

Identification of circular RNAs hsa_circ_0044235 and hsa_circ_0068367 as novel biomarkers for systemic lupus erythematosus

QING LUO^{1*}, LU ZHANG^{2*}, XUE LI², BIQI FU³, YANG GUO¹, ZIKUN HUANG¹ and JUNMING LI¹

¹Department of Clinical Laboratory, The First Affiliated Hospital of Nanchang University;

²Medical College of Nanchang University; ³Department of Rheumatology, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, P.R. China

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Abstract. Circular RNAs (circRNAs) have emerged as novel biomarkers for disease diagnosis. However, the expression profiles and clinical significance of circRNAs in peripheral blood mononuclear cells (PBMCs) from systemic lupus erythematosus (SLE) remain unclear. In the present study, the expression profile of circRNAs in PBMCs from patients with SLE and healthy controls (HCs) was detected by using microarray analysis and verified by reverse transcription-quantitative polymerase chain reaction. A total of 1,603 circRNAs were identified to be significantly aberrantly expressed in PBMCs from patients with SLE. Validation assays in 30 SLE patients and 20 HCs demonstrated that the levels of

hsa_circ_0044235 and hsa_circ_0068367 were significantly decreased in the patients with SLE. Receiver operating characteristic curve analysis suggested that hsa_circ_0044235 and hsa_circ_0068367 were significant for SLE diagnosis. Furthermore, the diagnostic potential of hsa_circ_0044235 and hsa_circ_0068367 for SLE was validated in an independent validation set with 45 patients with SLE, 38 HCs and 30 patients with rheumatoid arthritis. In addition, the level of hsa_circ_0044235 in the PBMCs from patients with SLE were identified to be significantly increased in new-onset SLE patients and in patients who were determined to be positive for anti-double-stranded DNA and anti-ribosomal protein P antibodies. Additionally, the level of a microRNA (miRNA) target of hsa_circ_0044235, hsa-miRNA-892a, was identified to be significantly increased in the PBMCs from patients with SLE. The present study suggested that the dysregulation of circRNAs may serve a role in SLE pathogenesis, and that the levels of hsa_circ_0044235 and hsa_circ_0068367 in PBMC have potential as biomarkers for SLE diagnosis.

Correspondence to: Dr Junming Li or Dr Zikun Huang, Department of Clinical Laboratory, The First Affiliated Hospital of Nanchang University, 17 Yongwai Zheng Jie, Nanchang, Jiangxi 330006, P.R. China
E-mail: lisir361@163.com
E-mail: 491353062@qq.com

*Contributed equally

Abbreviations: anti-dsDNA, anti-double-stranded DNA; anti-ENA, anti-extractable nuclear antigen; anti-nRNP/Sm, anti-nuclear ribonucleoprotein/Smith; anti-RIB-P, anti-ribosomal protein P; anti-Ro52, anti-tripartite motif-containing protein 21; anti-SSA, anti-Sjögren's-syndrome-related antigen A; anti-SSB, anti-Sjögren's-syndrome-related antigen B; anti-Sm, anti-Smith; AUC, area under the curve; C3, complement 3; C4, complement 4; CRP, C-reactive protein; circRNAs, circular RNAs; ESR, erythrocyte sedimentation rate; HCs, healthy controls; IgG, immunoglobulin G; miRNA, microRNA; PBMCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ROC, receiver operating characteristic; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index

Key words: systemic lupus erythematosus, circular RNAs, microarray assay

Introduction

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease characterized by multiple autoantibodies production, immune complex deposits and multiple organ damage (1,2). Early diagnosis and appropriate treatment may prevent serious clinical manifestations in patients with SLE. Although the effects of therapeutic regimens for SLE have made remarkable progress in recent years, the majority of patients experience relapse, due to the ambiguity of pathogenesis (3). Therefore, efforts into investigating the genetic and molecular abnormalities of SLE are urgently required and likely to be crucial for identifying new biomarkers for SLE diagnosis.

Circular RNAs (circRNAs) are a unique form of RNAs, which are composed primarily of transcripts from the exons (4). Compared to linear RNAs, circRNAs have a remarkable characteristic of non-canonical splicing without free 3' or 5' ends (5). This characteristic enables circRNAs to resist degradation by RNase R. In addition to having more stable characteristics, circRNAs often exhibit tissue/developmental stage-specific expression (6-9), which make them

more appropriate to be biomarkers compared with linear RNAs (6,10,11). Previous studies have identified that circRNAs may serve as ‘microRNA (miRNA) sponges’ by sequestering target microRNAs (miRNAs) and regulating RNA-binding proteins to control gene transcription (12,13). Accumulating evidence has indicated that circRNAs may be involved in neurological disorders, atherosclerotic vascular disease risk, prion diseases, cancer and autoimmune disease (14-17), supporting the hypothesis that circRNAs have potential to be new diagnostic and prognostic biomarkers, and novel therapy targets of diseases (18-20). However, little is known about circRNAs in peripheral blood mononuclear cells (PBMCs) in human SLE. The present study aimed to determine whether circRNAs in PBMCs may be used as novel diagnosis biomarkers for SLE.

Materials and methods

Patient variables. A total of 79 patients with SLE were admitted from the First Affiliated Hospital of Nanchang University from November 2016 to August 2018. All cases fulfilled the revised American College of Rheumatology criteria for SLE (21). Disease activity was assessed by the SLE disease activity index (SLEDAI) (22). Patients with SLE were classified into inactive (SLEDAI, 0-9) and active (SLEDAI, ≥ 10) groups, according to SLEDAI score. In the same time period, 62 healthy subjects who had no inflammatory or autoimmune diseases and genetically unrelated to the patients with SLE were selected as healthy controls (HC). A total of 30 rheumatoid arthritis (RA) patients admitted to the First Affiliated Hospital of Nanchang University from November 2016 to August 2018 were used as disease controls. All patients with RA fulfilled the revised American College of Rheumatology criteria for RA (23).

The demographic characteristics of the study population are demonstrated in Table I. In the discovery set, 4 patients with new-onset SLE and 4 sex- and age-matched HCs were registered for microarray analysis. An additional 30 patients with relapsed SLE and 20 HCs were included in a validation testing set for the validation of differentially expressed circRNAs and diagnostic model construction. An independent cohort consisting of 45 SLE patients (13 patients with new-onset SLE), 30 patients with RA and 38 HCs were enrolled in a double validation testing set for clinical evaluation of SLE diagnosis. Among the total 79 patients with SLE, 17 were newly diagnosed SLE patients with no history of immunosuppressive drugs or corticosteroids treatment prior to recruitment. The other patients with SLE were those with relapsed SLE that had been previously diagnosed with SLE and had received treatment prior to recruitment. The characteristics of patients with new-onset and relapsed SLE are summarized in Table SI. In addition, the characteristics of the patients with SLE and HCs in the validation testing set and the characteristics of the patients with SLE, HCs and patients with RA in the double validation testing set are summarized in Tables SII and SIII. The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (approval no. 2014003) and was performed according to the Declaration of Helsinki. Written informed consent was obtained from all participants.

