

Identification of circular RNAs hsa_circ_0044235 in peripheral blood as novel biomarkers for rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a chronic debilitating systemic autoimmune disease characterized by inflammation and destruction of the joints. Approximately 1% of the population suffers from RA, and many patients develop long-term joint damage, severe illness and disability [1]. Early diagnosis and proper treatment can relieve the pathogenetic condition effectively in RA patients. Current diagnostic methods, including American College of Rheumatology (ACR) classification criteria, show various disadvantages for the early diagnosis of RA. Therefore, new biomarkers aiming to improve the diagnosis and prognosis analysis of RA will be highly valuable.

Although great efforts have been made [2–5], the detailed mechanisms of RA pathogenesis and development are still unknown. Thus, in-depth study on the genetic and

Summary

Circular RNAs (circRNAs) are a new class of RNAs that can be used as biomarkers in clinical blood samples. However, little is known about circRNAs' diagnostic values for rheumatoid arthritis (RA). In this study, the hsa_circ_0054189, hsa_circ_0008675, hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0010932, hsa_circ_0002473 and hsa_circ_0044235 in peripheral blood were determined by quantitative reverse transcription–polymerase chain reaction (qRT–PCR). For hsa_circ_0044235, only one abnormal expression circRNAs in peripheral blood was selected as a targeted circRNA to explore the diagnostic value for RA. Our work demonstrated that the hsa_circ_0044235 in peripheral blood was decreased significantly in RA patients. The hsa_circ_0044235 in peripheral blood from RA patients did not correlate with C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) or disease activity score 28 (DAS28). Receiver operating characteristic (ROC) curve analysis suggested that the hsa_circ_0044235 in peripheral blood has significant value in the diagnosis of RA. The risk score based on hsa_circ_0044235 in peripheral blood also distinguished significantly the patients with RA from systemic lupus erythematosus (SLE). This study suggests that the hsa_circ_0044235 in peripheral blood may be a potential biomarker of patients with RA.

Keywords: biomarker, circular RNAs, rheumatoid arthritis

molecular abnormality of RA is necessary for the screening of new biomarkers. More than 80% of the genome in humans is transcribed into RNA transcripts without protein-coding potential [6]. However, recent studies have revealed that although non-coding RNAs do not code directly for proteins, they play a regulatory role in the transcription and translation of protein-coding genes [7]. According to the transcript structure, these so-called non-coding RNAs (ncRNAs) are divided loosely into two major classes: linear RNA, such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs). Previous studies have shown that miRNAs and lncRNA are involved in the pathogenesis of RA and can be used as biomarkers for the diagnosis of RA [8–11]. Increasing evidence reveals that circRNAs are used widely in various physiological and pathological conditions, such

Table 1. Clinical characters and laboratory measures of rheumatoid arthritis (RA)

Age (year), mean (s.d.)	54.99 ± 12.58
Sex (female/male)	63/14
DAS28, mean (s.d.)	5.09 ± 1.43
ESR (mm/h), mean (s.d.)	57.88 ± 39.22
CRP (mg/l), mean (s.d.)	34.62 ± 43.30
RF, positive (70 patients)	43
ACPA (RU/ml), mean (s.d.) (68 patients)	303.04 ± 428.83
WBC (10 ⁹ /l), mean (s.d.)	7.29 ± 2.53
RBC (10 ¹² /l), mean (s.d.)	3.91 ± 0.61
HGB (g/l), mean (s.d.)	110.38 ± 20.71
HCT (l/l), mean (s.d.)	0.34 ± 0.07
PLT (10 ⁹ /l), mean (s.d.)	290.02 ± 133.06
Lymphocytes (10 ⁹ /l), mean (s.d.)	1.64 ± 0.78
Lymphocytes (%), mean (s.d.)	23.55 ± 9.89
Monocytes (10 ⁹ /l), mean (s.d.)	0.44 ± 0.20
Monocytes (%), mean (s.d.)	6.16 ± 2.54
Neutrophils (10 ⁹ /l), mean (s.d.)	5.13 ± 2.24
Neutrophils (%), mean (s.d.)	68.91 ± 11.16

ACPA = anti-cyclic citrullinated peptide; DAS28 = disease activity score 28; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; HCT = haematocrit; HGB = haemoglobin; *Pglyrp1*/PGLYRP-1 = peptidoglycan recognition protein-1; PLT = platelets; RBC = red blood cell; RF = rheumatoid factor; WBC = white blood cell.

as atherosclerotic vascular diseases, neurological disorders, prion infection and cancer [12–15]. However, little is known about the roles of circRNAs in human RA.

