD4^+$ T cells of systemic lupus erythematous. *Clin Sci* (Lond) 2018; 132: 2285-98.
15. ZHAO Z, LI X, JIAN D *et al*.: Hsa_circ_0054633 in peripheral blood can be used as a diagnostic biomarker of pre-diabetes and type 2 diabetes mellitus. *Acta Diabetol* 2017; 54: 237-45.
16. ZHAO Z, LI X, GAO C *et al*.: Peripheral blood circular RNA hsa_circ_0124644 can be used as a diagnostic biomarker of coronary artery disease. *Sci Rep* 2017; 7: 39918.
17. ZHANG MY, WANG JB, ZHU ZW *et al*.: Differentially expressed circular RNAs in systemic lupus erythematosus and their clinical significance. *Biomed Pharmacother* 2018; 107: 1720-27.
18. LUO Q, ZHANG L, LI X *et al*.: Identification of circular RNAs hsa_circ_0044235 in peripheral blood as novel biomarkers for rheumatoid arthritis. *Clin Exp Immunol* 2018; 194: 118-24.
19. HOCHBERG MC: Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 1725.
20. LUO Q, HUANG Z, YE J *et al*.: PD-L1-expressing neutrophils as a novel indicator to assess disease activity and severity of systemic lupus erythematosus. *Arthritis Res Ther* 2016; 18: 47.
21. PETRI M, KASITANON N, LEE SS *et al*.: Systemic lupus international collaborating clinics renal activity/response exercise: development of a renal activity score and renal response index. *Arthritis Rheum* 2008; 58: 1784-8.
22. ERKELLER-YÜSEL F, HULSTAART F, HANNET I *et al*.: Lymphocyte subsets in a large cohort of patients with systemic lupus erythematosus. *Lupus* 1993; 2: 227-31.
23. BOMBARDIER C, GLADMAN DD, UROWITZ MB *et al*.: Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum* 1992; 35: 630-40.
24. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Assocaition 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
25. GÓMEZ-PUERTA JA, BURLINGAME RW, CERVERA R: Anti-chromatin (anti-nucleosome) antibodies: diagnostic and clinical value. *Autoimmun Rev* 2008; 7: 606-11.
26. MANSON JJ, MA A, ROGERS P *et al*.: Relationship between anti-dsDNA, anti-nucleosome and anti-alpha-actinin antibodies and markers of renal disease in patients with lupus nephritis: a prospective longitudinal study. *Arthritis Res Ther* 2009; 11: R154.
27. WATSON L, BERESFORD MW: Urine biomarkers in juvenile-onset SLE nephritis. *Pediatr Nephrol* 2013; 28: 363-74.
28. WANG X, ZHANG C, WU Z *et al*.: CircIBTK inhibits DNA demethylation and activation of AKT signaling pathway via miR-29b in peripheral blood mononuclear cells in systemic lupus erythematosus. *Arthritis Res Ther* 2018; 20: 118.
29. LI LJ, ZHU ZW, ZHAO W *et al*.: Circular RNA expression profile and potential function of hsa_circ_0045272 in systemic lupus erythematosus. *Immunology* 2018; 155: 137-49.