PBMCs preparation and total RNA extraction. PBMCs were isolated from EDTA anticoagulated blood samples from each

patient using Ficoll-Hypaque density gradients (Sigma-Aldrich; Merck KGaA) at 25°C. The cells were frozen in TRIzol® (Thermo Fisher Scientific, Inc.) at a concentration of 10⁶/ml and stored at -80°C. Following extraction of total RNA from PMBCs with TRIzol® reagent according to the manufacturer's protocol, the determination of RNA quantity and quality was assessed by absorbance spectrometry, measuring the absorbance ratios of A260/A280 and A260/A230 using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

Microarray analysis. Sample labeling and array hybridization were executed according to the manufacturer's protocol (Arraystar Inc.). The specific protocol was described previously by Ouyang *et al.* (24). The microarray analysis was performed by KangChen BioTech, Co., Ltd.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. cDNA was acquired by reverse transcription using a PrimeScript™ RT reagent kit (Takara Bio, Inc.). The relative expression of circRNAs was determined on an ABI 7500 Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR® Premix Ex Taq™ II (Takara Bio, Inc.), with the following PCR thermocycler protocol: Initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec (denaturation), 60°C for 1 min (annealing and elongation) and 72°C for 2 min (final extension). The primers sequences are listed in Table SIV. β -actin was used as an internal control and the relative expression of circRNAs were analyzed using the 2^{- $\Delta\Delta C_q$} method (25) normalized to the internal control, with $\Delta C_t = C_{t_{\text{target}}} - C_{t_{\text{reference}}}$.

Serum immunoglobulin G (IgG), complement 3 (C3), complement 4 (C4), C-reactive protein (CRP), autoantibody, erythrocyte sedimentation rate (ESR), and routine urine and blood analysis. The levels of serum C3, IgG, C4 and CRP were detected by nephelometry methods according to the manufacturer's protocol (IMMUNE800; Beckman Coulter, Inc.). Anti-extractable nuclear antigens (ENAs) antibodies including anti-Sjögren's-syndrome-related antigen A (anti-SSA), anti-Sjögren's-syndrome-related antigen B (anti-SSB), anti-tripartite motif-containing protein 21 (anti-Ro52), anti-Smith (anti-Sm), anti-nuclear ribonuclear protein/Smith (anti-nRNP/Sm), anti-ribosomal protein P (anti-RIB-P), and anti-nucleosome antibody were determined using an immunoenzyme dot assay (Euroimmun AG) according to the manufacturer's protocol. The results of the anti-ENAs detection were presented as negative (-) and positive (+; ++; +++) by EuroBlot One (Euroimmun AG).

The serum levels of anti-dsDNA were determined using a commercially available anti-dsDNA Enzyme Immunoassay Test kit (cat. no. ED180401; Shanghai Kexin Biotech Co., Ltd.) with the following protocol: Blood samples (5 ml) were collected from patients with SLE in a tube without anticoagulant and centrifuged at 2,000 x g for 10 min at normal temperature. The supernatants were carefully collected and stored at 80°C until use. Subsequently, the serum was used to detect the level of anti-dsDNA according to the manufacturer's protocol. Sodium citrate anti-coagulated blood, urine and K2-EDTA anti-coagulated blood were used to detect ESR, and analyze

Table I. Demographic characteristics of the study population.

Study set	Categories	SLE	HC	RA
Discovery	n	4	4	-
	Females, n (%)	4 (100.00)	4 (100.00)	-
	Age, mean (SD), years	30.50±17.53	34.00±7.21	-
	SLEDAI score, mean (SD)	10.25±6.76	-	-
Validation testing	n	30	20	-
	Females, n (%)	29 (96.67)	17 (85.00)	-
	Age, mean (SD), years	40.47±11.78	35.15±9.03	-
	SLEDAI score, mean (SD)	7.43±6.50	-	-
Double validation testing	n	45	38	30
	Females, n (%)	40 (89.79)	32 (85.71)	25 (83.33)
	Age, mean (SD), years	36.08±16.77	35.94±8.48	53.04±12.41
	SLEDAI score, mean (SD)	9.75±6.50	-	-

HC, healthy controls; RA, rheumatoid arthritis; SLE, Systemic lupus erythematosus; SLEDAI, SLE disease activity index; SD, standard deviation.

routine urine and blood parameters with a LBY-XC40 analyzer (Beijing Pulisheng Biotech Co., Ltd.), URIT-500B analyzer (Guilin Youlite Biotech Co., Ltd.) and Sysmex XE-2100 analyzer (Sysmex Corporation), respectively. Urine routine parameters included pH, nitrite, glucose, vitamin C, specific gravity, occult blood, proteinuria, bilirubin, urobilinogen, ketone body, leucocyte lipase, cylindruria, hematuria, pyuria and crystalluria. Blood routine parameters included white blood cell count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cells distribution width, platelet count, mean platelet volume, plateletcrit, platelet distributing width, numbers of lymphocytes, lymphocytes percentage, numbers of monocytes, monocytes percentage, numbers of neutrophils, neutrophils percentage, numbers of eosinophils, eosinophils percentage, numbers of basophil, basophils percentage and platelet large cell ratio.

Annotation for circRNA/miRNA interaction. The circRNA/miRNA interaction was predicted using Arraystar's home-made miRNA target prediction software based on TargetScan (26) and miRanda (27), and the differentially expressed circRNAs within all the comparisons were annotated in detail with the circRNA/miRNA interaction information.

Statistical analysis. All statistical analyses were performed using GraphPad Prism v5.0 software (GraphPad Software, Inc.) and SPSS version 16.0 software (SPSS, Inc.). Differences in circRNAs expression between two groups were analyzed using the Student's t-test or nonparametric Mann-Whitney test. Kruskal-Wallis test followed by Dunn's multiple comparison test was used for statistical analysis between three groups. The correlation analyses were performed using the nonparametric Spearman method. Receiver operating characteristic (ROC) curves were performed to evaluate the diagnostic value of circRNAs. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Dysregulated circRNAs expression profiling. To explore the differentially expressed circRNAs in SLE, PBMCs samples from 4 patients with SLE and 4 age- and sex-matched HC were selected to perform microarray analysis using an Arraystar Human circRNAs Microarray version 2.0. Based on the criteria of a fold change > 2.0 and $P < 0.05$ (Fig. 1A and B), 1,603 circRNAs were differentially expressed between the patients with SLE and HCs, of which the top 30 differently expressed circRNAs (15 were upregulated and 15 were downregulated) are listed in Table SV. Of the 1,603 differentially expressed circRNAs, 838 circRNAs were upregulated and 765 circRNAs were downregulated in the patients with SLE. A heatmap was constructed to group the circRNAs based on their expression levels among the samples (Fig. 1C).