Recently, two studies have revealed that circRNAs involved in the pathogenesis of RA [16,17] and hsa_circ_104871 in peripheral blood mononuclear cells (PBMCs) could be used as a potential biomarker of RA [16]. In one of our previous studies we found some differentially expressed circRNAs in the peripheral blood from SLE patients by circRNA microarray screening, which suggests that circRNAs might play a role in autoimmune diseases. Considering that both SLE and RA are common autoimmune diseases, some differentially expressed circRNAs between SLE and HCs were selected to investigate the possibility of being used as biomarkers for diagnosis of SLE and RA. In this study, hsa_circ_0044235 in peripheral blood was found to have potential for use as a new biomarker for RA diagnosis.

Materials and methods

Patient variables

A total of 77 patients who fulfilled the revised ACR criteria for RA [18] were recruited from the First Affiliated Hospital of Nanchang University from January to

September 2017. Among these, eight patients were new-onset rheumatoid arthritis (< 6 months of disease duration) [19]. RA disease activity was measured using the disease activity score 28 (DAS28) [20]. All patients received therapy with disease-modifying anti-rheumatic drugs (DMARDs). The patient characteristics of RA patients are shown in Table 1. In addition, this study included 50 healthy controls (HC) who were free from autoimmune or inflammatory diseases and who were unrelated to the patients. As an autoimmune disease control, 31 SLE patients fulfilled the revised ACR criteria for SLE [21] were also enrolled from the First Affiliated Hospital of Nanchang University from January to September 2017. All study protocols were approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (no. 019). All provided signed informed consent.

Collection of peripheral blood samples and total RNA extraction

Peripheral blood samples (2 ml) were drawn from the median cubital vein with an ethylenediamine tetraacetic acid (EDTA) anti-coagulated vacutainer and total RNA was extracted as soon as possible. Total RNA was extracted from 250 l blood using Trizol reagent, according to the manufacturer's instructions. The concentration and quality of the RNA were assessed by absorbance spectrometry measuring absorbance ratios of A260/A280 and A 260/A230 using a NanoDrop ND-1000 spectrophotometer (Agilent, Santa Clara, CA, USA).

Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) analysis

Reverse transcription (RT) reaction was performed using a PrimeScript™ RT reagent kit (Takara Bio Inc, Japan). The qPCR was then performed on an ABI 7500 Real-time PCR System (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's instructions. The primers used in qRT–PCR are shown in Supporting information, Table 1. -actin was used as an internal control. The 2^{-ΔCt} method was used to analyse the data.

ESR, CRP and autoantibody measurement

Erythrocyte sedimentation rate (ESR) was determined according to the instructions described by the manufacturer. C-reactive protein (CRP) and rheumatoid factor (RF) were measured by nephelometry. Anti-citrullinated protein antibodies (ACPA) of immunoglobulin (Ig)G in serum were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Kexin, Shanghai, China).

Statistical analysis

Statistical analysis and graphic presentation were carried out with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and spss version 16.0 (SPSS Inc., Chicago, IL, US). Student's *t*-test was used where the normality test passed; otherwise, the non-parametric Mann–Whitney test was used to analyse the data. Similarly, the Pearson method or the non-parametric Spearman's method was used for correlation analysis. Receiver operating characteristic (ROC) curves were carried out to assess the diagnostic value of dysregulated circRNAs. A value of $P < 0.05$ was considered as the significant difference.

Results

Screening of abnormal expression circRNA in peripheral blood from RA patients and HC

The seven selected circRNA (hsa_circ_0054189, hsa_circ_0008675, hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0010932, hsa_circ_0002473 and hsa_circ_0044235) were first tested in peripheral blood from 31 RA patients and 31 HC by qRT–PCR. Compared with HC, the expression level of hsa_circ_0044235 in peripheral blood from RA patients was down-regulated significantly ($P < 0.0001$), while the expression of hsa_circ_0054189, hsa_circ_0008675, hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0010932 and hsa_circ_0002473 in peripheral blood

did not show any remarkable differences between RA patients and the HC (Fig. 1).

Validation of the hsa_circ_0044235 in the second stage in peripheral blood from RA patients and HC

To verify the results of hsa_circ_0044235 in peripheral blood identified in the screening stage, we conducted a validation study in an independent cohort including 46 RA patients and 19 HC. From the data of all the RA patients and HC, the expression level of hsa_circ_0044235 in peripheral blood from 77 RA patients was decreased significantly compared to 50 HC (Fig. 2).

Potential diagnostic values of hsa_circ_0044235 in peripheral blood in RA

Next, we performed an analysis to evaluate the potential diagnostic value of hsa_circ_0044235 in peripheral blood, and we established a ROC curve. The area under the ROC curve (AUC) was up to 0.779 [95% confidence interval (CI) = 0.700–0.859; $P < 0.0001$] (Fig. 3). The sensitivity and specificity were 61.04 and 90.0%, respectively. We speculated that the hsa_circ_0044235 in peripheral blood might be used as a biomarker of RA.

To determine whether the hsa_circ_0044235 in peripheral blood from RA patients were relevant biomarkers for the severity of RA, the correlation test was analysed to evaluate the relationship between the clinical features of RA and the level of hsa_circ_0044235. However, the level of hsa_circ_0044235 in peripheral blood from patients

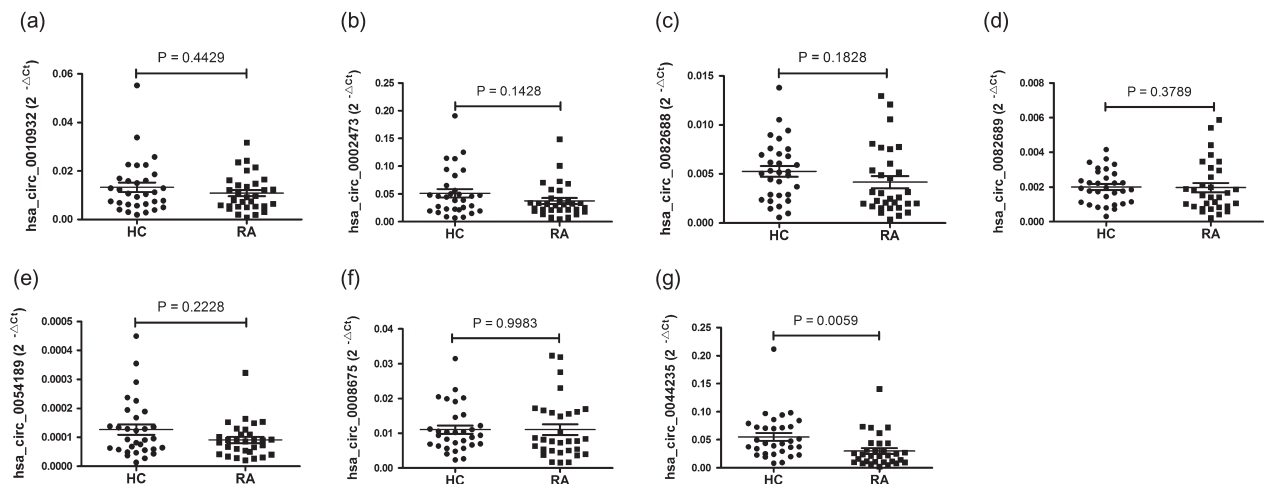


Fig. 1. Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) determined the relative expression levels of circular RNAs (circRNAs) in peripheral blood from rheumatoid arthritis (RA) patients and healthy controls (HC). (a) The average expression of hsa_circ_0010932 did not show any remarkable differences between patients with RA and the HC. (b) The average expression of hsa_circ_0002473 did not show any remarkable differences between patients with RA and the HC. (c) The average expression levels of hsa_circ_0082688 did not show any remarkable differences between patients with RA and the HC. (d) The average expression levels of hsa_circ_0082689 did not show any remarkable differences between patients with RA and the HC. (e) The average expression levels of hsa_circ_0054189 did not show any remarkable differences between patients with RA and the HC. (f) The average expression levels of hsa_circ_0008675 did not show any remarkable differences between patients with RA and the HC. (g) The average expression of hsa_circ_0044235 in patients with RA was significantly more decreased than those of the HC.