Validation of circRNAs expression. To verify the microarray data, 3 circRNAs (hsa_circ_0068367; hsa_circ_0037274; and hsa_circ_0044235) were selected for validation via RT-qPCR in a training set with 30 patients with SLE and 20 HCs. The 3 selected circRNAs were listed in the top 45 (fold change > 6) downregulated circRNAs, and had been demonstrated to be downregulated in peripheral blood from patients with SLE in our previous research (Luo *et al*, unpublished data). Consistent with the results of the microarray analysis, the levels of hsa_circ_0044235 and hsa_circ_0068367 in the PBMCs of patients with SLE were significantly decreased compared with those of the HCs, while the expression levels of hsa_circ_0037274 did not exhibit any remarkable differences between patients with SLE and the HCs (Fig. 2).

ROC curve analysis. To additionally evaluate the potential of these two differentially expressed circRNAs (hsa_circ_0044235 and hsa_circ_0068367) in SLE diagnosis, ROC curve analysis was performed. ROC curves indicated that the levels of hsa_circ_0044235 and hsa_circ_0068367

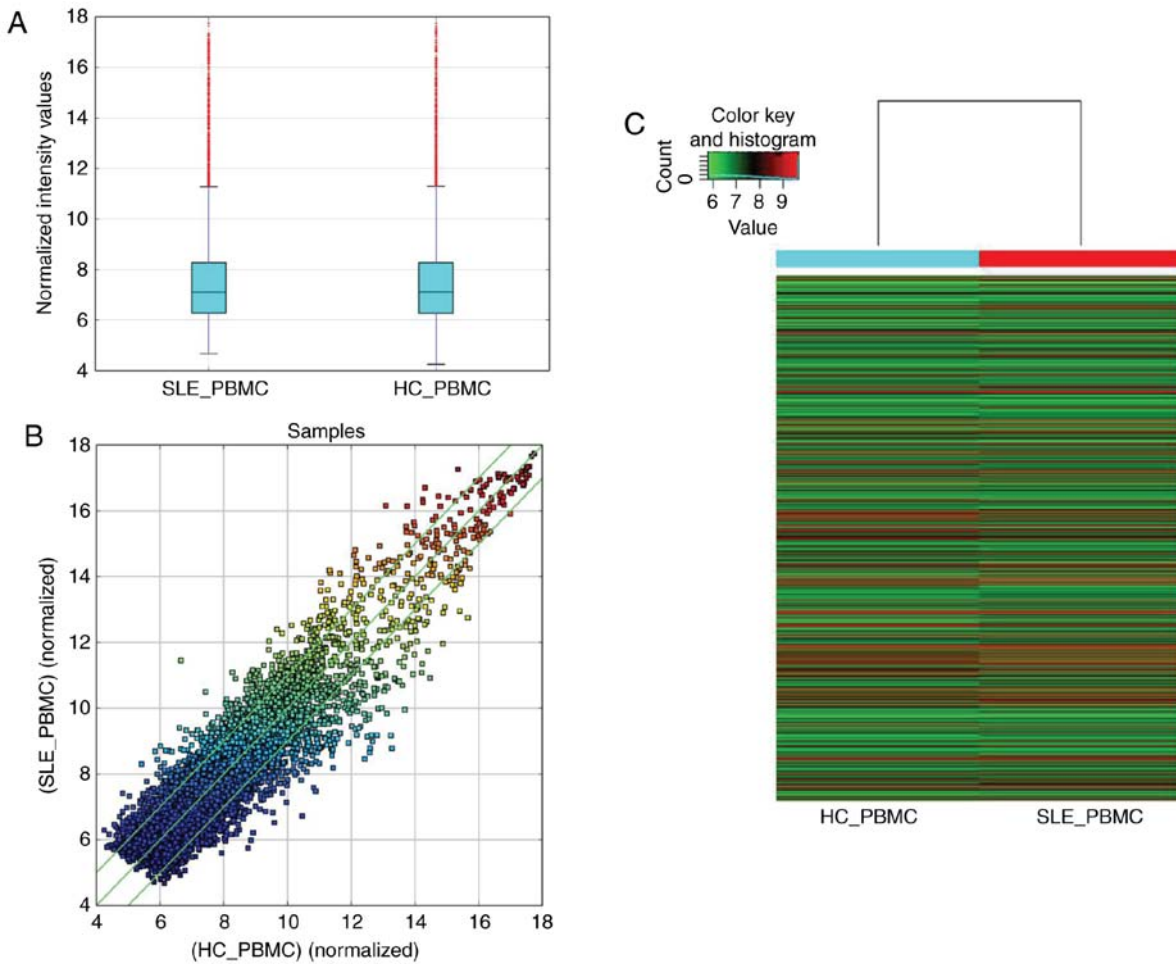


Figure 1. Determination of the circRNA expression profiles in PBMCs from 4 SLE patients and 4 HCs by microarray analysis. (A) Box plot visualization of the distributions of a dataset for the circRNAs profiles. (B) CircRNA scatter plot. Dots above the top green line and below the bottom green line indicate >1.5-fold changes in log-transformed circRNA data between the two compared groups. (C) Heatmap of differentially expressed circRNAs. circRNAs, circular RNAs; HCs, healthy controls; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus.

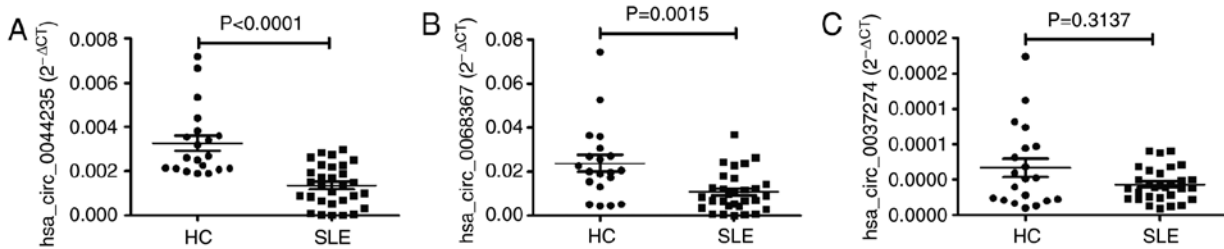


Figure 2. Determination of the relative expression levels of circRNAs in the training set in PBMCs from 30 patients with SLE and 20 HCs by reverse transcription quantitative polymerase chain reaction. (A) The average expression levels of hsa_circ_0044235 in the PBMCs of patients with SLE were significantly decreased compared with those of the HCs. (B) The average expression levels of hsa_circ_0068367 in the PBMCs of patients with SLE were significantly decreased compared with those of the HCs. (C) The expression of hsa_circ_0037274 did not exhibit any significant differences between patients with SLE and the HCs. circRNAs, circular RNAs; HCs, healthy controls; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus.

in PBMCs were able to distinguish between patients with SLE and the HCs. The highest area under the curve (AUC) was hsa_circ_0044235 [AUC = 0.873; 95% confidence interval (CI), 0.778-0.967; P < 0.0001; sensitivity = 70.00%, specificity = 100.00%; Fig. 3A], followed by hsa_circ_0068367 (AUC = 0.768; 95% CI, 0.629-0.907; P = 0.0014; sensitivity = 73.33%; specificity = 80.00%; Fig. 3A). The combined AUC of hsa_circ_0044235 and hsa_circ_0068367 (AUC = 0.876; 95% CI, 0.778-0.967; P < 0.0001; sensitivity = 70.00%;

specificity = 100.00%; Fig. 3B) was identical to the individual hsa_circ_0044235 AUC value (AUC = 0.873).

Double validation of the diagnostic value of differentially expressed circRNAs. To additionally evaluate the value of the aforementioned hsa_circ_0044235-only, hsa_circ_0068367-only and hsa_circ_0044235-hsa_circ_0068367 combination models in SLE diagnosis, an independent validation testing set consisting of 45 patients with SLE, 30 patients with

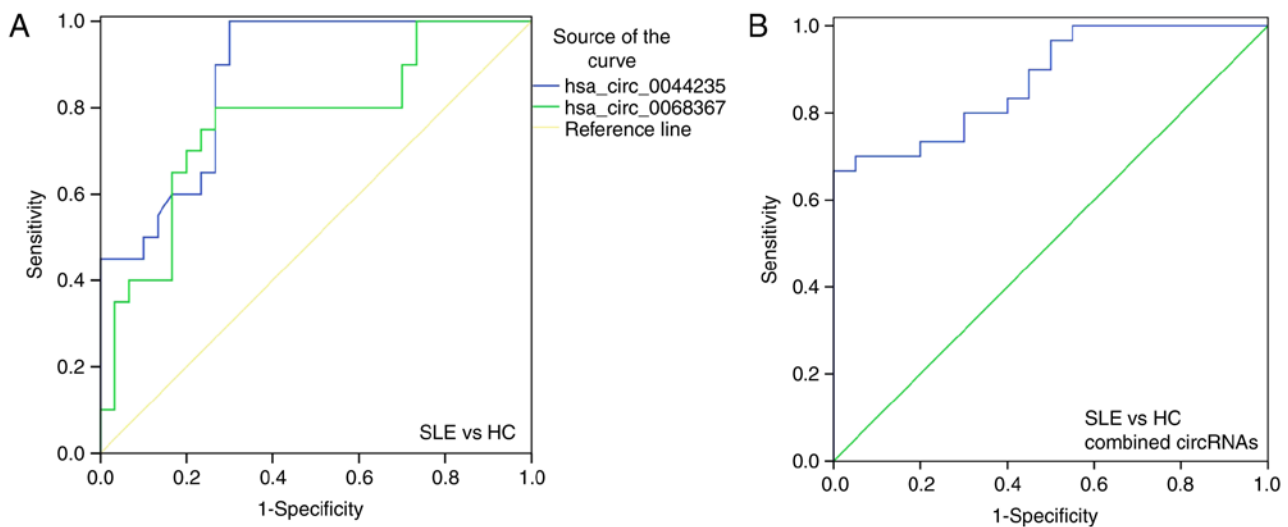


Figure 3. Receiver operating characteristic analysis of confirmed circRNAs in peripheral blood mononuclear cells from patients with SLE. (A) The largest AUC was identified for hsa_circ_0044235, followed by hsa_circ_0068367. (B) The AUC of combined circRNAs (hsa_circ_0044235 + hsa_circ_0068367) was 0.876. CircRNAs, circular RNAs; SLE, systemic lupus erythematosus; AUC, area under the curve.

RA and 38 HCs were enrolled and their circRNAs levels were determined. Similar to the training set, the results demonstrated that the levels of hsa_circ_0044235 and hsa_circ_0068367 were all significantly decreased in the patients with SLE compared with the patients with RA and HCs (all $P < 0.0001$) (Fig. 4A and B). In addition, ROC curves from the patients with SLE and HCs indicated that the AUC values of hsa_circ_0044235, hsa_circ_0068367 and hsa_circ_0044235-hsa_circ_0068367 were 0.861 (95% CI, 0.783-0.939; $P < 0.0001$; sensitivity = 86.67%; specificity = 71.05%), 0.707 (95% CI, 0.592-0.822; $P = 0.0010$; sensitivity = 80.00%; specificity = 60.53%) and 0.860 (95% CI, 0.781-0.938; $P < 0.0001$; sensitivity = 86.67%; specificity = 71.05%), respectively (Fig. 4C).

A risk score analysis based on hsa_circ_0044235 and hsa_circ_0068367 was subsequently performed in patients with SLE and all controls (HCs and patient with RA) (Fig. 4D). The AUC for the risk score based on hsa_circ_0044235 was 0.845 (95% CI: 0.774-0.916; $P < 0.0001$; sensitivity = 66.44%; specificity = 89.71%). The AUC for the risk score based on hsa_circ_0068367 was 0.789 (95% CI, 0.705-0.874; $P < 0.0001$; sensitivity = 82.22%; specificity = 69.12%), and the AUC for the risk score based on the combination of hsa_circ_0044235 and hsa_circ_0068367 was 0.874 (95% CI, 0.809-0.939; $P < 0.0001$; sensitivity = 93.33%; specificity = 66.18%). The risk score also significantly distinguished between the patients with SLE and the disease controls (patients with RA) (Fig. 4E); the AUC based on hsa_circ_0044235 was 0.825 (95% CI, 0.734-0.916; $P < 0.0001$; sensitivity = 64.44%; specificity = 86.67%); the AUC based on hsa_circ_0068367 was 0.893 (95% CI, 0.818-0.968; $P < 0.0001$; sensitivity = 82.22%; specificity = 83.33%); and the AUC based on the combination of hsa_circ_0044235 and hsa_circ_0068367 was 0.903 (95% CI, 0.834-0.972; $P < 0.0001$; sensitivity = 86.67%; specificity = 80.00%).