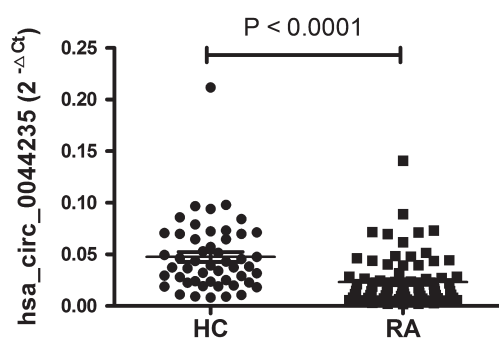


Fig. 2. The expression level of hsa_circ_0044235 in peripheral blood from 77 rheumatoid arthritis (RA) patients was decreased significantly compared to 50 healthy controls (HC).

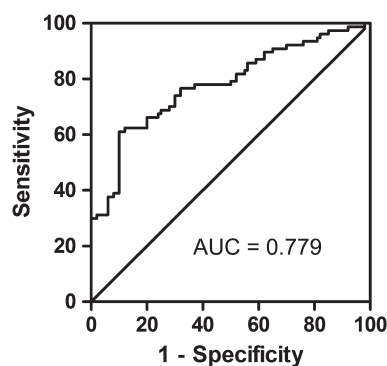


Fig. 3. Receiver operating characteristic (ROC) analysis of hsa_circ_0044235 in peripheral blood from rheumatoid arthritis (RA) patients. AUC = area under the curve.

with RA did not correlate with DAS28, CRP, ESR, RF or ACPA, which indicates the severity of the disease (data not shown).

Subsequently, we compared the hsa_circ_0044235 in peripheral blood between new- and late-onset RA patients. Results showed that hsa_circ_0044235 in peripheral blood was decreased in new-onset compared to late-onset RA patients, but the difference was not statistically significant (Fig. 4).

hsa_circ_0044235 in peripheral blood in patients with RA and SLE

Compared to SLE patients, the level of hsa_circ_0044235 in peripheral blood was clearly decreased in RA patients ($P = 0.0003$) (Fig. 5).

Subsequently, a risk score based on hsa_circ_0044235 in peripheral blood was analysed in RA patients and all controls (HC+SLE); the AUC for the risk score was 0.757 (95% CI = 0.682–0.832; $P < 0.0001$) (Fig. 5). The sensitivity and specificity were 61.04 and 82.72%, respectively. The risk score also distinguished RA patients from SLE

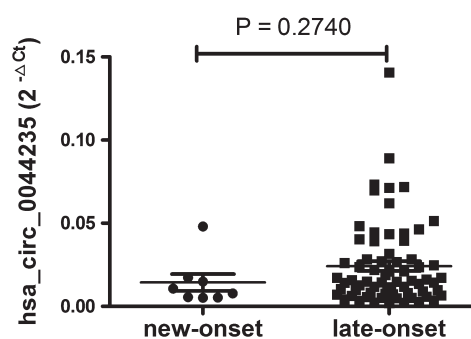


Fig. 4. The expression of hsa_circ_0044235 in peripheral blood did not show any remarkable differences between new- and late-onset rheumatoid arthritis (RA) patients.

patients significantly, and the AUC was 0.721 (95% CI = 0.612–0.831; $P = 0.0003$) (Fig. 5). The sensitivity and specificity were 90.91 and 45.16%, respectively.

MiRNAs targets of hsa_circ_0044235 prediction

To determine the function of hsa_circ_0044235, we predicted the target miRNA by aligning with the miRNA response elements (MREs) of hsa_circ_0044235 using miRanda software. Three putative miRNAs targets, hsa-miR-892a, hsa-miR-135b-5p and hsa-miR-135a-5p, were found. As shown in Fig. 6, the expression of hsa-miR-892a in peripheral blood was increased significantly in patients of RA than HC. However, the expression of hsa-miR-135b-5p and hsa-miR-135a-5p did not show any remarkable differences between patients with RA and HC.