Association of hsa_circ_0044235 and hsa_circ_0068367 levels in PBMCs with SLE clinical characteristics. To determine whether the hsa_circ_0044235 and hsa_circ_0068367 levels in the PBMCs from patients with SLE reflected the severity

of the disease, analysis was performed to assess the correlation between the clinical features of SLE and the levels of circRNAs (hsa_circ_0044235 and hsa_circ_0068367) in the double validation testing set. The data suggested that the expression levels of all confirmative circRNAs in the PBMCs from patients with SLE were not associated with SLEDAI, CRP, ESR, C3 or C4, which are established biomarkers used to measure the severity and activity of SLE (Table II). However, the level of hsa_circ_0044235 was negatively correlated with the numbers of monocytes ($r_s = -0.33$; $P = 0.02$), and the level of hsa_circ_0044235 was correlated with the level of hsa_circ_0068367 ($r_s = 0.42$; $P < 0.01$; Table II). Following the addition of the data from the validation testing set, the level of hsa_circ_0044235 was identified to be correlated with the numbers of monocytes ($r_s = -0.35$; $P < 0.01$) and the level of hsa_circ_0068367 ($r_s = 0.52$; $P < 0.01$; Table II).

SLE is a prototypical systemic autoimmune disease characterized by the production of autoantibodies, including anti-dsDNA and anti-ENAs. Therefore, the correlations between the levels of circRNAs (hsa_circ_0044235 and hsa_circ_0068367) and anti-dsDNA, anti-Sm, anti-SSB, anti-Ro52, anti-SSA, anti-nRNP/Sm, anti-nucleosome and anti-RIB-P were examined in patients with SLE in the double validation testing set. These analyses demonstrated that the levels of hsa_circ_0044235 were significantly increased in the patients with SLE who were positive for anti-dsDNA and anti-RIB-P antibodies compared with patients who were negative for anti-dsDNA and anti-RIB-P antibodies ($P = 0.0256$ and $P = 0.0186$, respectively; Fig. 5A and B). No marked correlation was observed between the levels of circRNAs (hsa_circ_0044235 and hsa_circ_0068367) and other autoantibodies (data not shown). Following the addition of the data from the validation testing, the levels of hsa_circ_0044235 remained significantly increased in patients with SLE who were positive for anti-dsDNA and anti-RIB-P antibodies compared with patients who were negative for anti-dsDNA and anti-RIB-P antibodies ($P = 0.0058$ and $P = 0.0266$, respectively; Fig. 5C and D).

The levels of circRNAs (hsa_circ_0044235 and hsa_circ_0068367) between patients with new-onset and relapsed

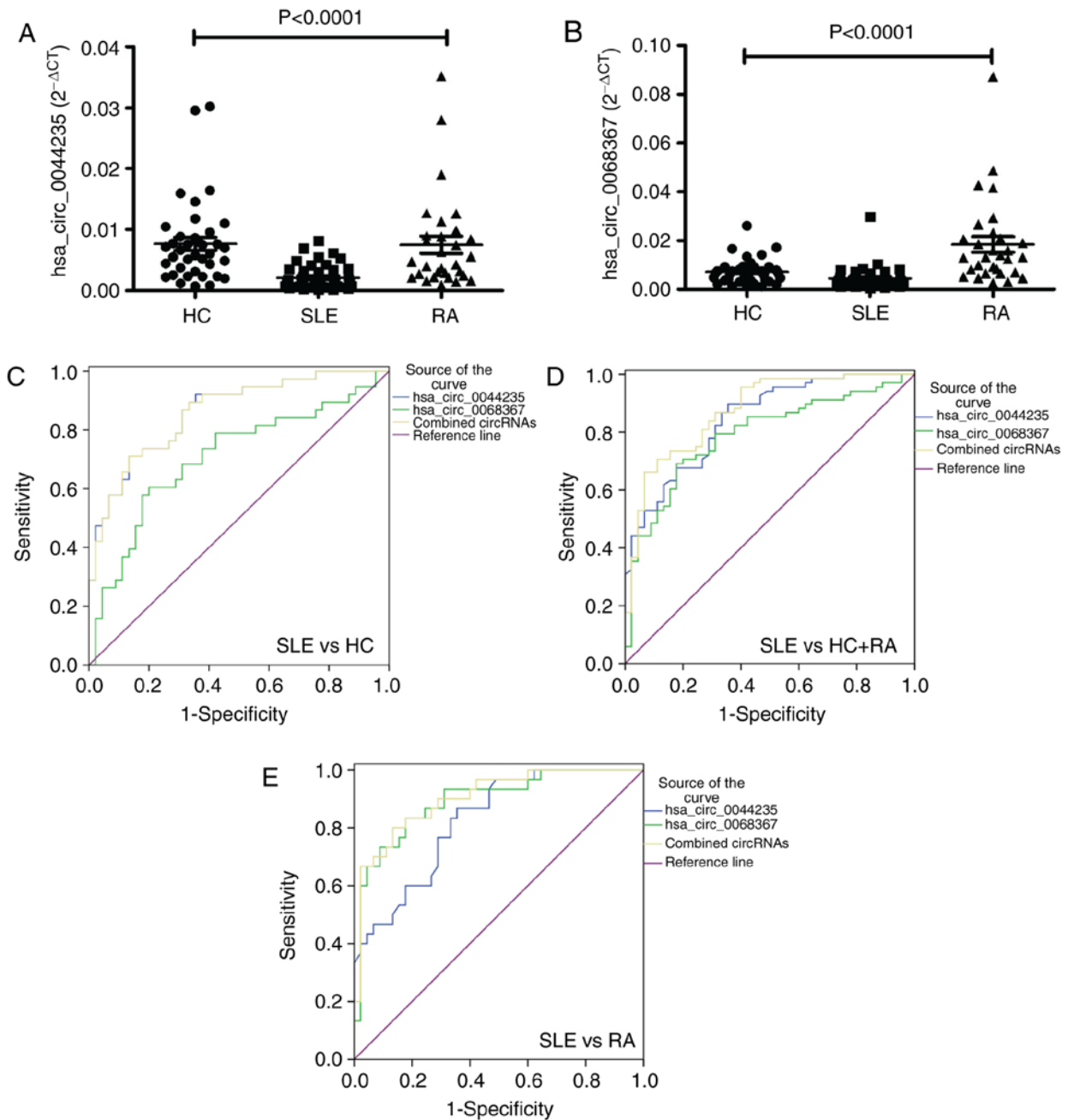


Figure 4. Double validation of the diagnostic value of differentially expressed PBMCs circRNAs. (A) The expression level of hsa_circ_0044235 was markedly decreased in the patients with SLE compared with patients with RA and HCs. (B) The expression level of hsa_circ_0068367 were markedly decreased in the patients with SLE compared with patients with RA and HCs. (C) ROC curve analysis of hsa_circ_0042345 and hsa_circ_0068367 in patients with SLE compared with HCs. (D) ROC curve analysis of hsa_circ_0042345 and hsa_circ_0068367 for the risk-score in SLE patients compared with all controls (HCs and RA). (E) ROC curve analysis of hsa_circ_0042345 and hsa_circ_0068367 for the risk-score in patients with SLE vs. RA. PBMCs, peripheral blood mononuclear cells; CircRNAs, circular RNAs; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; HCs, healthy controls; ROC, receiver operating characteristic.

SLE were also compared. The results demonstrated that the level of hsa_circ_0044235 was significantly increased in patients with new-onset disease compared with patients with relapsed SLE ($P=0.0081$; Fig. 6A), and the level of hsa_circ_0068367 were slightly increased in patients with new-onset disease, but the difference was not statistically significant ($P=0.0523$; Fig. 6B).

Next, the clinical features of patients with SLE, including lupus nephritis, cutaneous manifestations, alopecia, arthritis, effusion, neuropathic lupus and fever were recorded, and the

correlations between these features and the levels of circRNAs (hsa_circ_0044235 and hsa_circ_0068367) were analyzed. The results indicated that no correlation was identified between the clinical features of patients with SLE and the levels of circRNAs (hsa_circ_0044235 and hsa_circ_0068367) (data not shown).

Target miRNA prediction of hsa_circ_0044235 and hsa_circ_0068367. The field of circRNAs research is relatively new, and to the best of our knowledge, no studies have definitively

Table II. Associations of hsa_circ_0044235 and hsa_circ_0068367 levels in PBMCs with SLE clinical characteristics.

A, Double validation testing set					
hsa_circ_0044235			hsa_circ_0068367		
Categories	r _s	P-value	Categories	r _s	P-value
ESR	0.04	0.79	ESR	0.07	0.64
CRP	-0.12	0.46	CRP	-0.13	0.41
IgG	0.16	0.29	IgG	0.07	0.64
C3	-0.23	0.14	C3	-0.17	0.27
C4	-0.27	0.07	C4	-0.12	0.46
WBC	-0.16	0.30	WBC	-0.06	0.69
RBC	-0.07	0.64	RBC	0.02	0.90
HGB	-0.09	0.56	HGB	0.02	0.91
HCT	-0.07	0.64	HCT	0.03	0.84
PLT	-0.12	0.45	PLT	-0.07	0.64
Neutrophils	-0.09	0.57	Neutrophils	-0.12	0.45
Lymphocytes	-0.19	0.22	Lymphocytes	0.02	0.91
Monocytes	-0.33	0.02	Monocytes	-0.21	0.17
SLEDAI	-0.01	0.95	SLEDAI	0.10	0.52
hsa_circ_0068367	0.42	<0.01	hsa_circ_0044235	0.42	<0.01

B, Double validation testing and validation testing sets

hsa_circ_0044235			-	-	-
Categories	r _s	P-value	-	-	-
Monocytes	-0.35	<0.01	-	-	-
hsa_circ_0068367	0.52	<0.01	-	-	-

C3, complement 3; C4, complement 4; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HCT, hematocrit; HGB, hemoglobin; IgG, immunoglobulin G; PBMCs, Peripheral blood mononuclear cells; PLT, platelets; RBC, red blood cell; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; WBC, white blood cell.

demonstrated the bio-function of hsa_circ_0044235 and hsa_circ_0068367. It has been suggested that the miRNA response elements (MREs) of circRNAs may bind target miRNA, and thereby decrease miRNA-mediated post-transcriptional repression. To determine the potential functions of hsa_circ_0044235 and hsa_circ_0068367, target miRNA were predicted in the present study by aligning prospective target miRNA with the MREs of differentially expressed circRNAs using Arraystar's home-made miRNA target prediction software. For hsa_circ_0044235, 3 putative miRNAs targets (hsa-miR-892a; hsa-miR-135b-5; and hsa-miR-135a-5p) were identified. For hsa_circ_0068367, 3 putative miRNAs targets (hsa-miR-136-5p; hsa-miR-501-5p; and hsa-miR-5696) were identified. These putative miRNAs targets of hsa_circ_0044235 and hsa_circ_0068367 were detected in PBMCs from SLE and HC. It was identified that the levels of hsa-miR-135a-5p and hsa-miR-135b-5p did not exhibit any remarkable differences between patients of SLE and HC (Fig. 7A and B), while the level of hsa-miR-892a was increased significantly in PBMCs from patients of SLE compared with HC (Fig. 7C), suggesting that

hsa_circ_0044235 may serve a role in SLE by interacting with hsa-miR-892a. In addition, the present study identified that the level of hsa-miR-136-5p was decreased significantly in PBMCs from the patients with SLE compared with the HCs (Fig. 7D), whereas the level of hsa-miR-501-5p was not remarkably different between the patients with SLE and the HCs (Fig. 7E). However, the level of hsa-miR-5696 in PBMCs from the patients with SLE was not detectable. Due to the decreased level of hsa_circ_0068367 in the patients with SLE, hsa_circ_0068367 may serve a role in SLE, by not as hsa-miR-136-5p, hsa-miR-501-5p or hsa-miR-5696 sponges.

Discussion

SLE is a systemic autoimmune disease with indistinct etiology. The clinical manifestations in patients with SLE are heterogeneous and their disease progressions are unpredictable, which has prompted studies to explore novel biomarkers to achieve improved diagnoses and prognostic monitoring. CircRNAs are a special class of endogenous RNAs and generally expressed