Discussion

CircRNAs are a special class of endogenous RNAs. Recent studies have revealed that abnormal circRNAs are associated with several human diseases, such as atherosclerotic vascular disease, cancer and autoimmune disease [22–26]. Many dysregulated expression circRNAs are demonstrated to associate with RA [16,17]. Zheng *et al.* found that the levels of circRNAs (104194, 104593, 103334, 101407 and 102594) in PBMCs were expressed differentially in RA patients versus HC [17]. Ouyang *et al.* showed that the expression of hsa_circRNAs_092516, hsa_circRNAs_003524, hsa_circRNAs_103047, hsa_circRNAs_104871 and hsa_circRNAs_101873 was up-regulated significantly in PBMCs from RA patients [16]. However, little was known about the expression of circRNAs in peripheral blood in RA. In the present study, we utilized qRT-PCR to detect the expression of circRNAs (hsa_circ_0054189, hsa_circ_0008675, hsa_circ_0082689, hsa_circ_0082688, hsa_circ_0010932, hsa_circ_0002473 and hsa_circ_0044235) in peripheral blood in RA patients

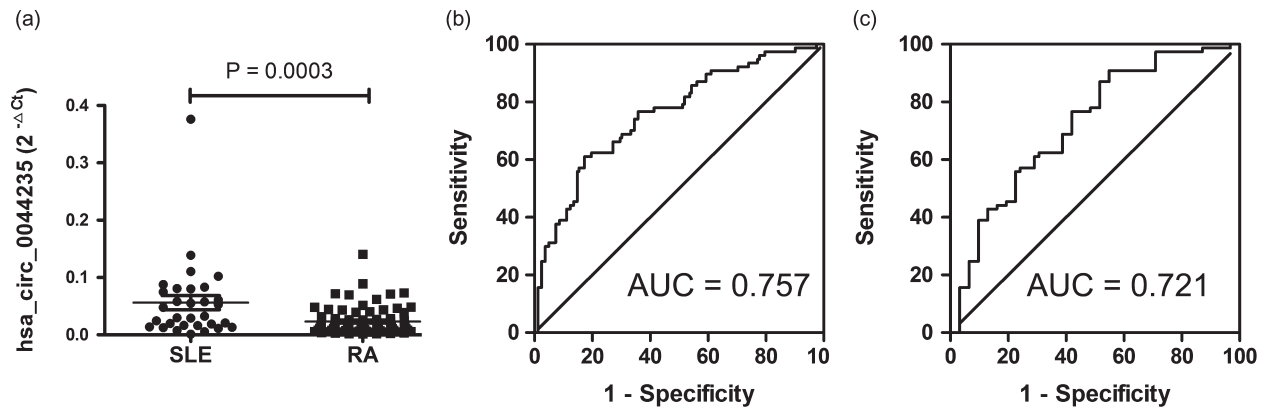


Fig. 5. Receiver operating characteristic (ROC) curve analysis of the risk-score of hsa_circ_0044235 in peripheral blood. (a) The expression level of hsa_circ_0044235 in peripheral blood in rheumatoid arthritis (RA) patients was decreased significantly compared to those of the systemic lupus erythematosus (SLE) controls. (b) ROC curve analysis hsa_circ_0044235 in peripheral blood for the risk-score in RA patients *versus* all controls (HC and SLE). (c) ROC curve analysis of hsa_circ_0044235 in peripheral blood for the risk-score in RA patients *versus* SLE patients. AUC = area under the curve;

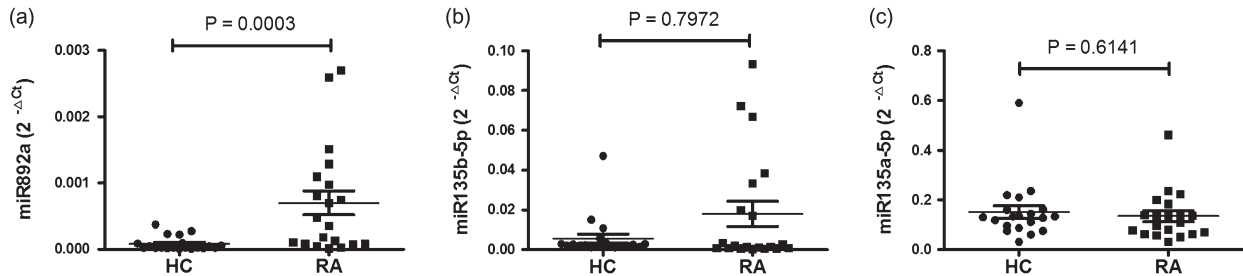


Fig. 6. Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) determined the relative expression levels of miRNAs in peripheral blood from rheumatoid arthritis (RA) patients and healthy controls (HC). (a) The expression of hsa-miR-892a in peripheral blood was increased significantly in RA patients than HC. (b) The expression of hsa-miR-135b-5p did not show any remarkable differences between patients with RA and HC. (c) The expression of hsa-miR-135a-5p did not show any remarkable differences between patients with RA and HC.

and HC, and only hsa_circ_0044235 in peripheral blood was decreased significantly in RA patients. The results indicated that hsa_circ_0044235 in peripheral blood associate with RA.