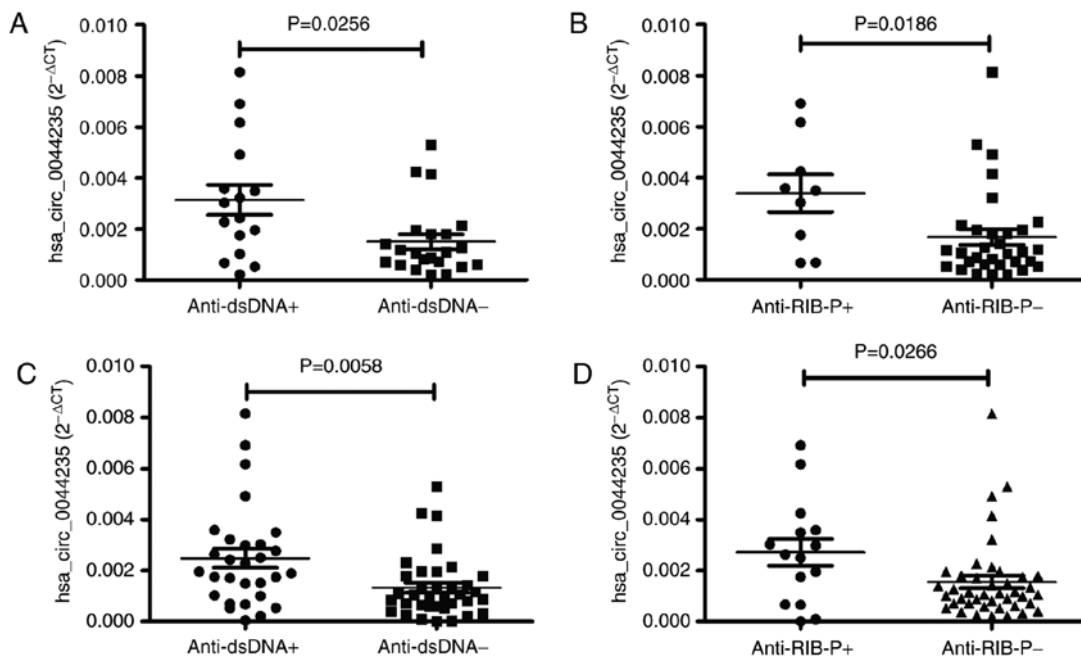


Figure 5. Correlations between the expression of hsa_circ_0044235 with autoantibodies. (A) The levels of hsa_circ_0044235 were significantly increased in patients who were positive for anti-dsDNA antibodies compared with patients who were negative for anti-dsDNA antibodies in the double validation testing set. (B) The levels of hsa_circ_0044235 was significantly increased in patients who were positive for anti-dsDNA antibodies compared with patients who were negative for anti-RIB-P antibodies in the double validation testing set. (C) The levels of hsa_circ_0044235 were significantly increased in patients who were positive for anti-dsDNA antibodies positive compared with patients who were negative for anti-dsDNA antibodies in the double validation and validation testing sets. (D) The levels of hsa_circ_0044235 were significantly increased in patients who were positive for anti-RIB-P antibodies compared with patients who were negative for anti-RIB-P antibodies in the double validation and validation testing sets. Anti-dsDNA, anti-double-stranded DNA; RIB-P, ribosomal protein P.

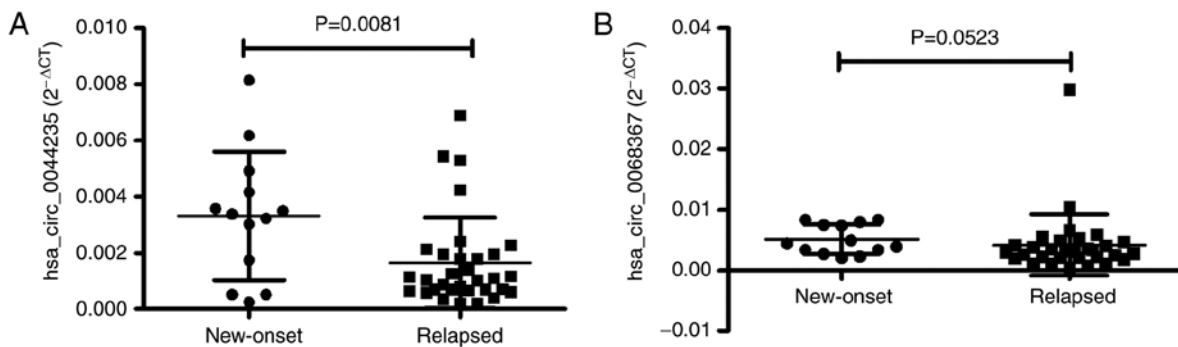


Figure 6. Comparison of the levels of circRNA (hsa_circ_0044235 and hsa_circ_0068367) between patients with new-onset and relapsed SLE conditions. (A) The level of hsa_circ_0044235 was significantly increased in patients with new-onset disease. (B) The level of hsa_circ_0068367 appeared to be increased in the patients with new-onset disease, but the difference was not significant. circRNA, circular RNA; SLE, systemic lupus erythematosus.

in human cells. It has been demonstrated that the expression levels of circRNAs may be ≥ 10 -fold compared with their corresponding linear isomers (11). In addition, circRNAs are more stable compared with long noncoding RNAs and miRNAs in mammalian cells (28). These characteristics give circRNAs the potential to be well-suited biomarkers for human diseases. Shang *et al* (29) identified that hsa_circ_0005075 may be used as a diagnostic biomarker for hepatocellular carcinoma and that the expression level of hsa_circ_0005075 was correlated with tumor size. Zhang *et al* (30) suggested that circ_101222 may be used as a predictive biomarker for pre-eclampsia. Ouyang *et al* (17) described 12 circRNAs that were differentially expressed in PBMCs from patients with RA, and demonstrated that circRNA_104871 may be used to diagnose

RA. Li *et al* (31) described the comprehensive expression profiles of circRNAs in plasma from patients with SLE and initiated the development of circRNAs as novel non-invasive biomarkers for SLE disease. However, the global expression profile of patients with SLE and the clinical significance of circRNAs in PBMCs from patients with SLE remain unknown.

To the best of our knowledge, the present study was the first to characterize the expression profiles of circRNA by comparing the transcriptome profiles of PBMCs from patients with SLE and those from HCs using circRNAs microarray analysis. When compared with HCs, the microarray data indicated a total of 1,603 circRNAs that were significantly dysregulated in patients with SLE. By using circRNAs sequencing, Wang *et al* (32) and Miao *et al* (33) also identified

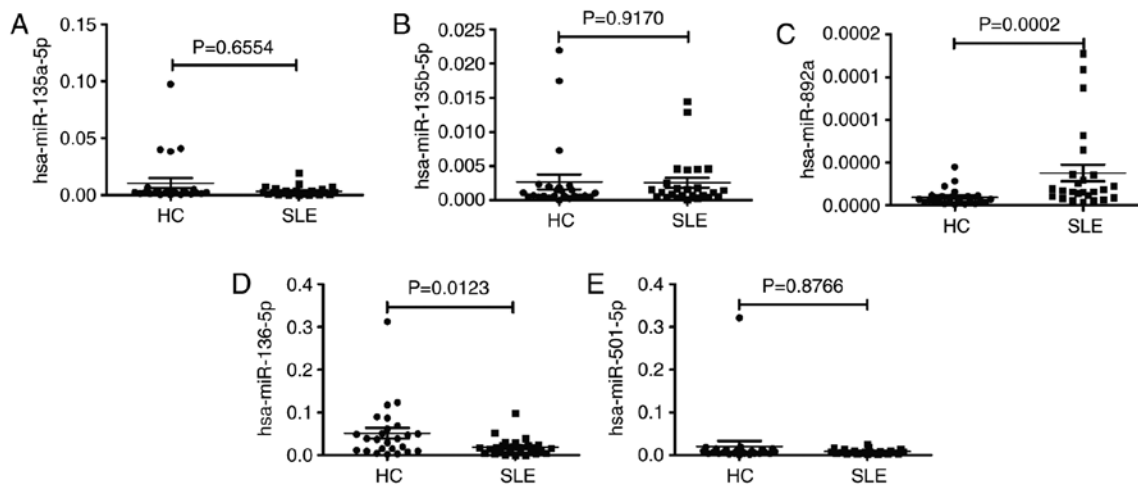


Figure 7. Comparison of the levels of miRNAs in PBMCs between patients with SLE and HCs. (A) The level of hsa-miR-135a-5p was not markedly different between patients with SLE and HCs. (B) The level of hsa-miR-135b-5p was not markedly different between patients with SLE and HCs. (C) The level of hsa-miR-892a was increased significantly in the PBMCs from patients with SLE compared with the HCs. (D) The level of hsa-miR-136-5p was decreased significantly in the PBMCs from patients with SLE compared with the HCs. (E) The level of hsa-miR-501-5p was not markedly different between the patients with SLE and HCs. miRNAs/miR, microRNAs; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus; HCs, healthy controls.

a number of differently expressed circRNAs in the PBMCs of patients with SLE. These data may assist future pathophysiology studies in SLE and assist in determining whether circRNAs in PBMCs may be used as novel non-invasive biomarkers for SLE diagnosis.