CircRNAs are a type of closed circular RNAs that do not have 5- or 3- ends; they are free of exonuclease-mediated degradation and more stable than most linear RNAs [27]. Also, circRNA often show tissue/developmental stage-specific expression [28]. These characteristics give circRNAs the potential of being ideal biomarkers for various diseases. For example, Zhao *et al.* [29] found that hsa_circ_0054633 in peripheral blood shows promise as a candidate biomarker for prediabetes and type 2 diabetes mellitus; Zhao *et al.* [30] identified that hsa_circ_0124644 in peripheral blood could be used as a new biomarker of coronary artery disease; and Ouyang *et al.* [16] determined circRNA_104871 in PBMCs as a potential biomarker

of RA. However, the diagnostic value of peripheral blood circRNAs in RA has never been explored. Our results showed the AUC of hsa_circ_0044235 in peripheral blood was up to 0.779. The risk score based on hsa_circ_0044235 in peripheral blood also demonstrated that it can distinguish RA patients effectively from SLE patients. This indicated that hsa_circ_0044235 in peripheral blood has potential as a diagnostic biomarker of RA.

In accordance with other reports showing that the expression of circRNAs was not correlated with biomarkers for disease severity [16], our study found that the hsa_circ_0044235 in peripheral blood of RA patients was not correlated with DAS28, CRP, ESR, RF or ACPA, which mirrors the severity of RA [31,32]. These results indicated that the hsa_circ_0044235 in peripheral blood may not be used as markers for systemic inflammation or disease activity in RA.

Bioinformatics analysis showed that hsa_circ_0044235 locates at chr17:45247282-45259003 and is spliced from cell division cycle 27 (cdc27) gene. It was reported that CDC27 might play a role in controlling the timing of mitosis by interacting with mitotic checkpoint proteins, including mitotic arrest deficient (Mad2), p55CDC and butyl bromide (BUBR) [34]. However, to our knowledge, no study on the role of CDC27 in RA has been published until now. Our preliminary study showed that the expression of cdc27 in peripheral blood was decreased significantly in RA patients when compared to HC (data not shown). However, the detailed role of CDC27 in RA needs further investigation.

It was reported that circRNAs could function as miRNA 'sponges', and thus naturally sequester or competitively suppress the activity of target miRNA [35]. To investigate the function of hsa_circ_0044235, the potential miRNA targets of hsa_circ_0044235 were predicted by using Arraystar's miRNA target prediction software. Three putative miRNAs targets of hsa_circ_0044235, hsa-miR-892a, hsa-miR-135b-5p and hsa-miR-135a-5p were found, and their levels in peripheral blood of RA patients and HCs were detected and compared. The data showed that the levels of hsa-miR-892a in peripheral blood were increased significantly in RA patients, suggesting that hsa_circ_0044235 might play a role in RA by interacting with hsa-miR-892a. However, further research is needed to confirm this hypothesis.

There were many limitations in this research. First, because of the relatively small sample size of new-onset RA patients, the study was restricted by the number of subjects from only one hospital, which may limit the universality of our results. Secondly, because of lack of information on RF and ACPA in HC, we were unable to assess the values of hsa_circ_0044235 in peripheral blood plus RF/ACPA in RA diagnosis. Thirdly, we did not investigate the exact role of hsa_circ_0044235 in RA pathogenesis.

Conclusions

For the first time, to our knowledge, our study detected circRNA expression in peripheral blood from RA patients and from HC, and indicated that the hsa_circ_0044235 in peripheral blood from RA was decreased. In addition, our results provides novel empirical evidence that the hsa_circ_0044235 in peripheral blood may specifically identify patients with RA and could provide better diagnostic accuracy.

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Disclosure

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table 1. Specific circRNA primers used for quantitative qRT-PCR analysis