In the present study, hsa_circ_0044235 and hsa_circ_0068367 were confirmed to be significantly down-regulated in the PBMCs of patients with SLE. It was also identified that the levels of hsa_circ_0044235 and hsa_circ_0068367 in the PBMCs of the patients with SLE were not correlated with SLEDAI, CRP, ESR, C3 or C4, indicating that these circRNAs may not be relevant biomarkers for disease severity, disease activity or systemic inflammation in SLE. However, it was revealed that the level of hsa_circ_0044235 were associated with anti-dsDNA and anti-RIB-P. Notably, the level of hsa_circ_0044235 was significantly increased in patients with new-onset disease compared with patients with relapsed conditions, indicating that hsa_circ_0044235 may serve a role in SLE pathogenesis. However, it was demonstrated that the level of hsa_circ_0044235 was correlated with the level of hsa_circ_0068367 in patients with SLE, but no correlation was identified between the levels of hsa_circ_0044235 and hsa_circ_0068367 in HC. Therefore, we hypothesize that hsa_circ_0044235 may interact with hsa_circ_0068367 directly or indirectly in patients with SLE, although future studies are required to verify this hypothesis.

Evidence from Zhang *et al* (34) have indicated that circRNAs in PBMCs may be potential biomarkers for SLE. To explore whether hsa_circ_0044235 and hsa_circ_0068367 in PBMCs may be used as diagnostic biomarkers for SLE, their expression levels were confirmed in a validation testing set and a double validation testing set by ROC curves. In the validation testing set, hsa_circ_0044235 had an AUC value of 0.873, hsa_circ_0068367 had an AUC value of 0.768 for SLE diagnosis, and the combination of hsa_circ_0044235 and hsa_circ_0068367 had an AUC value of 0.876. In the double validation testing set, these circRNAs yielded parallel AUC values in discriminating between the patients with SLE and

the HCs, indicating that the levels of hsa_circ_0044235 and hsa_circ_0068367 in PBMCs have potential diagnostic value for SLE. These diagnostic efficacies for SLE were quite comparable to those previously described circRNAs (24,32,34). In addition, the efficacies of hsa_circ_0044235 and hsa_circ_0068367 in distinguishing SLE from other autoimmune disease (RA) were assessed. The risk score demonstrated that hsa_circ_0044235 and hsa_circ_0068367 also distinguished between the patients with SLE and patients with RA.

The principal types of PBMCs are lymphocytes and monocytes. In the present study, the numbers of lymphocytes and monocytes were compared between the patients with SLE and the HCs, and it was identified that the numbers of lymphocytes were significantly decreased in SLE group ($P < 0.05$); no difference in the numbers of monocytes between SLE and HCs groups was observed. In addition, the correlation between the levels of circRNAs and the numbers of monocytes and the numbers of lymphocytes was determined. The results indicated that the level of hsa_circ_004235 was negatively correlated with the number of monocytes, suggesting that the difference in circRNAs expression was not the result of changes in the number of lymphocytes or monocytes in the PBMCs of patients with SLE.

Increasing evidence has demonstrated that circRNAs may perform biological functions by binding to target miRNAs. Li *et al* (35) suggested that hsa_circ_0045272 served an important role in pathogenesis of SLE by regulating the level of miR-6127 via directly binding. The present study revealed that hsa_circ_0044235 and hsa_circ_0068367 were significantly decreased in PBMCs of patients with SLE. To explore the potential roles of hsa_circ_0044235 and hsa_circ_0068367 in SLE, bioinformatics was employed in the present study to predict the potential target miRNAs, and 3 putative target miRNAs were obtained respectively for both hsa_circ_0044235 and hsa_circ_0068367. Among these 6 putative target miRNAs, hsa-miR-136-5p and hsa-miR-501-5p, putative targets of hsa_circ_006837, have been demonstrated to be associated with SLE (36,37). The functions of the other 4 miRNAs (hsa-miR-892a; hsa-miR-135b-5p; hsa-miR-135a-5p; and

hsa-miR-5696) have not been described previously, to the best of our knowledge. The levels of these putative target miRNAs in the PBMCs from patients with SLE and HCs were then detected and compared. The data demonstrated that the level of hsa-miR-892a in PBMCs was increased significantly in the patients with SLE, suggesting that hsa_circ_0044235 may serve a role in SLE by interacting with hsa-miR-892a. As the levels of hsa-miR-136-5p and hsa_circ_0068367 in PBMCs were both decreased in patients with SLE, the role of hsa_circ_0068367 in SLE may not be achieved by binding hsa-miR-136-5p. Nevertheless, the exact mechanisms of hsa_circ_0044235 and hsa_circ_0068367 in SLE require additional investigation.

Several limitations of the present study should be acknowledged. Firstly, the sample size of the patients with new-onset SLE was relatively small, and the samples were sourced from only one hospital, which may limit the universality of the results. Secondly, circRNAs were not detected in the serum or plasma of patients with SLE. Previous data have indicated that exonic circRNAs in serum are not stable, with a half-life of less than 15 sec (13), and that the circRNAs in serum constitute only a fraction of the total RNA. Thirdly, the specific role of circRNAs in SLE pathogenesis were not fully explored.

In conclusion, differentially expressed circRNAs were detected in PBMCs of patients with SLE. The data indicated that the levels of hsa_circ_0044235 and hsa_circ_0068367 levels in the PBMCs of patients with SLE were decreased and may represent novel biomarkers for SLE diagnosis. However, the molecular mechanisms and specific functions of these circRNAs in SLE require additional investigation.

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Availability of data and materials

The data used to support the results of this study are available from the corresponding author upon request.

Authors' contributions

QL, LZ and XL performed the experiments. BF, YG, ZH and JL analyzed and interpreted the data. QL and JL made substantial

contributions to the design supervision of the present study, and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was authorized by the Ethics Committee of the First Affiliated Hospital of Nanchang University. All participants provided written informed consent prior to the initiation of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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